Control of sarcoplasmic/endoplasmic-reticulum Ca^{2+} pump expression in cardiac and smooth muscle

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Cardiac muscle expresses sarcoplasmic/endoplasmic-reticulum Ca^{2+} pump isoform SERCA2a; stomach smooth muscle expresses SERCA2b. In 2-day-old rabbits, cardiac muscle contained levels of SERCA2 protein that were 100–200-fold those in the stomach smooth muscle. In nuclear run-on assays, the rate of SERCA2 gene transcription in heart nuclei was not significantly higher than in the stomach smooth-muscle nuclei. However, the SERCA2 mRNA levels (mean $\pm$ S.E.M.) were (29 ± 4)-fold higher in the heart. In both tissues the SERCA2 mRNA was associated with polyribosomes. In a sucrose-density-gradient sedimentation velocity experiment on polyribosomes, there was no difference in the sedimentation pattern of SERCA2 mRNA between the two tissues, suggesting that the translation efficiency of SERCA2 RNA in the two tissues is quite similar. Thus the main difference in the control of SERCA2 expression in the two tissues is post-transcriptional and pretranslational.

Key words: ATPase, calcium, differentiation, mRNA stability, transcription, translation.

INTRODUCTION

Three genes encode Ca^{2+} pumps in mammalian cells: the sarcoplasmic/endoplasmic-reticulum Ca^{2+} ATPases SERCA1, 2 and 3 [1–3]. Cardiac and smooth-muscle myocytes express the gene SERCA2. The primary SERCA2 transcript contains several cryptic exons that allow alternative splicing in its 3′ region [4–7]. All the splice variants are identical in the expression of exons 1–21. In one variant (SERCA2a), exon 21 is fused to exon 25. In the main, cardiac muscle and slow twitch muscle express SERCA2a. However, most tissues, including stomach smooth muscle, express SERCA2b, in which exons 22 and 23 follow exon 21. Some tissues also contain another splice variant (exon 24 in addition to exons 22 and 23) [5]. As a result, all the splices have a common 5′-untranslated region (UTR) and encode proteins with identical 993 N-terminal residues [2,4,6,8–11]. SERCA2a mRNA encodes four additional amino acid residues and SERCA2b encodes 49 different residues. These C-terminal differences in the two proteins might marginally affect the constitutive function or regulation of SERCA2 because both the proteins can be regulated by phospholamban [12]. SERCA2a and SERCA2b also differ in their 3′-UTRs.

Consistently with the need to generate greater force with a higher speed, the SERCA2 protein density is much higher in the cardiac muscle than in the stomach smooth muscle. What leads to this difference in SERCA2 expression? Phenotypic expression in eukaryotes is regulated at the level of transcription, nuclear export, splicing, polyadenylation, length and structure of mRNA, translation efficiency of mRNA, and post-translational events including the processing and protein stability [13–16]. The strength of polyadenylation sites has also been associated with alternative splicing of SERCA2 [17,18]. However, it is not known what leads to the different levels of SERCA2 expression in different tissues. To determine the nature of control of SERCA2 expression, we compared the following parameters for SERCA2 in cardiac muscle and stomach smooth muscle from 2-day-old rabbits: protein levels, transcription rates, mRNA levels and efficiency of mRNA binding to ribosomes.

EXPERIMENTAL

Membrane preparation

Rabbits (2 days old) were obtained from Reiman Fur Farms and anaesthetized with methoxyflurane (Metofane; Jannsen Pharmaceutica). The tissues were dissected and homogenized at 0–4 °C as described previously [10]. The homogenates were centrifuged at 1000 ***g*** for 10 min and the resulting supernatant was designated as postnuclear supernatant (PNS). PNS was stirred with 0.7 M KCl at 4 °C for 10 min and then centrifuged at 140000 ***g*** for 60 min to obtain a pellet designated as microsomes.

Western blots

A sample buffer was added to the PNS or microsome samples such that the final solutions contained 30 mM Tris/HCl, pH 6.8, 1.5% (w/v) SDS, 5% (v/v) glycerol and 0.2% Bromophenol Blue. The samples were stored frozen at −20 °C and used later in Western blots as described previously [10]. The primary antibody used was the anti-SERCA2 antibody IID8 (Affinity Bioreagents), which recognizes both SERCA2a and SERCA2b. The bound primary antibody was detected by enhanced chemiluminescence with an Amersham kit.

RNA isolation

All the buffers used for RNA were prepared in water treated with diethyl pyrocarbonate [19]. The rabbit left ventricle and stomach smooth muscle were dissected, minced in sterile chilled PBS and

Abbreviations used: DTT, dithiothreitol; PNS, postnuclear supernatant; RT–PCR, reverse-transcriptase-mediated PCR; SERCA, sarcoplasmic/endoplasmic-reticulum Ca^{2+} ATPase; UTR, untranslated region.

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used for RNA isolation with Trizol reagent (Gibco BRL) in accordance with the instructions of the manufacturer. The resulting RNA pellets were air-dried for 15–30 min at 20–23 °C and then stored at −70 °C until use. Poly(A)$^+$ RNA was isolated from total RNA with an Oligotex mRNA kit (Qiagen).

Northern blots

We used an anti-sense cRNA corresponding to the rabbit SERCA2 cDNA from 514 to 1282 bp [6] cloned in the *Eco*RI site of pSK$^+$BS (Stratagene). The plasmid was linearized with *Sma*I; the linear DNA was isolated by gel electrophoresis, purified with a PCR purification kit (Qiagen) and transcribed with T7 RNA polymerase (Pharmacia) to obtain a ^{32}P-labelled cRNA probe. Northern blots with total and poly(A)$^+$ RNA were performed as described previously [10]. The hybridized membranes were washed in 2 × SSC/0.1 % SDS at 20–23 °C for 5 min, 0.2 × SSC/0.1 % SDS for 90 min at 65 °C, 0.1 × SSC/0.1 % SDS for 60 min at 67 °C, and 2 × SSC/0.1 % SDS at 20–23 °C for 5 min.

Isolation of nuclei

Nuclei were isolated by a modification of a previously published method [20]. Fresh heart or stomach smooth-muscle tissue from up to ten 2-day-old rabbits was homogenized in 10 vol. of buffer MA [250 mM sucrose/10 mM Tris/HCl (pH 7.4)/10 mM $MgCl_2$/2.8 mM dithiothreitol (DTT)/0.1 mM PMSF, with 10 units/ml RNAguard] with a Polytron PT20 (setting 4.5) (Brinkman) for 12 s. The homogenate was centrifuged at 1000 ***g*** for 10 min. The pellet was resuspended in 10 vol. of buffer MA by using six strokes of a motorized Teflon homogenizer (clearance 0.15–0.23 mm) and the resulting suspension was filtered through a nylon mesh (size 320); the filtrate was centrifuged at 1000 ***g*** for 10 min. The resulting pellet was suspended in 10 ml of buffer MA/0.5 % (v/v) Triton X-100 by using a pipette, then centrifuged at 1000 ***g*** for 10 min. This pellet was resuspended in 10 vol. of buffer MB [2.2 M sucrose/10 mM Tris/HCl (pH 7.4)/10 mM $MgCl_2$/0.1 mM PMSF/2.8 mM DTT, with 14 units/ml RNAguard] by using two strokes of a motorized homogenizer. The suspension was then centrifuged at 110000 ***g*** for 60 min in an SW40 rotor. The pellet was resuspended in 5 ml of buffer MA and centrifuged at 1000 ***g*** for 20 min. The resulting pellet was resuspended in 1.5 ml buffer MA and centrifuged at 3000 ***g*** for 5 min. The final pellet was suspended in 215 μl of storage buffer [50 mM Tris/HCl (pH 8.3)/5 mM $MgCl_2$/0.1 mM EDTA/40 % (v/v) glycerol/2.8 mM DTT/0.1 mM PMSF, with 1 μl of RNAguard] to obtain (7–10) × 10^7 nuclei/ml.

Nuclear run-on assay

The plus strand of recombinant pSK$^+$BS containing 1088–2616 bp of rabbit anti-sense SERCA2 cDNA [6,9] was used as a probe and the vector alone was used as the control. Alternatively, we used the cRNA probe described above for the Northern blots. The probes (2 μg) were spotted on a positively charged membrane and dried; the membrane was then irradiated with UV. The membrane was prehybridized for 2–5 h at 65 °C in Church buffer [7 % (w/v) SDS/1 % (w/v) BSA/1 mM EDTA/250 mM NaH_2PO_4/NaOH (pH 7.2)] [21]. A run-on transcription mixture was prepared at 0 °C. It contained a matching number of cardiac or stomach nuclei [(1.5–2) × 10^7] in 300 μl of a solution containing 200 mM Tris/HCl, pH 8.0, 200 mM $MgCl_2$, 600 mM KCl, 4 mM ATP, 4 mM UTP, 4 mM GTP and 250 μCi of [α-^{32}P]CTP. The reaction mixture was incubated at 30 °C for 45 min, after which 5 μl of RNAse-free DNase I (10 units/μl) (Boehringer Mannheim) was added and the mixture was incubated for another 5 min. A further 5 μl of DNase I was added and incubation continued for a further 5 min. Each sample was mixed with the following and incubated at 42 °C for 45 min: 36 μl of SET buffer [10 % (w/v) SDS/100 mM Tris/HCl (pH 7.5)/50 mM EDTA] and 10 μl of 20 mg/ml proteinase K. The samples were then deproteinated with phenol/chloroform, mixed with 10 μl of 5 mg/ml yeast tRNA and precipitated with ammonium acetate and propan-2-ol. The resulting precipitate containing the labelled run-on transcript RNA was resuspended in 150 μl of Tris/EDTA buffer, incubated at 37 °C for 10 min and passed through a Sephadex G-25 spin column to obtain the purified RNA. The amount of radioactivity in a small aliquot of the purified RNA was determined. The radioactive RNA sample was mixed with Church buffer along with 2 mM DTT and single-stranded pSK$^+$BS DNA (2 μg), boiled for 5 min and cooled to 20–23 °C. Matching volumes of cardiac and smooth-muscle RNA samples containing 5 × 10^6 c.p.m./ml were placed in plastic bags containing the above membranes. The hybridization was performed at 65 °C for 48–65 h. After hybridization, each membrane was washed at 20–23 °C in 2 × SSC/0.1 % SDS for 15 min, at 65 °C in 0.1 × SSC/0.1 % SDS for 30 min, and again at 20–23 °C in 2 × SSC/0.1 % SDS for 15 min. The blots were then autoradiographed and quantified by image analysis.

Preparation and fractionation of polyribosomes

Left ventricles were isolated and minced in sterile Joklik medium (S-MEM, Joklik's modified; Gibco) as a modification of the previously published method [22]. The minced tissue was washed for 3 min in Joklik medium and then digested for 5 min in digestion buffer [Joklik medium/3 mg/ml collagenase (436 units/mg; Sigma)/1 mg/ml trypsin inhibitor/1 mg of DNase/0.2 mM $CaCl_2$/1 mg/ml BSA] containing cycloheximide (50 μg/ml) at 37 °C. The digestion medium was saved and each tissue was rinsed for 5 min with a solution containing Joklik medium, 0.2 mM $CaCl_2$, 1 mg/ml BSA and 50 μg/ml cycloheximide. The tissue was triturated and the medium saved. This 5 min rinse was repeated and the medium was saved again. The saved medium was pooled in 15 ml conical tubes and centrifuged at 50 ***g*** for 5 min to obtain single cardiac cells. Single cells were isolated from the smooth muscle of eight to ten stomachs by digestion in Joklik's medium/0.2 mM $CaCl_2$/1 mg/ml BSA/1 mg/ml trypsin inhibitor/1 mg/ml collagenase/50 μg/ml cycloheximide in a 31 °C shaking water bath for 50 min. The tissue was rinsed, digested and rinsed again. Finally the tissue was placed in collagenase-free medium, shaken for 30 min and triturated to obtain single cells [23]. The polyribosomes were isolated from the freshly isolated cardiac and stomach cells by a modification of a previously published method [24,25]. Pellets of freshly isolated cells were suspended in 2 ml of ribosome buffer [200 mM NaCl/2.5 mM magnesium acetate/0.5 % (v/v) Nonidet P40/0.1 mM PMSF/5 μM DTT/50 μg/ml cycloheximide/5 μl of RNAguard/10 mM Mops/NaOH (pH 7.2)], then homogenized manually with five strokes in a glass homogenizer. The samples were centrifuged at 11000 ***g*** for 5 min. The supernatants were centrifuged further, either in a sedimentation experiment or in a sedimentation velocity experiment with a sucrose density gradient. For the sedimentation experiment the supernatant was transferred into polycarbonate 3.5 ml ultracentrifuge tubes and centrifuged at 160000 ***g*** for 1 h in a TLA100 rotor. The subribosomal supernatant fractions were removed and 13 μg/ml tRNA was added. The polyribosomal pellet was suspended in 1 ml of Trizol reagent for RNA isolation. The subribosomal supernatant was precipitated with 2.5 vol. of ethanol overnight

at −20 °C; the pellet was collected by centrifugation for 20 min at 30000 ***g*** and suspended in 1 ml of Trizol reagent for RNA isolation. The RNA was suspended in 18 μl of water and stored at −70 °C. For fractionation by sedimentation velocity in a density gradient, the sample was layered on a 0–60 % sucrose density gradient and centrifuged for 2 h at 140000 ***g*** in an SW40 rotor. Fractions were then collected from the gradient and RNA was isolated from them as for the sedimentation experiment.

Estimation of poly(A)$^+$ and total RNA in polyribosomal fractions

Total RNA was estimated by dot-blot analysis as rRNA with a biotinylated anti-sense rabbit 18 S rRNA oligodeoxyribonucleotide probe (5′-CGT CTT CGA ACC TCC GAC TT-3′) [26]. The membranes were prehybridized at 45 °C for 1 h in a solution containing 0.25 M disodium phosphate, pH 7.2, 7 % (w/v) SDS and 1 mM EDTA, hybridized overnight at 45 °C in the same solution with 1.0 nM probe and then washed twice for 5 min at 20–23 °C in 2 × SSC/0.1 % SDS, twice for 15 min in 0.5 × SSC/0.1 % SDS and twice for 5 min in 2 × SSC at 20–23 °C. Poly(A)$^+$ RNA estimation was conducted in dot-blots by the same method except that biotinylated $(dT)_{20}$ (1 nM) (Boehringer Mannheim) was used as a probe, in accordance with the instructions of the manufacturer. The biotin label was detected by using the Southern Light system (Tropix). The data were analysed by image analysis followed by linear regression. SERCA2 estimation by dot-blots was performed as described for Northern blots.

Estimation of SERCA2 and actin mRNA in polyribosomal fractions

RNA from various fractions was estimated by reverse-transcriptase-mediated PCR (RT–PCR) or dot-blot analysis. For reverse transcription, RNA was first reverse-transcribed with the $(dT)_{15}$ primer (Promega) by using Superscript II (Gibco BRL) in accordance with the instructions of the manufacturer. To increase selectivity, nested PCR was performed in two steps: the reverse transcription mix was amplified by PCR with the primer SR2L3370D (5′-GTG ATC TGG AAG ATA AGC GGC A-3′, based on a sequence flanking an intron) [6,27] and an upstream primer SR2L3018U (5′-CGT TAC CTG GCT ATT GGC TGT T-3′), and then using SR2L3370D and an upstream primer internal to the first PCR product (SR2U3064, 5′-CTG CTG CGT GGT GGT TCA TT-3′) [26] to give a final PCR product of 306 bp. A typical reaction mixture for the first PCR (10 cycles) consisted of 5 μl of reverse transcript mix, 2 μl each of 1 μM SR2L3370D, 1 μM SR2L3018U, a dNTP mix containing 2.5 mM dATP, 2.5 mM dCTP, 2.5 mM dGTP and 2.5 mM dTTP, 25 mM $MgCl_2$, 10 × PCR buffer [100 mM Tris/HCl (pH 8.3 at 25 °C)/500 mM KCl], *Taq* DNA polymerase (2.5–5 units) (Perkin Elmer), and water to 20 μl. A PCR cycle consisted of denaturing at 94 °C for 30 s, annealing at 60 °C for 90 s and elongation at 74 °C for 90 s. The reaction mixture for the second PCR was similar except that the primers were 10 μM SR2L3370D and SR2U3064, 2 μl of the first amplification mixture was used as template and 5μCi ^{32}P-α-dATP (Amersham, 10 mCi/ml) was included. The same RNA samples were also used for amplifying actin. For cardiac actin the primers used were as reported previously [28] (caract140u, 5′-GAT TAT TGC TCC CCC TGA GCG-3′; caract366d, 5′- GCA CGT GTG TAA ACA AAC TG-3′) and gave a 246 bp product. PCR primers for smooth-muscle actin (smact508u, 5′-AAC TGG CAT TGT GCT GGA CTC-3′; smact642d, 5′-AGG AAT AGC CAC GCT CAG-3′) were based on rabbit smooth-muscle α-actin sequence [29] and gave a 155 bp product. For each type of experiment, PCR analysis was first conducted by using different cycles of PCR to determine the linearity of the relationship between the product obtained and the template. For direct detection, DNA obtained by PCR amplification of an authentic plasmid was added to each radioactive sample before electrophoresis in 9 % (w/v) polyacrylamide gels in 0.5 × TAE buffer [20 mM Tris/acetate (pH 8.5)/1 mM EDTA]. The gels were then stained with ethidium bromide and dried by using cellophane membrane backing (Bio-Rad) on both sides. The dried gels were either autoradiographed or fluorescence bands were cut and quantified by scintillation counting. SERCA2 estimation by dot-blots was performed as described for poly(A)$^+$ estimation in polyribosomal fractions.

RESULTS

To understand the control of expression of SERCA2 in heart muscle and stomach smooth muscle, we examined SERCA2 protein abundance, transcription, mRNA levels and mRNA translation in 2-day-old rabbits.

SERCA2 protein abundance

The abundance of SERCA2 protein was examined in PNS and microsomes from heart muscle and stomach smooth muscle from 2-day-old rabbits in Western blots with the monoclonal antibody IID8, which reacts with SERCA2a and SERCA2b (Figure 1A). Figure 1(B) shows the analysis of data from gels in four experiments similar to that shown in Figure 1(A). On the basis of this analysis, the relative intensity of the 115 kDa antibody reactivity band for PNS from the heart muscle (mean ± S.E.M.) was determined as being (119 ± 10)-fold that of the PNS from the stomach smooth muscle. Similarly for microsomes, the relative intensity in the heart muscle (mean ± S.E.M.) was (250 ± 22)-fold that of the stomach smooth muscle. Thus the heart muscle contained levels of SERCA2 protein that were 100–200-fold those of the stomach smooth muscle, depending on whether the PNS or microsome fractions were used for the analysis.

SERCA2 mRNA levels

The abundance of SERCA2 mRNA was examined in poly(A)$^+$ and total RNA with a riboprobe that reacted with SERCA2a and SERCA2b. Figure 2 is an autoradiogram of a Northern blot showing SERCA2 levels observed with different amounts of cardiac poly(A)$^+$ RNA and 3 μg of stomach poly(A)$^+$ RNA. On the basis of 17 values such as that derived from Figure 2, the SERCA2 mRNA abundance in the heart muscle was (29 ± 4)-fold that in the stomach smooth muscle. The gels with total RNA were stained with ethidium bromide (results not shown) and they showed two sharp bands representing 28 S and 18 S RNA, indicating that the total RNA samples had not been degraded. Northern blots from total RNA showed a much higher background than those with poly(A)$^+$ RNA (results not shown) and showed that the level of SERCA2 mRNA in the heart muscle was 39-fold that in the stomach smooth muscle.

Nuclear run-on assays

Nuclei were isolated from the heart muscle and the stomach smooth muscle; the rates of SERCA2 transcription *in vivo* were monitored with an unlabelled riboprobe (the same probe as used in the Northern blots) and a single-stranded plasmid DNA probe containing SERCA2 cDNA downstream of the riboprobe (see the Experimental section). In each experiment a matching number of nuclei was used for the heart muscle and stomach smooth-

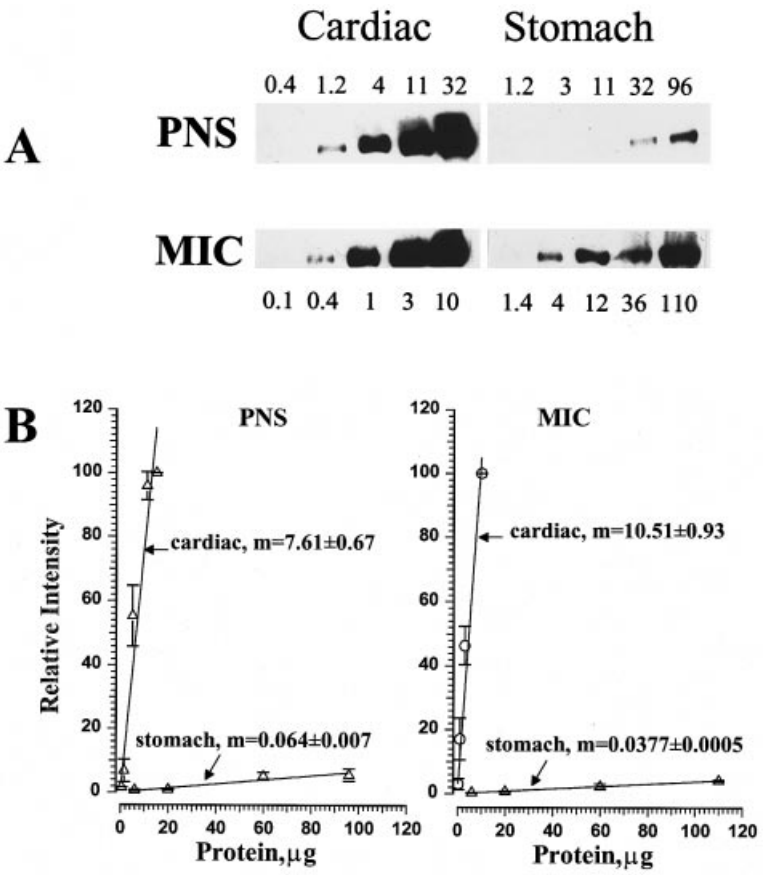

Figure 1 Estimation of SERCA2 protein

(**A**) Autoradiogram of a Western blot showing estimation of SERCA2 protein. The number along each lane represents the amount (in μg) of cardiac or stomach PNS or microsome (MIC) protein loaded in that lane. (**B**) Quantification of SERCA2 protein levels. Attenuance × number of pixels for each band and the attenuance of the background were determined from autoradiograms such as that shown in (**A**). In each PNS gel from a single animal, the relative densities were calculated by taking the value for the 15 μg band to be 100%, as follows: 100 × (attenuance of band − attenuance of blank) × pixel size of sample/[(attenuance of 15 μg PNS band − attenuance of blank) × pixel size of 15 μg PNS band]. Similarly, in each microsomes gel from a single animal, the relative densities were calculated by taking the attenuance of 10 μg of cardiac microsomes band to be 100%. The values shown are means ± S.E.M. for four animals. Best-fit lines by linear regression are shown.

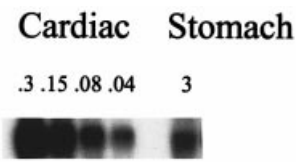

Figure 2 Autoradiogram of Northern blot showing estimation of SERCA2 mRNA

The indicated amounts (in μg) of cardiac muscle poly(A)$^+$ RNA and 3 μg of stomach smooth-muscle poly(A)$^+$ RNA were loaded in each lane.

muscle cells. The total amount of radioactivity incorporated in heart nuclei in eight experiments was (0.81 ± 0.34)-fold that obtained with stomach smooth-muscle nuclei. Thus the total rate of transcription in the two tissues did not differ significantly ($P > 0.05$). Matching counts for heart and stomach nuclei were used in each hybridization experiment to determine the SERCA2 transcription rates. Figure 3 shows dot-blots with the single-stranded DNA of recombinant pSK$^+$BS containing SERCA2 cDNA. The parent plasmid without the SERCA2 cDNA, used as a control, showed a very low intensity, indicating specificity of the signal. The rate of SERCA2 transcription in heart nuclei was (1.34 ± 0.3)-fold that obtained with stomach smooth-muscle nuclei (Figure 3). Figure 3 also shows an analysis from similar experiments in which the riboprobe was used to trap the transcripts. The rate of SERCA2 transcription in heart nuclei, on the basis of the riboprobe, was (0.96 ± 0.01)-fold that obtained with stomach smooth-muscle nuclei. Thus, with the use of two different types of probe, the rates of SERCA2 transcription in heart muscle and stomach smooth-muscle nuclei did not differ significantly ($P > 0.05$).

Polyribosome binding of SERCA2 mRNA

Total RNA was isolated from polyribosomal pellet and sub-polyribosomal supernatant from heart muscle and stomach smooth muscle, and analysed by using RT–PCR and dot-blots. For RT–PCR, the samples were analysed for different number of cycles with primers for SERCA2 and cardiac or smooth-muscle actin. Figure 4(A) shows the amplification of SERCA2 and actin from smooth muscle for different numbers of cycles. There was no product in lanes in which reverse transcription had not been

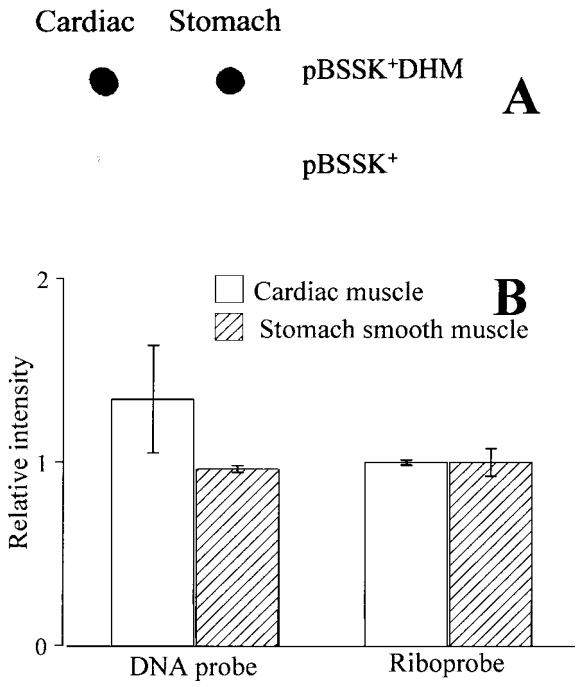

Figure 3 Nuclear run-on transcription

(**A**) Autoradiogram showing nuclear run-on transcription results. Matching numbers of nuclei (1.5×10^7) from cardiac muscle and stomach smooth muscle were used in the nuclear run-on assay with [α-^{32}P]CTP as described in the Experimental section. Radioactive RNA was isolated, suspended in 120 μl of buffer and the radioactivity was determined for both cardiac (1.09×10^5 c.p.m./μl) and stomach (1.12×10^5 c.p.m./μl) samples. Blots were hybridized with 2 μg of single-stranded DNA of pSK$^+$BSDHM4 or pSK$^+$BS with 3 ml of the RNA suspension containing matching amounts of radioactivity (3000 c.p.m./μl) for cardiac muscle and smooth-muscle nuclei. After hybridization, each membrane was washed at 20–23 °C in 2 × SSC/0.1% SDS for 15 min, at 65 °C in 0.1 × SSC/0.1% SDS for 30 min, and again at 20–23 °C in 2 × SSC/0.1% SDS for 15 min. (**B**) Quantification of nuclear run-on transcription levels. For each experiment the relative intensity for stomach was taken as 1 and the mean relative intensity for heart muscle was calculated. The calculation is also corrected for the attenuance of the blanks and the pixel size of the dots. The values shown are means ± S.E.M. for three experiments.

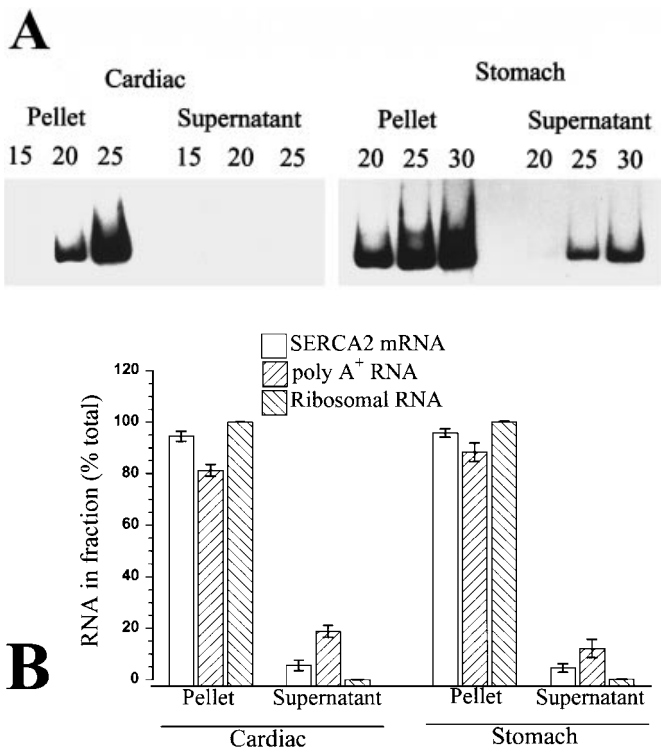

Figure 4 SERCA2 mRNA distribution in polysomal and subpolysomal fractions

(**A**) Autoradiograms showing RT–PCR of mRNA in polysomal pellet and subpolysomal supernatant of stomach smooth-muscle samples. Reverse transcription was performed by using 6 μl of each sample with Superscript II as described in the Experimental section. For amplifying SERCA2, 2 μl of the reverse transcript was amplified by PCR with the primers SR2L3370D and SR2L3018U, and the product of this PCR was amplified further for the specified number of cycles with the primers SR2L3370D and primer SR2U3064, to give a final PCR product of 306 bp. (**B**) Quantification of SERCA2 mRNA, poly(A)$^+$ RNA and total rRNA in polysomal pellet and subpolysomal supernatant. The values are means ± S.E.M. for five samples for SERCA2, and for three samples for poly(A)$^+$ and total RNA.

performed and only the PCR reaction had been conducted (results not shown). RT–PCR was performed on several preparations with the number of PCR cycles optimized for each preparation. The results are shown in Figure 4(B). Most of the SERCA2 mRNA and actin mRNA were in the pellets, indicating that both the mRNA species were translated very efficiently. There was no significant difference ($P > 0.05$) between (1) the percentage of SERCA2 mRNA in the pellet in heart muscle compared with stomach smooth muscle, (2) the percentage of actin mRNA in the pellet in heart muscle compared with stomach smooth muscle, (3) the percentage of SERCA2 compared with actin mRNA in the pellet in heart muscle and (4) the percentage of SERCA2 compared with actin mRNA in the pellet in stomach smooth muscle. These fractions were also analysed by using dot-blot analysis for SERCA2 mRNA, poly(A)$^+$ RNA and total RNA. The percentages of SERCA2 mRNA in the pellet were not significantly different ($P > 0.05$) in heart muscle compared with stomach smooth muscle (Figure 4B). The results obtained with the dot-blot were also consistent with those obtained with RT–PCR. For instance, heart SERCA2 mRNA in the pellet was 97.6 ± 0.6% in the PCR experiment and 94.5 ± 2.0% in the dot-blot experiment. An anti-sense oligonucleotide against 18 S RNA was used as a probe for total RNA. Whereas an exposure of 2 min was sufficient to detect the total RNA in the pellets, none was detected in the supernatants even after a 30 min exposure, indicating that fractionation resulted in the migration of all the ribosomes into the pellet. In contrast, a significant amount (12–19%) of the poly(A)$^+$ RNA (monitored with an oligo-dT probe) remained in the supernatant (Figure 4B), suggesting that not all of the poly(A)$^+$ RNA was associated with the ribosomes. Thus more of the SERCA2 or actin mRNA was associated with polyribosomes than some of the other mRNA species. Figure 5 shows the migration patterns of SERCA2 mRNA and rRNA in a sedimentation velocity experiment with sucrose density gradients. The migration patterns were very similar, suggesting that, on the basis of ribosome association, SERCA2 mRNA translation rates are similar in the two tissues.

DISCUSSION

The results presented here show that heart muscle and stomach smooth-muscle cells transcribe the SERCA2 gene at the same rate but there is significantly more SERCA2 mRNA in the heart muscle than in the stomach smooth muscle. The SERCA2 mRNA translation efficiencies are similar in the two tissues. This section focuses on the methods used and on the possible significance of these results.

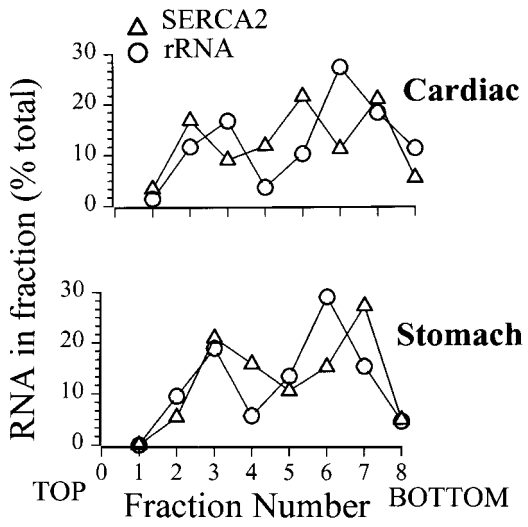

Figure 5 Distribution of SERCA2 mRNA and rRNA in polysomal continuous gradient fractions

Each conclusion presented here was based on results that used either different methods or more than one probe. Nuclear run-on assays were conducted with two probes: a riboprobe and a single-stranded cDNA probe. The two spanned slightly different regions of the SERCA2 gene and showed that the two tissues did not differ in their SERCA2 transcription rates. The results for translational efficiency measured by using RT–PCR or dot-blots are consistent with each other in that the major population of SERCA2 mRNA is translated. Further, the gradient experiments showed that there is no clear difference in the sedimentation patterns of the polyribosomes. This is the first study to compare the transcription and translation of SERCA2 in heart and smooth muscle *in vivo*. Consistently with this study, it was previously reported, on the basis of RT–PCR studies, that the levels of SERCA2 heteronuclear RNA are not different in the adult heart muscle and the stomach smooth muscle [27]. The higher levels of SERCA2 protein and mRNA were reported here for newborns but we and others have reported that adult heart expresses SERCA2 protein and mRNA at substantially higher levels than various smooth muscles [3,10,27,30].

On the basis of these results we conclude that the main control in expression leading to the differential expression of SERCA2 in the two tissues is post-transcriptional but pretranslational. Thus the surprising result is not that the heart contains more of the SERCA2 protein but how this is achieved. The simplest way to think about the control of SERCA2 expression is that heart has more sarcoplasmic reticulum and hence more SERCA2 protein can be inserted in it, and that in the stomach smooth muscle there is not as much sarcoplasmic reticulum and so the extra SERCA2 protein is degraded. However, our results suggest that it is the SERCA2 mRNA stability that controls the protein abundance. Energetically, it makes sense for it to happen; however, energetically it makes even more sense to have this control at the level of transcription so as not to waste resources in synthesizing mRNA that is going to be degraded anyway.

Because heart muscle expresses the splice SERCA2a and stomach smooth muscle expresses SERCA2b, the *cis*- and/or *trans*-acting factors controlling this expression might be splice-dependent or tissue-specific. The SERCA2 mRNA in various tissues represents four different splices that yield unique 3′-UTRs [5]. A 3′-UTR can regulate mRNA stability by diverse mechanisms [31,32]. RNA stability might depend on the length of the poly(A) tail, RNA binding to specific proteins and hairpin configurations in RNA structure [25,33,34]. Specific proteins can bind AUUUA repeats in the 3′-UTR of lymphokine mRNA and decrease its stability [35]. Similarly, binding of a 32 kDa protein to AU-rich domains in the 3′-UTR of granulocyte/macrophage colony-stimulating factor, c-Fos and c-Myc mRNA decreases their stability [36]. Phosphorylation of an RNA-binding protein by growth-factor-dependent protein kinase C stabilizes GAP43 mRNA [37]. The *cis*-acting AUUUA repeats are absent from SERCA2a and SERCA2b 3′-UTR. Therefore we do not know whether the control in mRNA stability depends on novel *cis*-acting elements in the splice variants and/or on tissue-specific *trans*-acting elements. We also do not know whether the site of mRNA degradation is cytoplasmic or nuclear. The 3′-UTR might also alter the nuclear export of RNA and the unexported RNA might be degraded in the nucleus or in the cytoplasm.

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REFERENCES

1 Burk, S. E., Lytton, J., MacLennan, D. H. and Shull, G. E. (1989) J. Biol. Chem. **264**, 18561–18568
2 Grover, A. K. and Khan, I. (1992) Cell Calcium **13**, 9–17
3 Wu, K. D., Lee, W. S., Wey, J., Bungard, D. and Lytton, J. (1995) Am. J. Physiol. **269**, C775–C784
4 Eggermont, J. A., Wuytack, F., De Jaegere, S., Nelles, L. and Casteels, R. (1989) Biochem. J. **260**, 757–761
5 Eggermont, J. A., Wuytack, F. and Casteels, R. (1991) Biochim. Biophys. Acta **1088**, 448–451
6 Lytton, J., Zarain-Herzberg, A., Periasamy, M. and MacLennan, D. H. (1989) J. Biol. Chem. **264**, 7059–7065
7 Zarain-Herzberg, A., Rupp, H., Elimban, V. and Dhalla, N. S. (1996) FASEB J. **10**, 1303–1309
8 Gunteski-Hamblin, A. M., Greeb, J. and Shull, G. E. (1988) J. Biol. Chem. **263**, 15032–15040
9 Khan, I. and Grover, A. K. (1990) Nucleic Acids Res. **18**, 4026
10 Khan, I., Spencer, G. G., Samson, S. E., Crine, P., Boileau, G. and Grover, A. K. (1990) Biochem. J. **268**, 415–419
11 Lytton, J. and MacLennan, D. H. (1988) J. Biol. Chem. **263**, 15024–15031
12 Verboomen, H., Wuytack, F., De Smedt, H., Himpens, B. and Casteels, R. (1992) Biochem. J. **286**, 591–595
13 Brooks, D. A. (1997) FEBS Lett. **409**, 115–120
14 Curatola, A. M., Nadal, M. S. and Schneider, R. J. (1995) Mol. Cell. Biol. **15**, 6331–6340
15 Pain, V. M. (1996) Eur. J. Biochem. **236**, 747–771
16 Saini, K. S., Summerhayes, I. C. and Thomas, P. (1990) Mol. Cell. Biochem. **96**, 15–23
17 Mertens, L., Van Den Bosch, L., Verboomen, H., Wuytack, F., De Smedt, H. and Eggermont, J. (1995) J. Biol. Chem. **270**, 11004–11011
18 Van Den Bosch, L., Mertens, L., Gijsbers, S., Heyen, M. V., Wuytack, F. and Eggermont, J. (1997) Biochem. J. **322**, 885–891
19 Ausubel, F. M. (1987) Current Protocols in Molecular Biology, vols. 1 and 2, John Wiley and Sons, New York
20 Boheler, K. R., Chassagne, C., Martin, X., Wisnewsky, C. and Schwartz, K. (1992) J. Biol. Chem. **267**, 12979–12985
21 Cheung, P., Panning, B. and Smiley, J. R. (1997) J. Virol. **71**, 1784–1793
22 Weissberg, P. L., Little, P. J., Cragoe, E. J. J. and Bobik, A. (1989) Circ. Res. **64**, 676–685
23 Finkle, T. J. and Grover, A. K. (1988) Biochem. Biophys. Res. Commun. **151**, 473–479
24 Adamou, J. and Bag, J. (1992) Eur. J. Biochem. **209**, 803–812

25 Bag, J. and Wu, J. (1996) Eur. J. Biochem. **237**, 143–152
26 Rairkar, A., Rubino, H. M. and Lockard, R. E. (1988) Nucleic Acids Res. **16**, 3113
27 Khan, I. and Grover, A. K. (1993) Cell Calcium **14**, 17–23
28 Ito, H., Miller, S. C., Akimoto, H., Torti, S. V., Taylor, A., Billingham, M. E. and Torti, F. M. (1991) J. Mol. Cell. Cardiol. **23**, 1117–1125
29 Harris, D. E., Warshaw, D. M. and Periasamy, M. (1992) Gene **112**, 265–266
30 Wuytack, F., Raeymaekers, L., Verbist, J., De Smedt, H. and Casteels, R. (1984) Biochem. J. **224**, 445–451
31 Carter, B. Z. and Malter, J. S. (1991) Lab. Invest. **65**, 610–621
32 Sachs, A. B. (1993) Cell **74**, 413–421
33 Heus, H. A. and Pardi, A. (1991) Science **253**, 191–194
34 Young, L. S., Dunstan, H. M., Witte, P. R., Smith, T. P., Ottonello, S. and Sprague, K. U. (1991) Science **252**, 542–546
35 DeMaria, C. T. and Brewer, G. (1996) J. Biol. Chem. **271**, 12179–12184
36 Rajagopalan, L. E. and Malter, J. S. (1996) J. Biol. Chem. **71**, 19871–19876
37 Perrone-Bizzozero, N. I., Cansino, V. V. and Kohn, D. T. (1993) J. Cell Biol. **120**, 1263–1270

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