Simultaneous reduction of the sarcolemmal and SR calcium ATPase activities and gene expression in cardiomyopathic hamster

Tuan H. Kuo 1, Wayne Tsang 1, Kevin K.W. Wang 1 and Leon Carbeck 2

1 Department of Pathology, Wayne State University, School of Medicine, Detroit, MI U.S.A. and 2 Department of Molecular Biology, Wayne State University, School of Medicine, Detroit, MI U.S.A.

(Received 22 July 1991)

Key words: Cardiac ATPase; Gene expression; Cardiac plasma membrane; sarcoplasmic reticulum function; Cardiomyopathy

Altered calcium regulation is a prominent feature in the hereditary cardiomyopathy of the Syrian hamster. However, the activity of the two systems necessary for intracellular calcium homeostasis in the heart, the sarcolemmal and sarcoplasmic reticulum calcium ATPase pumps, have not been correlated. Using age- and pair-matched myopathic and control hamsters, a simultaneous reduction in gene expression and enzyme activity for these two pumps has been demonstrated. The concommitant alteration in gene expression as early as 1 month of age, preceding noticeable myopathy suggests that the depressed activity in these two calcium ATPase systems is not due to cell necrosis but at least in part due to reduction in their mRNA levels. Reduced capacity of the calcium pumps would result in calcium overload as well as impaired contractility that leads to the eventual heart failure in this animal model.

Introduction

The cardiac plasma membrane (sarcolemma) and sarcoplasmic reticulum (SR) are the principal membrane components controlling intracellular Ca2+ concentration in the heart. The existence of the Ca2+-ATPase pumps in these two membrane compartments has been well recognized. It is thought that the function of the sarcolemmal Ca2+ pump is to maintain the resting level of intracellular Ca2+ by extruding excess Ca2+ out of the cell [1], while that of the SR Ca2+pump is to participate in the contraction-relaxation cycle of the myocardium by sequestering Ca2+ from cytosol into SR [2]. Biochemical evidence has shown that these two Ca2+-ATPases are distinct enzymes, encoded by different gene families. Recent cDNA cloning experiments indicate that there are at least two major isoforms for the plasma membrane Ca2+pump [3,4] and three isoforms for the intracellular Ca2+pump [5,6]. Since both sarcolemmal and SR Ca2+pumps participate in the reduction of cytosolic Ca2+ levels, it is conceivable that their actions may be coordinated so that intracellular Ca2+ homeostasis can be maintained efficiently. However, no information is available regarding the interrelationship between these two ATPases.

The Syrian cardiomyopathic hamster has a hereditary disease characterized by a progressive myocarditis, necrosis and intracellular calcium overload [7,8]. Previous work has shown that a selective decrease of sarcoplasmic Ca2+-ATPase activity in the heart of 40-day-old myopathic hamsters occurs, while the Na+-Ca2+ exchange system and the calcium-sensitive Na+/K+-ATPase activity remain intact [9]. Furthermore, depressed SR Ca2+ uptake in heart homogenates from 9-month-old myopathic hamsters was reported [10]. In the present study, using samples obtained from various aged myopathic animals, we have examined the mRNA levels as well as the ATPase activity of these two pumps. We report a synchronized reduction in the sarcolemmal and SR Ca2+-ATPase activities and their mRNA expression in cardiomyopathic hamster heart at 1 month of age.

Materials and Methods

Animals. In order to maintain consistency in disease characteristics, littermates used in the present study were specially bred by the University of Toronto (Or. M. Sele) and named as the 14.6-To strain. There were no differences in disease characteristics between the
current strain Bio 14:6-To and previously used strain Bio 53:58 [9]. However, over the years, the Bio 53:58 strain has developed a milder form of the disease with a longer life span. In order to correct this situation, specific breeding of the Bio 14.6 was carried out by the Toronto group [8]. The control hamsters used here were heterogenous F1B.

Membrane preparations and assays. Cardiac sarcolemma and SR membranes were prepared from five pooled hearts (for each age) according to Ref. 9. After sucrose density gradient centrifugation, the top band (density 1.13) and lower band (density 1.15) corresponding to the respective sarcosomal and SR fractions were used for enzyme assays. The assay of the sarcosomal Ca²⁺-ATPase activity (1 μM vanadate-sensitive) was described previously [9]. Sarcosomal vesicles (2 μg of protein) were incubated at 37°C in 1 ml of medium containing 160 mM KCl, 20 mM Hepes (pH 7.4), 1 mM MgCl₂, 1 mM EGTA, 0.96 mM CaCl₂ (free Ca²⁺ ~ 1.15 μM), 1 μg oligomycin, 10 μM dithiothreitol, 5 μM A23187. The reaction was started by the addition of 1 mM ATP. The P, released was assayed by colorimetric determination. The assay for SR activity (ouabain-facilitated Ca²⁺-uptake) was according to Ref. 11. The reaction medium contains 100 mM KCl, 50 mM K-Hepes (pH 6.8), 3 mM MgCl₂, 1 μg oligomycin, 10 μM dithiothreitol, 50 μM 45CaCl₂, 3 mM oxalate, 20 μg/ml of SR membranes and the reaction was initiated by addition of 3 mM ATP. Radioactive Ca²⁺ accumulation at each time point was determined by filtration.

Measurements of the phosphorylated intermediate of the enzyme (E-P). Because of the relative abundance of the SR ATPase in the crude membranes (cardiac membrane fraction that has not been separated by sucrose density gradient), it is possible to detect the adenylyl phosphate intermediate of this ATPase and use it to estimate the amount of active enzyme present in the membranes [12]. On the other hand, signals from the E-P of the sarcosomal ATPase are invariably too weak to be detected. Ca²⁺-dependent phosphorylation of the SR ATPase was carried out at 37°C as previously described [13] with modification, in 1 ml of a medium containing (mM) KCl 100, Tris-HCl (pH 7.4), and either CaCl₂ 1, or EGTA 1. The reaction was initiated by the addition of 20 μg of the crude membrane and quenched after 15 s and the phosphorylated membranes were electroblotted in SDS gel at pH 6.5 as previously described [14]. Dried gel slabs were exposed to X-ray film for autoradiography. The E = P formed was characterized by its sensitivity to hydroxyamine and to specific SR Ca²⁺-ATPase inhibitors, DTBHQ (D-tret-butylhydroquinone).

cDNA probes and RNA blot analysis. Isoform specific probes used in Fig. 1 were isolated from the 3′ untranslated regions of the corresponding rat cDNA clones 4-1, a 692 bp HpaI-PstI fragment and 1-2, the 790 bp SpeI-AxlI fragment (provided by Dr. G. Shull, University of Cincinnati, see Ref. 3). A previously undefined 1200 bp cDNA clone (c10) was isolated in this laboratory and used in Fig. 1c as an internal control. We have sequenced c10 and found that it did not match with any existent sequence in the GenBank. Since c10 can detect messages in various tissues, it is assumed to be a housekeeping gene. The probe used in Fig. 2A was a 1600 bp EcoRI fragment isolated from a partial Ca²⁺-ATPase cDNA clone. This probe which was isolated from a hamster cardiac library, corresponds to the 3′ coding sequence of rat 1-1 (nucleotide 2903 to 3996). To obtain a cardiac SR Ca²⁺-ATPase specific probe, the KS 16-17′ 3′ untranslated region, a 308 bp Clal-Xhol fragment was used (provided by Dr. G. Shull), see Ref. 5. To study Na⁺/K⁺-ATPase gene expression, a probe for the α isoform, the 332 bp NotI-SmaI fragment, was used (provided by Dr. J. Lingel).

Each preparation of total RNA was isolated from five pooled hearts by the guanidinium thiocyanate method [15], as described previously [16]. 20 μg of total RNA were denatured, size-fractionated on 1% agarose gels, transferred to GeneScreen membrane (Du Pont), prehybridized and hybridized according to manufacturer’s protocol. All probes used for hybridization had similar concentration (1-10 probe/μl) and specific activity (1-10 probe/μl). Methylated DNA was washed in 2× SSC, 1× SSC at 65°C, twice for 30 min and 1× SSC at room temperature, twice for 30 min and processed for autoradiography (−70°C) and densitometry as described in Ref. 16.

Densitometry and data analysis. Autoradiograms were quantified by scanning with a video densitometer (model 620, Bio-Rad). Similar results were also obtained by using a flat-bed scanner (model 730 GS, Xerox) with the Image 1.22 software developed by NIH. Each experiment presented is representative of at least two experiments performed independently. Data were analyzed by Student’s t-test and significance defined as a P value of <0.05.

Results and Discussion

The sarcosomal Ca²⁺-ATPase isoforms. We first examined the mRNA expression of the sarcosomal Ca²⁺-ATPase. Since recent cloning efforts have defined two major isoforms of the plasma membrane Ca²⁺-ATPase in the rat brain [3], it was necessary to assay control hamster mRNA with gene specific probes to define which isoforms were expressed in the heart of this animal. Fig. 1 shows Northern blots of total RNA from various tissues of 1-month-old animals, hybridized with isoform 1 and 2 specific probes. The
The 7.5 probe (Fig. 1A) detected two messages (7.5 and 5.5 kb) in brain (B), kidney (K), heart (H) and skeletal muscle (SK) of control hamsters. The 7.2 probe (Fig. 1B) detected a specific 7.2 kb message in brain, kidney, heart and liver (L). The non-specific hybridization of this probe to the homoeo-genes (not shown). In rat brain, an additional 8 kb message was seen. Rehybridization of this filter with a Ca2+-ATPase coding sequence probe demonstrated that the message was not derived from I-1 or -2 (not shown). The integrity of each mRNA sample was determined by hybridization with the homoeogeneic gene c10 isolated from our laboratory (Fig. 1C and see Materials and Methods). This probe detected an abundant 1.2 kb message in all tissues. Since the exposure time for autoradiography of the c10 message in Fig. 1C was only 16 h, while that for the I-1 and -2 messages in Fig. 1A and B required 1 week, this indicates the very low abundance of the plasma membrane (PM) Ca2+-ATPase messages. This finding is consistent with the previous report [1] suggesting that PM Ca2+-ATPase accounts for less than 0.1% of total membrane protein. Our Northern analysis results indicate that while both isoforms are expressed in the brain as well as in the kidney, only 1 isoform is expressed in the heart and skeletal muscle and only isoform 2 is expressed in the liver to any significant extent. This isoform expression pattern in hamster tissues is consistent with the pattern seen in the rat [17]. This tissue specific expression suggests that the isoform 1 gene produces the majority of the cognate mRNA in the heart and is likely to encode the bulk of the enzyme activity. In addition, comparing the control and myopathic animals shows that the same isoform is expressed in normal and diseased states without any gross alteration in the isoform pattern (not shown).

Sarcoclemmal and SR Ca2+-ATPase mRNA expression in cardiomyopathy. Fig. 2A (upper panel) shows a Northern analysis of RNA prepared from control (lanes 1, 3, 5, 7) and myopathic (lanes 2, 4, 6, 8) hamster hearts at 1, 2, 3 and 4 months of age. A sarcoclemmal Ca2+-ATPase coding sequence probe, isolated from a hamster cardiac cDNA clone, was used to detect the I-1 specific mRNAs. A decrease of sarcoclemmal Ca2+-ATPase mRNA expression was observed in the hearts of myopathic animals when com-

Fig. 1. Assay of sarcoclemmal Ca2+-ATPase homoeogene expression in 1-mo-old control hamster tissues. (A) The isoform 1 (0.15 kb) the isoform 2 (0.25 kb) (C) the homoeogeneic gene (6 kb). Brain (B), kidney (K), heart (H), skeletal muscle (SK) and liver (L).

Fig. 2. Northern analysis of Ca2+-ATPase mRNA in heart tissue of post-mitotic control and myopathic hamsters of various ages. (A) Upper panel: sarcoclemmal ATPase mRNA was assayed in hearts of control (lanes 1, 3, 5, 7) and myopathic (lanes 2, 4, 6, 8) hamsters at 1, 2, 3 and 4 months of age. A Ca2+-ATPase coding region probe (isolated from hamster cardiac library) was used to detect the I-1 specific mRNAs in total RNA. Lower panel shows the ethidium bromide staining of the gel samples in the upper panel. 20 µg of total RNA were applied in each lane. Sample from 4-month-old myopathic heart (lane 8) always shows some degradation due to necrosis of the tissue. (B) The sarcolemmal reticulum (SR) Ca2+-ATPase mRNA was assayed using a 3' unlabelled probe derived from rat clone RS 8-17, a cDNA clone of the major cardiac SR ATPase. Total RNA from 1, 2 and 3-month-old control (lanes 1, 3, 5) and myopathic (lanes 2, 4, 6) hamsters was hybridized with this probe.
pared to age matched controls. While Fig. 2A represented a single experiment with RNA preparations derived from five pooled hearts for each age, the average of several experiments indicated a 20 to 40% decrease (n=6, four different preparations, P<0.05) in myocardial sample by 1 month and a maximal decrease of 40 to 60% (n=4, three different preparations, P<0.01) after 3 months of age. The ethidium bromide staining of these samples (Fig. 2A, lower panel) indicated equal amounts of ribosomal RNA loaded per lane (from lane 1–7). This decrease in the sarcocellular gene expression from 1 month to 3 months of age, is not a generalized event, since the expression levels of several other genes, including the Na+/K+-ATPase a isoform and the housekeeping gene c10 (not shown), are unchanged during this time period. Furthermore, this decrease was limited to the diseased heart and no change in the calcium pump mRNA levels was observed in other tissues. It should be pointed out that by 4 months of age, nonspecific effects due to necrosis of the myopathic heart may cause some RNA degradation as indicated in lane 8 of the ethidium bromide stained gel (Fig. 2A). Fig. 2B shows a similar analysis for the SR Ca2+-ATPase expression using a cardiac specific probe [5]. Since the SR ATPase messages are more abundant, the exposure time for the autoradiogram in Fig. 2B required only 24 h as opposed to 1 week for the PM ATPase messages in Fig. 2A. An age-dependent decrease in the amount of the SR mRNA was also observed (Fig. 2B). Densitometric analysis showed that on the average, a 30–40% decrease (n=3, three different preparations, P<0.05) in mRNA levels occurred by 1 month of age and that this decrease continued such that by 3 months of age, the amount of SR Ca2+-ATPase mRNA in the myopathic heart was only 40–50% of the control (n=3, three preparations, P<0.01). These results (reproduced with total RNA and poly A+RNA) demonstrate that the mRNA levels for the sarcocellular and SR Ca2+-ATPases are both reduced in the myopathic hamster heart between 1 and 4 months of age. The absolute quantitation of this reduction sometime varies, especially in different batches of animals, possibly reflecting a variable penetrance for this recessive disorder. Despite these variations, a reduction of the mRNA levels for both the PM and SR Ca2+-ATPases was consistently observed in the 1-month-old myopathic animals.

Recently, a decrease in the myocardial level of the mRNA encoding the SR Ca2+-ATPase has been reported in rats with hypothyroid condition [18], or with pressure overload [12,19,20]. Altered SR Ca2+-ATPase gene expression has also been reported in hamsters with end-stage heart failure [21]. However, the present study demonstrates for the first time that the mRNA level of the sarcocellular Ca2+-pump is also reduced in hearts under myopathic conditions.

Sarcocellular and SR Ca2+-ATPase activities in cardiomyopathy. To determine the effect of the reduced mRNA levels on these enzyme activities, parallel experiments were conducted using the same batches of heart. Littersates to compare the sarcocellular and SR Ca2+-ATPase enzyme activities with the RNA bioactivity results. Fig. 3A shows the time-course of Ca2+-ATPase activity in sarcocellular membranes prepared from 1–4-month-old control (C) and myopathic (M) hamsters. A progressive decrease in sarcocellular activity was observed that began approximately at 1 month, and reached a minimum between 3 and 4 month of age during which time only 30% of the original activity remained (three experiments and five animals for each age). The present data confirm our previous report showing a similar regression in the sarcocellular calcium pump activity of myopathic hamster and its apparent correlation with the lesion development [9].

![Graph A](image1)

![Graph B](image2)

---

Fig. 3. (A) Sarcocellular Ca2+-ATPase activity vs. age of hamsters. Heart sarcocellular preparations were obtained from control (C) and myopathic (M) hamsters at various ages and tested for 3-H Ca2+ uptake. (B) SR Ca2+ uptake vs. reaction time. Heart SR preparations were obtained from control (C) and myopathic (M) hamsters at various ages from 1 to 4 months (indicated by globe symbols) and assayed for calcium-induced Ca2+ uptake. Since results of control samples do not vary with age, only a representative plot (C) is shown here for the purpose of clarity. Data are representative of experiments performed with three membrane preparations.
Using heart SR membranes prepared from control and myopathic animals, the activity of the SR Ca\textsuperscript{2+}-ATPase was assayed as a function of oxalate-facilitated Ca\textsuperscript{2+} transport. Fig. 3B shows the Ca\textsuperscript{2+} uptake by SR vesicles versus reaction time. Typically, the calcium accumulation by SR vesicles was a linear function during the first 2 min, with a steady state attained after 9 min. This steady state level has been used as a measure of the calcium loading or 'capacity' for calcium transport [10].

Fig. 3B shows a progressive decrease in SR activity in the myopathic group as compared to controls (three experiments and five animals for each age). Both the initial rate and the extent of uptake were reduced with samples from 1–4-month-old myopathic animals while no changes with age were observed in control groups (For the purpose of clarity, only a representative plot of 1 C is shown here). This reduction in the velocity and capacity of the SR ATPase may represent either a quantitative or a qualitative defect, i.e., a decrease in the number of pump units or an altered catalytic activity (K_m). These two possibilities can be distinguished on the basis of the K_m values which are estimated by taking the ratio of velocity to capacity [10]. From the data of Fig. 3B, calculating K_m values for the control 0.25 ± 0.04 min\textsuperscript{-1} and myopathic groups 0.24 ± 0.03 min\textsuperscript{-1}, n = 3 shows no significant difference between these two groups at all ages tested. This suggests that a major defect in the myopathic group may be a decrease in the number of SR calcium transport sites with no change in the enzyme characteristics. This is consistent with a previous report showing a similar decrease in SR pump capacity in the 9-month-old myopathic hamster [10].

Using the crude membrane fraction, it was possible to identify the acylphosphatase intermediate (E = P) formation of the SR pump, judging from its apparent molecular weight, its calcium dependence, and sensitivity to a SR pump inhibitor, D-totri-butylinhydroquinone (Fig. 4A). On the other hand, the E = P formation of the sarcoplasmic pump was not observed probably due to its very low catalysis in the myofibril (see Materials and Methods). This E = P which represents the active enzyme molecule, has been used as an estimate of the functional SR pump molecule present in the myofibril [12]. Further study determined that the E = P level of the SR pump from 3-month-old myopathic hamster heart was 40% lower than that of the control hamster heart (Fig. 4B), based on equal loading of the protein samples on the SDS gel (Fig. 4C). This evidence together with the above K_m analysis suggest that the depressed SR ATPase activity in the myopathic heart is mainly due to a reduction in the number of active ATPase molecules.

Comparison of the ATPase activity with the mRNA levels. Fig. 5 summarizes the data concerning Ca\textsuperscript{2+} pump activity and densitometry analysis of the mRNA levels in myopathic animals of various ages, expressed as percent of their age-matched controls. For both the sarcoplasmic pump (Fig. 5A) and the SR pump (Fig. 5B), there was a good correlation between the ATPase activity and the quantity of the specific message at 1
month of age. However, the decline in the ATPase activity after 1 month of age became much faster than the decline in the corresponding mRNA levels. These results suggest the involvement of other regulatory mechanisms in the inactivation of the pumps, such as changes in protein phosphorylation [16] or in membrane phospholipid composition in the myopathic heart [9]. The present data also suggest that decreased Ca\textsuperscript{2+}-ATPase activities of the sarcomembran and SR pump in the heart of myopathic hamster are at least in part, due to decreased levels of the respective message. This reduction in the mRNA levels is probably due to a depression of gene expression and/or a decrease in mRNA stability.

In addition to the abnormalities in the Ca\textsuperscript{2+}-ATPase pumps, an increase of the Ca\textsuperscript{2+} channel receptor num-

Acknowledgments

We thank Drs. G. Shull and J.B. Lingrel for providing gene specific probes; Dr. C.C. Liew for the hamster cardiac cDNA library. Supported by NIH grant HL 39441 and a grant-in-aid from the American Heart Association of Michigan (THK).

References
