Nucleotide Sequences of Avian Cardiac and Brain SR/ER Ca\(^{2+}\)-ATPases and Functional Comparisons with Fast Twitch Ca\(^{2+}\)-ATPase

CALCIUM AFFINITIES AND INHIBITOR EFFECTS

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Two similar forms of the cardiac/slow Ca\(^{2+}\)-ATPase (SERCA2a and SERCA2b), differing in sodium dodecyl sulfate-polyacrylamide gel electrophoresis mobility, are expressed in chicken heart and brain (Kaprielian, Z., Campbell, A. M., and Fambrough, D. M. (1989) Mol. Brain Res. 6, 55–60). In the current study, cDNAs encoding each form were cloned and sequenced. Chicken SERCA2a is 94% identical to its rabbit homologue, while SERCA2b has an extended carboxyl terminus with 38 of 49 amino acids identical to mammalian homologues. SERCA2b mRNA contains the SERCA2a encoding sequence within its 3'-untranslated region. Chicken genomic DNA sequence reveals that the alternate RNA splicing used to produce SERCA2a and SERCA2b subtypes involves a splice site within an exon. Tissue culture cells expressing the avian SERCA2a, SERCA2b, and SERCA1, each targeting to the endoplasmic reticulum, were used to measure Ca\(^{2+}\) affinities and inhibitor effects; no differences among the three pumps were detected.

The concentration of free Ca\(^{2+}\) ions in the cytosol is maintained at about 0.1 \(\mu M\) while the extracellular level is 10,000-fold higher. Cells tightly control their cytosolic calcium levels by extruding Ca\(^{2+}\) across the plasma membrane as well as by sequestering calcium in internal stores (for review see Bronner and Shamo, 1985). These internal stores of Ca\(^{2+}\) are filled around 110 kDa that pump Ca\(^{2+}\) into membrane-bound compartments. Mammalian SERCA2 is expressed as two different subtypes, SERCA2a, which predominates in cardiac and slow twitch skeletal muscle, and SERCA2b, which is found in a variety of tissues (Lytton and MacLennan, 1988, Guinetki-Hamblin et al., 1988, Lyttton et al., 1989, Eggermont et al., 1989). The two subtypes are identical except that the four carboxyl-terminal amino acids of SERCA2a are replaced by 49 or 50 different residues in SERCA2b. The significance of these two forms, generated from alternate splicing of primary transcripts from the same gene, is unknown.

If the different carboxyl termini found in mammals have functionally significant roles, one might expect this to be evolutionarily conserved. Based on immunological data, our laboratory found that two slightly different forms of SERCA2 are expressed in the chicken heart and brain (Kaprielian et al., 1989). One purpose of this study was to identify these different forms of chicken SERCA2 at the molecular level. Specifically, are the differences in the avian Ca\(^{2+}\)-ATPase subtypes homologous to the alternately spliced products seen in mammals? If so, are there any functional differences between the two alternate forms of SERCA2 (e.g. inhibitor sensitivity or Ca\(^{2+}\) affinity)?

MATERIALS AND METHODS

Enzymes and Radioisotopes—Restriction enzymes were purchased from Pharmacia LKB Biotechnology Inc., Amersham Corp., and New England Biolabs. Radioisotopes ([\(\alpha\)-\(\beta\)]dATP, [\(\alpha\)-\(\beta\)]dCTP, and deoxyadenosine [\(\beta\)]dATP) were purchased from Du Pont-New England Nuclear.

cDNA Library Construction and Screening—Total RNA was extracted from the heart and brain of one adult chicken in guanidine thiocyanate as described in Taborino and Fambrough (1990). The poly(A) RNA was converted to oligo (dT)-primed double-stranded cDNA, methylated, coupled with EcoRI linkers, ligated into lambdaZAP phage vector and packaged (Stratagene Cloning Systems). (However, based on examination of several different proteins encoding several different proteins, it has become apparent that the cDNA used to make this library had been incompletely methylated. Many clones terminate at internal EcoRI sites and some have unrelated sequences ligated adjacent to the clones of interest.) For screening the libraries, the coding region of rat stomach SERCA2a (Guinetki-Hamblin et al., 1988) was excised with PstI and isolated as described (Davis et al., 1986). The resulting probe was labeled with \(^{32}\)P by the method of Feinberg and Vogelstein (1983). Hybridization of probe to nitrocellulose filter lifts of the plated library was performed overnight in a solution of 120 mM Tris, pH 8, 600 mM NaCl, 4 mM EDTA, and 50% formamide at 68 °C. Forty and 20 positive clones from the brain and heart cDNA libraries, respectively, were rescreened with the same rat probe. Five clones from each library were isolated by an in vitro excision method involving the helper phage R408 (Stratagene Cloning Systems).

cDNA Sequencing and Analysis—All but the 107 5' most nucleotides (noncoding) of SERCA2 were sequenced on both strands by the dideoxyribonucleotide termination method (Sanger et al., 1977) with the U. S. Biochemical Corporation Sequenase kit. Some clones were sequenced after subcloning restriction fragments. Synthetic oligonucleotide sequencing primers were made on the 391 DNA Synthesizer (Applied Biosystems) and used to sequence other clones. By the procedure in Sambrook et al. (1989), nested deletions were produced.
with exonuclease III to sequence one clone. The GAP program of GCG (a software package from the University of Wisconsin) was used for computer comparisons of cDNA sequence similarity across species.

**RNA Blot Analysis**—Total RNA was isolated from adult and embryonic heart and brain tissues by the RNAzol B method (Cinna/Alzet, Inc.) and electrophoresed on a 1% agarose gel containing formaldehyde. Probe A in Fig. 3A was derived from the 1.3-kb EcoRI fragment of clone B13° and labeled with 3H. The remaining probes were generated by a PCR (Perkin-Elmer Cetus) procedure with primers which were designed to amplify specific regions of chicken SERCA2a. The primers are as follows: probe A, 5' primer ACTCTGGCTGGTGGAGC (an EcoRI linker plus bases 2788–2804) and 3' primer GGAATTCATATCACTAAAGTAG (an EcoRI linker plus bases 3101–3117). 1.5 pg of chicken genomic DNA were used as template in reaction conditions outlined above but with 0.5 M MgCl₂, 0.1% SDS, and 1% formaldehyde at room temperature) for 10 min and permeabilized with 0.5% Saponin. The cells were then incubated with a chicken-specific monoclonal antibody to SERCA2 (Cas-3H2, Kapoor and Fambrough, 1987) or SERCA1 (CaF3-5C3, Karin et al., 1989) followed by a rhodamine-conjugated goat anti-mouse secondary antibody and photographed on an epifluorescence microscope.

**Microsome Preparation**—As in Clarke, et al. (1990), twenty 150 × 25-mm dishes of transfected COS-1 cells were washed twice with 10 ml of phosphate-buffered saline, harvested in 80 ml of 5 mM EDTA in phosphate-buffered saline, and washed with 40 ml of phosphate-buffered saline. The cells were resuspended in 16 ml of 10 mM MOPS, pH 7.0, 0.5 mM MgCl₂, 20 KIU/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride and, after 10 min to allow for hypotonic swelling of cells, homogenized with Dounce homogenizer. The suspension was diluted with 0.5 M sucrose/2 mM mercaptoethanol/40 μM CaCl₂/300 mM KCl/20 mM MOPS, pH 7.0, and centrifuged at 18,000 × g for 20 min. The supernatant was adjusted to 800 mM KCl and centrifuged at 100,000 × g for 60 min. The pellet was resuspended with 50 mM MOPS, pH 7.0, 10% sucrose and frozen in liquid nitrogen for later use.

**Calcium Uptake Assay**—5.0 ml of the reaction mixture (20 mM MOPS, pH 7.0, 80 mM KCl, 5 mM MgCl₂, 5 mM sodium oxalate, 0.2 mM EDTA, 2.5 mM ATP, 0.2 mM CaCl₂ (with 0.4 μCi/ml ⁴⁰CaCl₂) and 0.1% BSA in microsomal membranes diluted in 2°C for 5 min. 1.0 ml aliquots were filtered (0.45-μm pore size; Millipore) and washed with 12 ml of 10 mM MOPS, pH 7.0, 2 mM CaCl₂. A liquid scintillation counter was used to determine amounts of ⁴⁰Ca in each aliquot. For the cyclopiazonic acid and thapsigargin studies, the inhibitors were present during the 5-min incubation period. For the calcium concentration-dependence studies, free calcium concentration was varied as estimated from total CaCl₂ and EGTA in solution (Fernandez-Belda et al., 1984). The data points in Figs. 6 and 7 represent averaged results obtained from two or three independent transfections and microsome preparations.

**RESULTS**

**cDNA Sequence and Analysis**—A cDNA probe encoding rat SERCA2a was used to screen chicken heart and brain cDNA libraries at high stringency (68 °C, 50% formamide). A number of overlapping cDNA clones from each library were excised *in vivo* (see "Materials and Methods") and sequenced to yield complete nucleotide sequences encoding chicken SERCA2a and SERCA2b. Clone B13° did not contain the complete coding sequence but it was of particular interest since it was the only clone isolated from the brain cDNA library which encoded the SERCA2a carboxyl terminus. No poly(A) tails were found in any of the clones, perhaps due to the presence of an EcoRI site between the polyadenylation signal and the poly(A) tail in combination with partial methylation of the cDNA during construction of both libraries (see Materials and Methods).

The nucleotide and deduced amino acid sequences of chicken SERCA2a are shown in Fig. 1A. The amino acid sequence is 94% identical to mammalian homologues (MacLennan et al., 1985, Lytton and MacLennan, 1988, and Guntski-Hamblin et al., 1988, Eggemont et al., 1989). Of the variant amino acids, conservative changes account for nearly half of the substitutions.

In addition to the cDNA clones that encode SERCA2a, the avian cDNA homologue of mammalian SERCA2b was also sequenced. The nucleotide and deduced amino acid sequences of SERCA2b are presented in Fig. 1A. The 44 additional carboxyl-terminal amino acids account for the difference in apparent molecular mass between SERCA2a and SERCA2b seen by SDS-PAGE (110 kDa). When the carboxyl terminus of avian SERCA2b is compared to homologous mammalian sequences, 38 of 49 amino acids are identical with six out of the 11 substitutions being conservative ones. Among

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2 The abbreviations used are: PCR, polymerase chain reaction; kb, kilobases; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MOPS, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; 3'-UT, 3'-untranslated; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ethylenebis(oxyethylenenitrilo)tetraacetic acid; TG, thapsigargin; CPA, cyclopiazonic acid; ER, endoplasmic reticulum; SR, sarcoplasmic reticulum; Clones beginning with the letter "B" are from the brain cDNA library while those with an "H" are derived from the heart library.
A

-120

Fig. 1. Nucleotide and deduced amino acid sequences of chicken SERCA2 cDNAs. Panel A, nucleotides are numbered on the left and amino acids on the right. Amino acids are identified by single letter abbreviations below their codons, stars denote stop codons, and consensus polyadenylation signals are underlined. The alternate splice site occurs at base 2980. The SERCA2b unique sequence is located between the two (V A) symbols and is absent from SERCA2a mRNA as a result of alternate splicing. The SERCA2a carboxyl terminus is written below its encoding sequence with the first base of the first codon located 5′ of the alternate splice site. Panel B, the diagram depicts mRNAs transcribed from SERCA2. The black boxes represent coding sequences of SERCA2 with open boxes indicating the 5′ portions. The dashed lines indicate the relationship of the SERCA2b unique sequence to the SERCA2a mRNA.

B

Fig. 1. Nucleotide and deduced amino acid sequences of chicken SERCA2 cDNAs. Panel A, nucleotides are numbered on the left and amino acids on the right. Amino acids are identified by single letter abbreviations below their codons, stars denote stop codons, and consensus polyadenylation signals are underlined. The alternate splice site occurs at base 2980. The SERCA2b unique sequence is located between the two (V A) symbols and is absent from SERCA2a mRNA as a result of alternate splicing. The SERCA2a carboxyl terminus is written below its encoding sequence with the first base of the first codon located 5′ of the alternate splice site. Panel B, the diagram depicts mRNAs transcribed from SERCA2. The black boxes represent coding sequences of SERCA2 with open boxes indicating the 5′ portions. The dashed lines indicate the relationship of the SERCA2b unique sequence to the SERCA2a mRNA.
the various chicken cDNA clones, there are three base substitutions occurring in the coding region. These changes do not alter the primary structure of the protein and are probably caused by two different alleles being expressed by a heterozygous animal used for construction of the libraries. The changes are from C1014 to A, T1097 to C and C7807 to A.

Previous publications of SERCA2 sequences (Lytton and MacLennan, 1988, Gunteski-Hamblin et al., 1988, Lytton et al., 1989, and Eggermont et al., 1989) had not shown the correct relationship between SERCA2a and SERCA2b mRNA. The fact that the 3'-UT region of SERCA2b message also contained the sequence which encodes the SERCA2a carboxyl terminus was not realized. But as shown in Fig. 1, A and B, the SERCA2a terminal encoding cDNA is downstream of the SERCA2b cDNA. In order to translate SERCA2a, the primary transcript must be spliced so that all of the SERCA2b unique sequence is excised. By removing the SERCA2b unique sequence, the encoding sequence of the four terminal amino acids of SERCA2a becomes contiguous with the bulk of the coding region, thus allowing SERCA2a translation. Therefore, primary transcripts of the SERCA2 gene contain the encoding sequences for both SERCA2a and SERCA2b. After processing the RNA, the 3'-UT region of SERCA2b mRNA still contains the SERCA2a-terminal encoding sequence while SERCA2a mRNA has had its SERCA2b unique portion excised.

mRNA Processing—To delineate exact intron/exon boundaries surrounding the chicken alternate splice site within the gene, PCR was employed to amplify a portion of the gene. The deduced gene structure and RNA splicing pattern are shown in Fig. 2. Primers were designed to amplify both the alternate splice site and the intron upstream of the SERCA2b unique sequence. The resulting PCR product was cloned and sequenced. Between nucleotides G2493 and A2504 is a 119-base pair intron* which begins with GT and ends in AG. An intron sequence at 4048 since the band detected with probe H has an apparent size 1 kb greater than that predicted for messages terminating at 4048. In RNA blots from both heart and brain, bands are evident when probe C was used but not probe D. These data show that for both SERCA2a and SERCA2b mRNAs, the AATAAA at 5158 is used as the predominant polyadenylation signal rather than the consensus sequence at position 5473. It is interesting to note that the rarely used polyadenylation signal sequence at 5473 is not present in homologous mammalian cDNAs. Heart transcripts occasionally use the signal sequence at 5473 (or at some position further downstream) since one cardiac cDNA clone (H14) was found to contain the sequence downstream of the predominantly used polyadenylation signal at base 5158. (A faint signal in the heart RNA lane was detected when probe D was used but was too faint to appear in the photograph.) There is no evidence that brain messages ever use the polyadenylation signal at nucleotide 5473. Probe E, an oligonucleotide which spans the alternate splice site and is specific for SERCA2a mature mRNA, hybridizes to RNA of similar size in both brain and heart lanes. This verifies that brain does express SERCA2a but at a much lower level than SERCA2b. The brain SERCA2a mRNA was not detected in panels A or C probably because of the low expression level and smeared signal. In summary, both brain and heart transcribe messages which predominantly use the polyadenylation signal at nucleotide 5158, though heart infrequently uses the polyadenylation signal at nucleotide 5473. SERCA2a mRNA was detectable in both brain and cardiac lanes while SERCA2b message was seen in brain RNA only.

Expression and Analysis of cDNA Clones in Tissue Culture—In order to examine functional differences among Ca" pumps, cDNAs encoding each SERCA subtype as well as a chicken SERCA1 were expressed in tissue culture (see "Material and Methods"). Full-length constructs encoding either SERCA2a, SERCA2b, or SERCA1 were transiently expressed in COS-1 cells. The transfected cells were fixed, permeabilized, and labeled with a chicken specific anti-SERCA2 or anti-SERCA1 monoclonal antibody and rhodamine-conjugated secondary antibody. High levels of expression were
obtained for all three avian Ca\textsuperscript{2+}-ATPases. An immunofluorescent staining pattern indicative of the endoplasmic reticulum was observed. This is best seen at the thin edges of cells as shown in Fig. 4, A-C. These results show that SERCA1 and the SERCA2 subtypes are capable of targeting to the appropriate organelle when transfected into non-muscle tissue cultured cells. Microsomes made from similarly transfected cells were analyzed by SDS-PAGE and immunoblots. Using protein blots from 6% polyacrylamide gel electrophoresis and probing with avian-specific monoclonal antibodies, it was possible to demonstrate clearly the expressed avian Ca\textsuperscript{2+}-ATPases (Fig. 4D).

To ensure that the ER localization was not merely due to accumulation of misfolded protein, microsomes of cells transfected with SERCA1, SERCA2a, or SERCA2b were assayed for their ability to sequester \textsuperscript{45}Ca\textsuperscript{2+}. Fig. 5 shows that all three pumps are functional. The apparent lower activity of SERCA2a is due to lower yields of SERCA2a protein/milligram of total microsomal protein (see Fig. 4D). Equal amounts of total microsomal protein were analyzed by immunoblots to quantify relative amounts of SERCA2a and SERCA2b. There is 1.7-fold less SERCA2a than SERCA2b in the respective microsomes (data not shown). Therefore, with a factor of 1.7 to correct for the lower expression of SERCA2a, all three pumps sequester about 1100 nmol of Ca\textsuperscript{2+}/mg protein/h. This is an order of magnitude greater than the rate of Ca\textsuperscript{2+} sequestration by microsomes from nontransfected COS-1 cells or cells transfected with SERCA2a cDNA cloned into the expression vector in the reverse orientation.

There are two toxins reported to be SR/ER Ca\textsuperscript{2+} pump inhibitors. Both thapsigargin (TG, Thastrup et al., 1990) and cyclopiazonic acid (CPA, Seidler et al., 1989) are believed to act upon SERCA-type ATPases but not the plasma membrane calcium pumps. When calcium uptake was measured for SERCA2a, SERCA2b, and SERCA1 over a range of inhibitor concentrations, no significant differences were detected in the sensitivity of the three isoforms to the inhibitors (Fig. 6).

Finally, the apparent Ca\textsuperscript{2+} affinity for each isoform was determined. Equal amounts of microsomes were incubated with varying free Ca\textsuperscript{2+} concentrations. There was no appreciable difference in the Ca\textsuperscript{2+} activation patterns of the Ca\textsuperscript{2+}-ATPases as shown in Fig. 7.

**DISCUSSION**

Previous work has shown that two subtypes of SERCA2 with different $M_\text{r}$ are expressed in the chicken (Kaprielian et al., 1989). This paper demonstrates that the difference is due to alternate splicing at an intronexonic donor site in the primary transcript. SERCA2a can only be expressed when a splice site donor, which occurs within the exon coding for the carboxyl terminus of SERCA2b, is used for RNA processing. Only SERCA2a was detected in heart, while both forms of SERCA2 were expressed in brain with SERCA2b being the
predominant form. This means that the internal RNA splice site donor is used much less often in brain and that the carboxyl-terminal coding sequence of SERCA2a usually appears within the 3'-UT region of SERCA2b mRNA. A similar, though more complex, splicing pattern of RNA has recently been reported for mammals (Piessens et al., 1991). Unlike mammalian SERCA2 mRNA expression, there are only two forms of avian SERCA2 mRNA. The only detected SERCA2b mRNA always contained within its 3'-UT region the SERCA2a terminal encoding nucleotides. Therefore, alternate splicing via an internal donor site appears to be the mechanism to produce alternate carboxyl termini in avian as well as mammalian SERCA2.

When SERCA2a and SERCA2b were expressed in COS-1 cells, the Ca\(^{2+}\)-ATPases were targeted to the endoplasmic reticulum as evident by the immunofluorescent staining pattern (Fig. 4, A-C). This localization is not due to accumulation of misfolded protein since similarly transfected cells synthesized functional enzymes. In the photomicrographs, there is some punctate staining in addition to the reticular network. This could be due to incomplete fixation and vesiculization of the ER and/or capping of the Ca\(^{2+}\)-ATPases within the ER. The antibody's epitope is glutaraldehyde and methanol sensitive so other fixation protocols were unsuccessful. The possibility of lateral mobility within the ER of SERCA2 proteins is under investigation.

We have compared the expression and activities of the three chicken isoforms in a number of ways. When analyzed by immunoblotting (Fig. 4D), the bands in the SERCA2 lanes appear as broad bands. These data, in addition to some preliminary data, suggest that the calcium pump might be a glycoprotein. Functionally, the three Ca\(^{2+}\)-ATPases are very similar in their sensitivity to Ca\(^{2+}\) as an activator and to CPA and TG as inhibitors. Since SERCA2a and SERCA2b differ only at their carboxyl termini, it is not surprising that the ATPases are indistinguishable in their apparent Ca\(^{2+}\) affinities and inhibitor sensitivities. Although there is a 15% amino acid sequence difference between SERCA1 and SERCA2, the similar effects of TG and CPA suggest that neither inhibitor interacts with isoform-specific residues. By comparing primary sequences and pharmacological sensitivities of SERCA-type pumps from a variety of species, it may be possible to predict which regions interact with TG and CPA.

In order to understand Ca\(^{2+}\)-ATPases more fully, it is helpful to compare primary sequences across a wide range of species. Chicken SERCA2a is 94% identical to its mammalian homologue while the carboxyl terminus of SERCA2b is also highly conserved. A series of mutagenesis studies has furthered our understanding of the structure/function relationship (e.g. Clarke et al., 1989a, 1989b; Maruyama et al., 1989; Vilsen et al.; 1989, Andersen et al., 1989; Clarke, et al., 1990). Of the residues shown by other laboratories to be required for the function of the Ca\(^{2+}\)-ATPase, all are completely conserved in chicken SERCA2. There is no evidence which shows that the carboxyl terminus of a calcium pump is vital for function and yet diverse species have conserved, through millions of years, alternate SERCA2a and SERCA2b termini. It remains to be determined why there is a selective advantage for birds and mammals to retain multiple isoforms of the Ca\(^{2+}\)-ATPase.

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REFERENCES


Fig. 7. Calcium concentration dependence of initial rates of calcium uptake. Reaction conditions are the same as used in Fig. 6 except the concentration of free calcium was varied as described under "Materials and Methods." The results are standardized as fraction of maximal rate and plotted against free calcium concentration. The curves are labeled as follows: A, SERCA2a; O, SERCA2b; C, SERCA1. Experimental points were fitted with the Hill equation, assuming 4.17 x 10^{-7} M for the Ca^{2+} concentration yielding half-maximal activation.

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