Molecular Cloning of the Mammalian Smooth Muscle Sarco(endo)plasmic Reticulum Ca\(^{2+}\)-ATPase*

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We have isolated and sequenced full-length cDNA clones from a rabbit uterine library which encode the smooth muscle sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase. These cDNAs resulted from an alternative splice of the cardiac/slow-twitch Ca\(^{2+}\)-ATPase gene transcript, and encoded a protein identical to rabbit cardiac/slow-twitch Ca\(^{2+}\)-ATPase except for the replacement of the carboxyl-terminal four amino acids with an extended and relatively hydrophobic sequence of 49 amino acids. This cDNA was virtually identical to the alternatively spliced product of the cardiac/slow-twitch Ca\(^{2+}\)-ATPase gene recently identified in human kidney (Lytton, J., and MacLennan, D. H. (1988) J. Biol. Chem. 263, 15024-15031) and rat non-muscle tissues (Gunter-Hamblin, A.-M., Greed, J., and Shull, G. (1988) J. Biol. Chem. 263, 15032-15040).

S1 nuclease mapping of total cellular RNA from a variety of tissues demonstrated that cardiac muscle expressed the cardiac/slow-twitch isoform almost exclusively, most smooth muscle and non-muscle tissues expressed the alternatively spliced smooth/non-muscle isoform almost exclusively, and a few tissues expressed both isoforms in varying amounts. Thus, regulation of alternative splicing of the cardiac/slow-twitch Ca\(^{2+}\)-ATPase gene transcript is tissue-specific. The expression of the smooth/non-muscle isoform in every tissue tested supports the hypothesis that this molecule represents the "housekeeping" endoplasmic reticulum Ca\(^{2+}\)-ATPase.

Intracellular calcium plays an essential role in excitation-contraction coupling of smooth muscle, as it does in striated muscle, since contraction can occur in vitro in the virtual absence of extracellular calcium (Bond et al., 1984). Excitation-contraction coupling in smooth muscle can be initiated by plasmalemmal depolarizations (electromechanical coupling), as well as by various chemical agonists which do not induce concomitant membrane depolarization (pharmacomechanical coupling) (Castells, 1980). The latter group of agents also induce the rapid formation of inositol 1,4,5-trisphosphate (Baron et al., 1984; Hashimoto et al., 1986), which has been shown to release calcium from intracellular stores in both smooth muscle (Somlyo et al., 1985) and other cell types (Berridge and Irvine, 1984), and may well be the physiological messenger of pharmacomechanical excitation-contraction coupling in vivo (Somlyo, 1985). Depolarization, on the other hand, does not induce phosphatidylinositol turnover or inositol 1,4,5-trisphosphate production (Baron et al., 1984) and presumably results in calcium release through a different mechanism, perhaps similar to excitation-contraction coupling in striated muscle.

As an interesting parallel to these two mechanisms of excitation-contraction coupling, Iino and colleagues (Iino, 1987; Iino et al., 1988) have recently described two intracellular pools of calcium in smooth muscle. One pool can be released only by inositol 1,4,5-trisphosphate, whereas the other can be released either by inositol 1,4,5-trisphosphate or by caffeine. The caffeine-induced release has properties (including ryanodine sensitivity) similar to calcium release through the channel of striated muscle sarcoplasmic reticulum terminal cisternae. The calcium release channel has recently been shown to be the ryanodine receptor and the "foot structures" visualized as spanning the junctional space in triad preparations (Imura et al., 1987; Imagawa et al., 1987; Hynce et al., 1988; Lai et al., 1988; Saito et al., 1988).

It is tempting to speculate that independent calcium stores reside in different sub-specializations of the sarco(endo)plasmic reticulum. The smooth muscle sarco(endo)plasmic reticulum has been shown to be the major site of intracellular calcium storage using electron probe X-ray microanalysis (Somlyo, 1985b). Morphologically, it can be separated into a central region and a peripheral region in close apposition to the plasmalemma. The peripheral region appears to contain an electron-dense luminal content reminiscent of calsequestrin, as well as a sarco(endo)plasmic reticulum-to-surface coupling analogous to the foot structures of striated muscle terminal cisternae (Somlyo and Franzini-Armstrong, 1985). In fact, smooth muscle sarco(endo)plasmic reticulum has been demonstrated to contain molecules very similar, if not identical, to cardiac calsequestrin and phospholamban (Wuytack et al., 1987; Raeymaeckers and Jones, 1986; Ferguson et al., 1988).

In addition to these well characterized molecules of striated sarcoplasmic reticulum, smooth muscle has also been shown to contain two Ca\(^{2+}\)-ATPase molecules. One is localized in the plasmalemma and is clearly identical to the calmodulin-regulated calcium pump best characterized in erythrocytes.
(Niggli et al., 1979); the other is a molecule which bears structural and immunological similarity to the Ca\textsuperscript{2+}-ATPase of cardiac sarcoplasmic reticulum (Eggermont et al., 1988; Wuytack et al., 1984). Recently de la Bastie et al. (1988) have demonstrated that nucleotide probes from the cardiac Ca\textsuperscript{2+}-ATPase are partially protected from S1 nuclease digestion by smooth muscle mRNA. This suggested the possibility that the sarc(o)endoplasmic reticulum Ca\textsuperscript{2+}-ATPase message expressed in smooth muscle could be an alternatively spliced product of the cardiac/slow-twitch Ca\textsuperscript{2+}-ATPase gene. The data of de la Bastie et al. (1988) indicated that the point of difference would be close to the carboxyl terminus of the encoded protein.

We and others (Lytton and MacLennan, 1988; Gunteski-Hamblin et al., 1988) have recently demonstrated by cDNA cloning that the non-muscle endoplasmic reticulum Ca\textsuperscript{2+}-ATPase is identical to the cardiac/slow-twitch enzyme except for an extended carboxyl-terminal end which results from alternative splicing. In order to determine precisely which form(s) of the sarco(endo)plasmic reticulum enzyme are expressed in smooth muscle, we have isolated and characterized clones from a rabbit uterine smooth muscle cDNA library. The phenotypic expression of these clones was analyzed in several smooth muscle and non-muscle tissues by S1 nuclease protection.

MATERIALS AND METHODS

RNA Isolation—Various tissues from 7- to 10-week-old male New Zealand rabbits were frozen in liquid nitrogen immediately following dissection from the animals. The uterus from a gravid rabbit of 30 days gestation was removed and placed in a sterile saline solution. Total cellular RNA was isolated from uterine total RNA by the oligo(dT) cellulose (Pharmacia LKB Biotechnology Inc.) column procedure (Aviv and Leder, 1972).

Library Construction—cDNA was synthesized from 10 µg of poly(A)* mRNA using a cDNA synthesis kit obtained from Pharmacia LKB Biotechnology Inc. Following the addition of the EcoRI adapters, one-half of the cDNA was separated by 1% agarose gel electrophoresis, and the regions corresponding to 2-5 and 5-10 kilobase pairs were excised and electroeluted using an apparatus purchased from IBI Inc. The eluate was precipitated with ethanol, phenol/chloroform-extracted, and re-prepared. An aliquot of the unfractionated cDNA (~100 ng), all of the 2-5-kilobase pair fraction (~100 ng) and all of the 5-10-kilobase pair fraction (~40 ng) were ligated overnight at 13 °C with 1 µg of XZAP arms (Stratagene Cloning Systems, La Jolla, CA) using 4 units of T4 DNA ligase (Pharmacia LKB Biotechnology Inc.) in a final volume of 5 µl. Each sample was packaged using a single Gigapack Plus reaction (Stratagene Cloning Systems) and plated on BB4 cells, resulting in libraries of 500,000, 250,000, and 70,000 independent clones, respectively. The unfractionated library was screened directly, whereas the other two were amplified once.

Screening—The unfractionated smooth muscle cDNA library was screened at low stringency using nucleotide probes from the rabbit fast-twitch skeletal muscle and cardiac/slow-twitch muscle Ca\textsuperscript{2+}-ATPase cDNAs as described by Lytton and MacLennan (1988). Full-length clones were obtained by screening both size-fractionated libraries at high stringency using a probe from the rabbit cardiac/slow-twitch Ca\textsuperscript{2+}-ATPase cDNA (MacLennan et al., 1985; Brandi et al., 1987; nucleotides 218-0). The XZAP system allows easy recovery of the entire cloned insert in the Bluescript vector following co-infection of XL-1 Blue cells with the XZAP phage containing the clone and with the helper phage, R408.

A rabbit brain cDNA library in Agt1, purchased from Clontech Laboratories Inc., Palo Alto, CA, was screened sequentially, as described by Lytton and MacLennan (1988), first at low stringency with a nucleotide probe derived from the alternatively spliced 3' end of clone HK1 (Lytton and MacLennan, 1988; nucleotides 3253-3952); then, following removal of radioactive filters, at a high stringency with a nucleotide probe from the rabbit cardiac/slow-twitch Ca\textsuperscript{2+}-ATPase cDNA (MacLennan et al., 1985; nucleotides 2155-2222). A screen of 250,000 plaques yielded a total of four clones, each of which was positive with probe HK1. Three of the clones had lost one of the EcoRI sites used to insert the cDNA into the vector and could not be subcloned. The fourth had a single insert of ~2.4 kilobase pairs which was subcloned into the pTZ vectors.

All probes were labeled using the random primed synthesis method of Feinberg and Vogelstein (1983), either with [\textsuperscript{32}P]dCTP or [\textsuperscript{35}S]dATP with modified T7 polymerase obtained in kit form, either from USB Inc. (Sequenase) or from Pharmacia LKB Biotechnology. The sequencing strategies for the various clones are shown in Fig. 1.

Nucleic Acid Sequencing—Fractions of the different clones obtained by suitable restriction endonuclease digestions were digested into the pTZ vectors. Additional fragments were generated using the exonuclease III/mung bean nuclease kit from Stratagene Cloning Systems. Sequencing was performed on single-stranded templates by the deoxyribonucleotide chain termination procedure of Sanger et al. (1977) using either [\textsuperscript{35}S]dCTP or [\textsuperscript{32}P]dATP with modified T7 polymerase obtained in kit form, either from USB Inc. (Sequenase) or from Pharmacia LKB Biotechnology. The sequencing strategies for the various clones are shown in Fig. 1.

S1 Nuclease Protection Assay—This was performed according to a modification of the method of Berk and Sharp (1977). Briefly, 30 µg of total cellular RNA were dissolved in 22 µl of hybridization buffer (80% formamide, 1 mM EDTA, 1% sodium dodecyl sulfate, 10 mM Tris-Cl, pH 7.4), heated to 65 °C, and combined with 3 µl (~106 cpm) of heat-denatured, single-stranded probe in hybridization buffer. NaCl was added to a final concentration of 0.4 M from a 5 M stock, the reaction was cooled slowly to 42 °C, and allowed to anneal at that temperature overnight. The annealed complexes were then digested with 300 µl of 150 mM NaCl, 3 mM ZnSO\textsubscript{4}, 30 mM sodium acetate, pH 5, containing 200 units of S1 nuclease (Boehringer Mannheim, Canada), and digestion was allowed to proceed for 60 min at 25 °C. The reactions were stopped with 33 µl of 0.1 M EDTA and ethanol precipitated. The pellets were dissolved in 15 µl of 80% formamide, 1 mM EDTA, 0.5% sodium deoxycholate, heated for 5 min in a boiling water bath, and separated on a 5% polyacrylamide/area sequencing style gel. The DNA fragments used for the S1 analysis were probe A (5359 bp) from the cardiac/slow-twitch sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase (MacLennan et al., 1985); probe B (5361 bp) from clone M1bBr4; and probe C, NolI(4)-NolI(660) from the cardiac/slow-twitch sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase (MacLennan et al., 1985). Probe D, EcoRI-linker(517)-NolI(3) from the 5'-untranslated region of the cardiac/slow-twitch sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase cDNA clone XCA,\textsuperscript{1} (see Figs. 1 and 2). Probes A and B were labeled at the 5'-hydroxyl ends using terminal transferase (Boehringer Mannheim Biochemicals) and d-\textsuperscript{32}P-cystidine (Du Pont-New England Nuclear); Probes C and D were labeled at the 5'-hydroxyl using T4 polynucleotide kinase (Bethesda Research Laboratories) and \textsuperscript{32}P-PATP (Du Pont-New England Nuclear). The noncoding strand of each probe was prepared by electroelution from a strand-separating gel as described by Smith and Birnstiel (1976).

RESULTS

A recent report by de la Bastie et al. (1988) suggested that an alternatively spliced version of the cardiac/slow-twitch Ca\textsuperscript{2+}-ATPase is expressed in smooth muscle. We were interested both in the structure of this spliced product and in identifying any other candidate molecules for the sarco(endo)plasmic reticulum Ca\textsuperscript{2+}-ATPase of smooth muscle. We constructed a cDNA library from rabbit uterus. All efforts were made to remove adventitious tissue from this smooth muscle preparation (see “Materials and Methods”). However, the presence of a small amount of vascular tissue cannot be discounted. As a result, although this library is predominantly of uterine smooth muscle origin, it may contain 1\textsuperscript{1} The abbreviations used are: dCTP, deoxycytidine 5'-\textsuperscript{32}P thiotriphosphate; dATP, deoxyadenosine 5'-\textsuperscript{32}P thiotriphosphate.
2 A Zarain-Herzberg, D. H. MacLennan, and M. Periasamy, manuscript in preparation.
tain some clones derived from vascular smooth muscle. The library was screened with nucleotide probes from the rabbit sarcoplasmic reticulum Ca\(^{2+}\)-ATPase cDNA, under conditions of reduced stringency, in order to identify any similar, cross-reactive clones.

A screen of 500,000 independent, unamplified plaques yielded 20 positive signals, of which eight were false positives. The remaining clones were divided into four groups, containing six, four, one, and one members, on the basis of detailed analysis by Southern blotting, restriction endonuclease digestion, and nucleic acid sequencing (data not shown). The class with four members contained different length clones for the Na\(^+\),K\(^+\)-ATPase \(\alpha\) subunit. One of the classes with a single member corresponded to a partial length clone from the 3′ end of the cardiac/slow-twitch sarcoplasmic reticulum Ca\(^{2+}\)-ATPase isoform. The other class with only a single member remains unidentified.

A full-length version of the most abundant class of cDNAs was isolated from the libraries constructed from size fractionated cDNA (see Fig. 1). Nucleotide sequencing of the full-length clone displayed a molecule identical to the cardiac/slow-twitch sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (MacLennan et al., 1985) from the 5′ end to nucleotide 2980, at which point the sequences diverged completely, suggesting an alternative splice. This cDNA encoded a protein identical with the one from cardiac/slow-twitch muscle, except for the replacement of the carboxy-terminal four amino acids with an extended sequence of 49 amino acids (see Fig. 2). A very similar molecule was recently described using molecular cloning techniques from rat non-muscle tissues (Gunterski-Hamblin et al., 1988) and from human kidney (Lyton and MacLennan, 1988). Through analysis of the human gene, we demonstrated that the major transcript from human kidney (which encodes the unique carboxyl terminus of the non-muscle isoform) is generated from the cardiac/slow-twitch Ca\(^{2+}\)-ATPase gene by the retention of an exon which is replaced with an alternative exon in the major transcript generated in cardiac muscle or slow-twitch muscle (Lyton and MacLennan, 1988).

In order to demonstrate unequivocally that the smooth muscle Ca\(^{2+}\)-ATPase isozone was the same one expressed in rabbit non-muscle tissues, we isolated clones from a rabbit brain library. Four clones were identified, all of which hybridized to both a probe from the cardiac/slow-twitch Ca\(^{2+}\)-ATPase coding region and to a probe from the 3′-untranslated region of HK1. One of these cDNAs (ArBrBr4) was subcloned and sequenced and demonstrated to be identical to nucleotides 1444–3885 of the uterine clones (see Figs. 1 and 2).

Fig. 3 shows a comparison of the deduced amino acid sequence from the alternatively spliced carboxy-terminal tail of rabbit, rat, and human smooth/non-muscle Ca\(^{2+}\)-ATPases. Although the level of nonidentity is low (10 out of 50 differences between rabbit and rat, 3 between rat and human, and 10 between rabbit and human), this is still much higher than in the remainder of the molecule common between the cardiac/slow-twitch and smooth/non-muscle isoforms (8 out of 994 differences between rabbit and rat, 7 between rat and human, and 6 between rabbit and human). On the other hand, most of these changes are conservative replacements.

In order to assess the true level of expression of message for either isoform of the cardiac/slow-twitch Ca\(^{2+}\)-ATPase gene, we performed S1 analyses on total RNA isolated from a variety of smooth muscle and non-muscle tissues, as well as from slow-twitch skeletal and cardiac muscles. Figs. 4 and 5 show the results of S1 nuclease digestion using probes from the alternative splice site region of cardiac/slow-twitch muscle (probe A) and smooth/non-muscle (probe B) isoforms, respectively. As expected, probe A was protected for its full-length when hybridized with either slow-twitch or cardiac muscle RNA, although low levels of a 363-nucleotide protected band were also visible. However, this 363-nucleotide band was the predominant protected species in non-muscle tissues and various smooth muscle types. Esophageal was the only tissue other than cardiac and slow-twitch muscle in which the fully protected species was more abundant than the partially protected form. Since it is known that esophageal tissue contains slow-twitch skeletal muscle fibres as well as smooth muscle (Nagai et al., 1985), this result is not surprising.

The partially protected fragment (363 nucleotides) observed with probe A corresponds to an RNA species which differs from the cardiac/slow-twitch muscle Ca\(^{2+}\)-ATPase at the point where the smooth/non-muscle Ca\(^{2+}\)-ATPase sequence diverges from the cardiac/slow-twitch one. If, in fact, this species corresponds to the alternatively spliced smooth/non-muscle isoform, then probe B should show full protection. This is confirmed by the data of Fig. 5, where all smooth and non-muscle RNAs (except esophagus) reveal predominantly the fully protected species. Conversely, one would predict that in cardiac muscle, slow-twitch muscle, and esophagus (where the fully protected probe A is most abundant) hybridization

![Fig. 1. Partial restriction endonuclease map and sequencing strategies for the cDNA clones obtained from rabbit uterus and brain libraries.](attachment://image.png)

- **Fig. 1.** Partial restriction endonuclease map and sequencing strategies for the cDNA clones obtained from rabbit uterus and brain libraries. A linear composite map of the cDNA is shown, with the coding region indicated by the open box and the positions of several diagnostic restriction endonuclease sites marked. The vertical arrow at about 3 kilobase pairs indicates the point after which sequence in the 3′ direction is different from that of the cardiac/slow-twitch Ca\(^{2+}\)-ATPase isozone (MacLennan et al., 1985). Each of the separate clones analyzed is shown by a solid bar below the map. RBU denotes a clone isolated from rabbit uterus, RbBr, from rabbit brain. The direction and extent of sequencing of each clone is indicated by the horizontal arrows.
Fig. 2. The complete sequence of the smooth muscle Ca²⁺-ATPase clone. The composite nucleotide sequence is shown above its translation. Numbering in the positive direction begins with the first nucleotide in the in-frame initiation codon. Clone \( xRuB2-1 \) extends from nucleotide -192 to -391; clone \( xRuB6 \) from about nucleotide 250-384; clone \( xRuB4 \) from nucleotide 1444-3885. The nucleotide sequence is identical to that reported by MacLennan et al. (1985) and Brandi et al. (1987) from the 5' end up to nucleotide 2980, after which it differs completely. The vertical arrow indicates the point of divergence from the cardiac/slow-twitch clone. The asterisk and underline indicate a few allelic variations. Our uterine clones substitute a G at position -168 where the cardiac/slow-twitch clone has a C, and a G for a T at position 1137. In addition, clone \( xRuB4 \) substitutes at position 2469 where both uterine and cardiac/slow-twitch clones have an A and a T at position 3112 where the uterine clone has a C. None of these substitutions results in an amino acid replacement.
**DISCUSSION**

We have used molecular cloning techniques to demonstrate that the sarco(endo)plasmic reticulum Ca²⁺-ATPase of smooth muscle is identical to the alternatively spliced cardiac/slow-twitch Ca²⁺-ATPase gene product expressed in non-muscle tissues (Lytton and MacLennan, 1988; Gunteski-
Hamblin et al., 1988). Two lines of evidence support the view that an alternatively spliced smooth/non-muscle transcript of the cardiac/slow-twitch Ca\(^{2+}\)-ATPase gene is the predominant Ca\(^{2+}\)-ATPase message expressed in a variety of smooth muscle types. In the first place, we have screened our uterine library exhaustively and have isolated many cDNAs encoding the smooth/non-muscle isoform, a single clone of the cardiac/slow-twitch isoform, and no other Ca\(^{2+}\)-ATPase candidates. If another gene product or isoform had been expressed in either uterine smooth muscle or in the vascular smooth muscle which was present in the tissue used to prepare the library, it would have appeared in these screens under the low stringency conditions which were used. Second, we have used an S1 nuclease digestion assay to demonstrate that the mRNA expressed in all of the major smooth muscle types and in several other non-muscle tissues protects probes from the two alternatively spliced isoforms in a ratio greatly favoring the smooth/non-muscle isoform over the cardiac/slow-twitch isoform.

From these data it is clear that the alternative splicing event which generates cardiac/slow-twitch or smooth/non-muscle isoforms of the Ca\(^{2+}\)-ATPase is regulated in a tissue-specific fashion. Thus, cardiac and slow-twitch muscles express the cardiac/slow-twitch isoform almost exclusively, esophagus, brain, testis, aorta, and urinary bladder express both isoforms in varying amounts, and the remainder of the tissues examined express the smooth/non-muscle isoform almost exclusively. It remains unclear, however, whether both isoforms are expressed within the same cell, since non-muscle tissues are, in general, composed of many different cell types. Although we have delineated the arrangement of the exons which give rise to the alternatively spliced products of this gene in human tissue (Lytton and MacLennan, 1988) this still gives no clue how the event is regulated.

It is also unclear whether the low relative expression of the smooth/non-muscle form compared to the cardiac/slow-twitch form in cardiac and slow-twitch muscles is physiologically significant. This pattern might be explained by contamination of preparations from these tissues with vascular smooth muscle and non-muscle cells (fibroblasts). It should be noted, however, that the absolute expression of the smooth/non-muscle form in cardiac and slow-twitch muscle is actually not much different from that in smooth muscle or non-muscle tissues. By virtue of the universal nature of its expression, one might hypothesize that the smooth/non-muscle isoform represents an endoplasmic reticulum housekeeping enzyme, cardiac and skeletal muscles having evolved new isoforms of the enzyme to satisfy their special requirements for calcium regulation during muscle contraction. This being the case, it is interesting that smooth muscle uses the housekeeping enzyme for regulation of contraction rather than a separate isoform. It is possible that the vastly slower kinetics of smooth muscle contraction compared to cardiac or skeletal muscle contraction obviate the need for a specialized system.

The functional significance of the extended carboxyl terminus of the smooth/non-muscle Ca\(^{2+}\)-ATPase isoform is equally unclear at this time. It is interesting to note, however, that phospholamban, a phosphorylated sarcoplasmic reticulum-associated protein (Tada et al., 1974), is expressed in cardiac, slow-twitch skeletal and smooth muscles (Jorgensen and Jones, 1987; Jorgensen and Jones, 1986; Raeymaekers and Jones, 1986). Phospholamban is thought to mediate the regulatory effects of catecholamines and other agonists on calcium pumping through a direct interaction with the Ca\(^{2+}\)-ATPase (Inui et al., 1986; Kirchberger et al., 1986; Suzuki and Wang, 1986), and, indeed, the effects of cAMP on calcium pumping have been observed in smooth muscle (Nishikori and Maeno, 1979; Saida and Van Bremman, 1984). Since cardiac muscle and smooth muscle express different isoforms of the calcium pump, the interaction of phospholamban with the enzyme must not be influenced, at least in any major way, by the expression of the alternatively spliced carboxyl-terminal sequences.

It is possible that the extended carboxyl terminus of the smooth/non-muscle Ca\(^{2+}\)-ATPase plays a role in the localization of the enzyme within the cell or represents the binding site for a putative regulatory molecule other than phospholamban. Antibodies against the tail, combined with expression studies, should provide answers to some of these questions.

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