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Abundance of sarcoplasmic reticulum calcium pump isoforms in stomach and cardiac muscles

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Rabbit stomach smooth muscle contains mRNA for an internal Ca^{2+} pump identical in sequence to that reported for rabbit uterus [Lytton, Zarain-Herzberg, Periasamy & MacLennan (1988) J. Biol. Chem. **264**, 7059–7065]. This is an alternatively spliced form (Is) of the cardiac muscle sarcoplasmic reticulum Ca^{2+} pump (Ic). The splicing results in replacement of the last 4 amino acids (Ala-Ile-Leu-Glu) present in Ic by 49 amino acids and by a different 3′-non-coding region. Using cDNA probes against the conserved and the alternatively spliced regions, we determined that poly(A^+) RNA isolated from rabbit stomach smooth muscle did not contain any transcripts for Ic. The poly(A^+) RNA from cardiac muscle contained transcripts mostly for Ic, but also some for Is. The abundance of the Ca^{2+}-pump transcripts as measured by the binding of a cDNA probe against the conserved region to poly(A^+) RNA was 6–8 times higher in cardiac than in smooth muscle. The amount of the corresponding pump protein, measured using two antibodies, was 60–80 times higher in cardiac membranes than in smooth muscle membranes. Thus the protein-to-transcript level was approx. 10-fold higher in the cardiac muscle. We conclude that the regulation of the abundance of this protein occurs at steps leading to the formation of the mature mRNA for the two splices, which may differ in their translation efficiency.

INTRODUCTION

The relaxation of smooth muscle requires attainment and maintenance of submicromolar cytoplasmic Ca^{2+} concentrations, although Ca^{2+} concentrations in the extracellular space and in the endoplasmic reticulum (ER) may exceed 1 mM (Daniel *et al.*, 1983; Schatzmann, 1989). Based on physiological experiments and biochemical evidence, we have suggested that the smooth muscle contains two Ca^{2+} pumps, one in the plasma membrane (PM), which may be important for maintenance of Ca^{2+} homeostasis, and the other in the ER, which may be important in the attainment of homeostasis (Grover, 1985; Grover & Samson, 1986). The presence of the two Ca^{2+} pumps in smooth muscle has now been confirmed independently from several laboratories (Wuytack *et al.*, 1982, 1984). In the presence of Ca^{2+} the PM Ca^{2+} pump forms an acid-stable hydroxylamine-sensitive acyl-phosphate intermediate of 135 kDa, whereas the ER Ca^{2+} pump forms a similar intermediate of approx. 100 kDa (Wuytack *et al.*, 1984; Grover & Samson, 1988; Grover *et al.*, 1988). Antibodies against the Ca^{2+}-pump protein isolated from erythrocyte ghosts react with the 135 kDa protein in various tissues (Wuytack *et al.*, 1984; Grover, 1988). The cDNA encoding this protein has been cloned, and it has been shown that several isoforms of this protein exist (Wibo *et al.*, 1981; Shull & Greeb, 1988). The 100 kDa protein of the smooth muscle ER Ca^{2+} pump does not react with antibodies against the PM Ca^{2+} pump. In addition, it reacts poorly with antibodies against skeletal muscle sarcoplasmic reticulum (SR) Ca^{2+} pump, and very well with those against the cardiac SR enzyme (Grover *et al.*, 1988). It may also be regulated by phospholamban, as in cardiac muscle. Recently it has been reported that the 100 kDa Ca^{2+} pump present in several tissues is an isoform (Is) (Bastie *et al.*, 1988; Gunteski-Hamblin *et al.*, 1988; Lytton & MacLennan, 1988; Lytton *et al.*, 1988; Eggermont *et al.*, 1989) resulting from alternate splicing of the gene that is responsible for the cardiac isoform (Ic) (MacLennan *et al.*, 1985). In Is the four amino acids at the *C*-terminus of Ic are replaced by 49 or 50 amino acids, and the Is isoform also continues into a different 3′-non-coding region. However, it is unclear whether the various smooth muscles produce only Is or whether they contain Ic as well. It is also notable that, among the tissues which contain either product of this gene, only the cardiac muscle is rich in the Ic isoform of the Ca^{2+} pump protein. We have previously reported that the abundance of the 100 kDa Ca^{2+} pump in stomach smooth muscle membranes is very low (Grover *et al.*, 1988). The physiological significance of this discrepancy in the levels of this pump between the heart and other tissues may be that cardiac tissue has a very fast relaxation rate compared with smooth muscle. It is not known whether regulation occurs in the steps leading to the production of the mature mRNA for the two splices or at the level of translation.

In this study we report that the sequence of the Ca^{2+} pump isoform present in rabbit stomach smooth muscle (Is) is identical to the sequence reported in rabbit uterus (Lytton & MacLennan, 1988). Using Northern and Western blots we show that the Ca^{2+} pump protein/transcript level ratio in cardiac muscle is higher by a factor of approx. 10 than that in stomach smooth muscle. We discuss the role of alternate splicing in the regulation of the levels of the Ca^{2+} pump present in the stomach and cardiac muscles.

EXPERIMENTAL

RNA and library preparation

Albino rabbits weighing 1–2 kg were killed with pentobarbital and the stomachs were dissected immediately and processed for the preparation of RNA (Chirgwin *et al.*, 1979). To obtain the smooth muscle layer, the stomachs were cut open, the contents were removed by three rinses with ice-cold diethyl pyrocarbonate-treated water and then the stomachs were dissected to remove

Abbreviations used: ER, endoplasmic reticulum; SR, sarcoplasmic reticulum; Ic, cardiac isoform of sarcoplasmic reticulum Ca^{2+} pump; Is, stomach isoform; PM, plasma membrane; SSC, 0.015 M-sodium citrate/0.15 M-NaCl, pH 7.0.

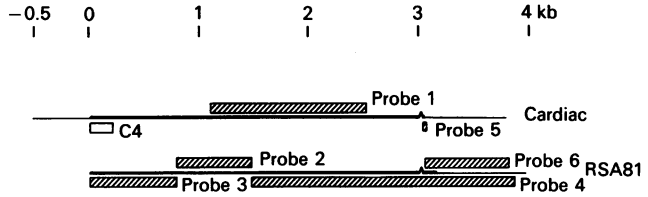

Fig. 1. Probes and clones

A schematic representation of the cardiac SR cDNA (MacLennan *et al.*, 1985) is shown, along with clones obtained and probes used. The sequence of the longest clone obtained, RSA81, aligns with the cardiac muscle cDNA as shown. The sequences were identical until the break marked by ^. Bold lines indicate the protein-coding region. Locations of the various probes used in this work are also shown. Probe 1 was the 1.5 kb *Pst*I fragment from cardiac muscle used for screening the stomach smooth muscle library. Probes 2, 3 and 4 are subclones of RSA81. Probe 5 was a chemically synthesized deoxyoligonucleotide (TTTAGGAAGCGGTTACACCAGTATTG) selective against the cardiac splice and probe 6, an *Nla*IV–*Ban*II fragment of probe 4, was the selective probe for the splice Is. C4 was a polyclonal antibody against the peptide shown here.

adhering fat, mucosa, submucosa and serosa (Grover *et al.* 1988). Poly(A$^+$) RNA was purified from the total smooth muscle RNA by oligo-dT chromatography (Aviv & Leder, 1972), and cDNA was prepared by random plus oligo-dT priming. Libraries were constructed into the *Eco*RI site of λgt11 by Clonetech. The library was screened (Maniatis *et al.*, 1982) with a ^{32}P-labelled 1.5 kb *Pst*I fragment of rabbit cardiac cDNA (MacLennan *et al.*, 1985; probe 1 in Fig. 1) kindly provided by Dr. D. H. MacLennan, Best Institute, Toronto, Canada. Both strands of the longest clone (Fig. 1) and single strands of seven other clones were sequenced (Sanger *et al.*, 1977).

Northern blotting

For Northern blots, total RNA was prepared as described above from stomach smooth muscle and from cardiac tissue. Poly(A$^+$) RNA was isolated by oligo-dT chromatography, and then electrophoresed and blotted electrophoretically on nylon membranes (Gelman) (Maniatis *et al.*, 1982). The filters were exposed to u.v. light for 4 min and prehybridized and hybridized as for the screening described above. Probes 3 and 6 were labelled with [α-^{32}P]dATP by nick translation (Maniatis *et al.*, 1982). Probe 5, which was a synthetic 26-mer anti-sense deoxyoligonucleotide to the initial alternate Ic splice (see Fig. 1), was end-labelled (Maniatis *et al.*, 1982) using [γ-^{32}P]ATP. For hybridization with the nick-translated probes, 50% formamide was used at 42 °C and the filters were washed in 0.1 × SSC at 65 °C for 2 × 30 min. For the oligonucleotide probe the formamide was omitted during the hybridization and the filters were washed in 0.1 × SSC at 42 °C for 2 × 15 min.

Western blotting

Crude membranes were prepared as described previously (Daniel *et al.*, 1983; Grover *et al.*, 1988) by centrifuging the post-nuclear supernatant fraction at 200000 *g* for 60 min. These membranes were used for SDS/PAGE followed by electrotransfer of the proteins to nitrocellulose (Verma *et al.*, 1988). A rabbit polyclonal antibody (C4) against the conserved region of the amino acid sequence as shown in Fig. 1 (Lytton & MacLennan, 1988), was a gift from Dr. J. Lytton, Best Institute, Toronto. A mouse monoclonal antibody IID8 (Grover *et al.*, 1988) was a gift from Dr. K. P. Campbell, University of Iowa. The blots were treated with these antibodies and then with the appropriate ^{125}I-labelled anti-IgGs. The blots were autoradiographed to reveal the positions of the bands, which were excised and counted for radioactivity in a gamma counter.

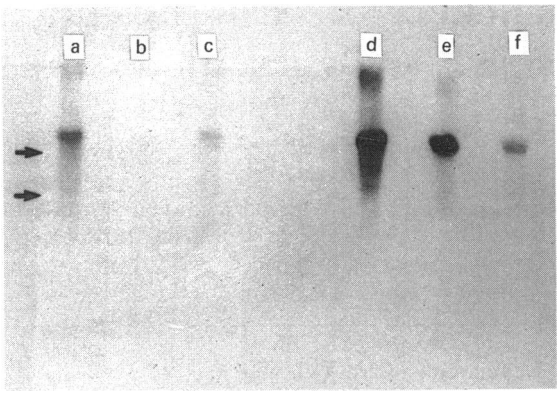

Fig. 2. Isoforms of transcripts present in poly(A$^+$) RNA prepared from rabbit stomach smooth muscle (lanes a–c) and cardiac muscle (lanes d–f)

A 6 μg portion of poly(A$^+$) RNA was loaded in each lane. The probes used for the various lanes were: cDNA probe 3 corresponding to a conserved sequence (lanes a and d); probe 5 selective for the splice Ic (lanes b and e) and probe 6 selective for the splice Is (lanes c and f).

RESULTS

Cloning and sequencing of the rabbit stomach Ca^{2+}-pump cDNA

The complete cDNA sequence of the longest clone RSA81 and of the seven partial clones showed 99% identity with the cDNA sequence for the internal Ca^{2+} pump in rabbit uterus (Lytton & MacLennan, 1988) and is therefore not repeated in this communication. The sequence up to base 2981 of RSA81 showed 100% identity with the sequence up to base 2981 of Ic (MacLennan *et al.*, 1985). However, from this point on, the RSA81 sequence differed from that of Ic such that (*a*) the last four amino acids in Ic were replaced by 49 amino acids in the stomach isoform (Is), and (*b*) the 3′-non-coding sequences were completely different, with Ic cDNA being rich in bases G and C and Is DNA rich in A and T and containing several stretches of poly(T). As reported by Lytton *et al.* (1988), the same gene encodes for at least two isoforms, namely Ic, reported originally in the cardiac muscle, and Is, present in other tissues.

Isoforms present and their abundance

Fig. 2 shows the initial experiment on the types and levels of transcripts for the isoforms present in the stomach and the cardiac muscles. In this experiment three probes were used: probe 3 against the conserved region, probe 5, selective for isoform Ic, and probe 6, selective for isoform Is. Fig. 2 shows that the stomach smooth muscle poly(A$^+$) RNA hybridized with the probe against the conserved region as well as with the probe for the splice Is. The cardiac poly(A$^+$) RNA hybridized strongly with the probes for the conserved region and for the Ic splice, but it also bound weakly to probe 6 for the Is splice. On cutting and counting this blot, it was determined that the total amount of the transcript for cardiac muscle as determined with the conserved region probe was approx. 4 times greater than that for stomach muscle. With the probe for isoform Is the counts were similar in the cardiac and the stomach muscles (Fig. 2).

The quantity of the transcript was determined further using different amounts of poly(A$^+$) RNA from preparations from three different rabbits, as shown for one of the animals in Fig. 3(*a*). The binding of the probe against the conserved region [per μg of poly(A$^+$) RNA] was 6–8 times higher in cardiac muscle than in stomach smooth muscle. The amount of protein corresponding to the Ca^{2+} pump was determined using two different

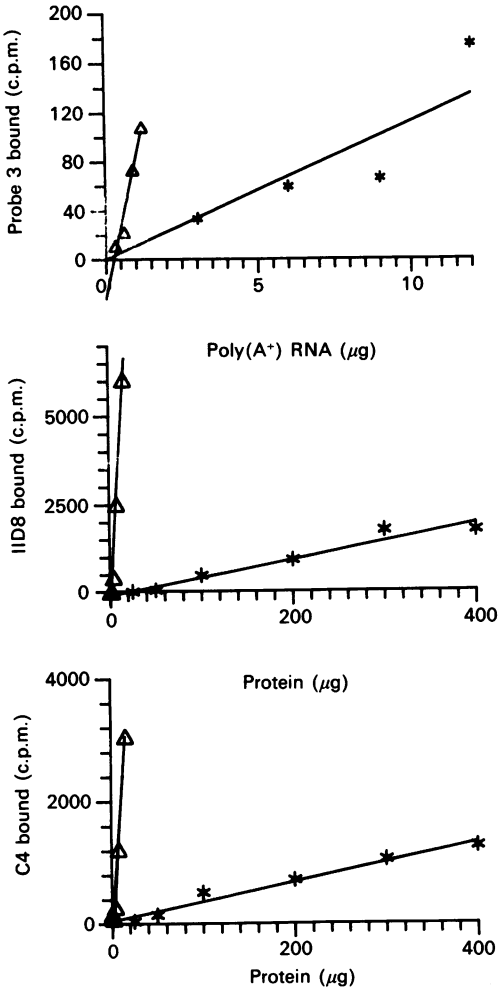

Fig. 3. Levels of transcripts and Ca^{2+} pumps in rabbit stomach (*) and cardiac (△) muscles

The probe used for the determination of the level of transcripts was the conserved region cDNA probe 3. The antibodies IID8 and C4 were used for determining the amounts of the Ca^{2+} pump protein. Note that different secondary antibodies were required for IID8 and C4, and this may explain the difference in the absolute c.p.m. The degree of binding in (*a*) was 11 and 69 c.p.m./μg of poly(A^+) RNA for the cardiac and the stomach muscles respectively. In (*b*), the corresponding values were 404 and 5.1 c.p.m./μg of membrane protein, and for (*c*) these were 195 and 3.2 c.p.m./μg of membrane protein.

antibodies as shown in Figs. 3(*b*) and 3(*c*). One of these antibodies (IID8) is a monoclonal antibody against the dog heart SR Ca^{2+} pump and the other (C4) is a polyclonal antibody against a peptide in the conserved region (see Fig. 1). Fig. 3 shows that the amount of the Ca^{2+} pump protein in the cardiac muscle membranes is 60–80 times higher than in the stomach smooth muscle membranes. Repeating such experiments with two more rabbits gave similar results. The crude membranes in this study represented approx. 20% of the total protein in the homogenate in both heart and stomach. The amount of protein obtained in homogenates of the two tissues was also very similar (28.6 ± 1.2 mg/g of tissue). Therefore the Ca^{2+} pump protein was 60–80 times more abundant in the cardiac muscle than in the stomach muscle whether total crude membrane protein, total homogenate protein or tissue wet weight were used for normalization. We conclude that the ratio of protein/mRNA in cardiac muscle is approx. 10 times higher than in stomach smooth muscle.

DISCUSSION

We have presented results which show that the stomach smooth muscle cDNA library contains clones whose deduced sequence corresponds to an isoform (Is) of the previously reported Ca^{2+} pump from cardiac muscle sarcoplasmic reticulum (Ic) (MacLennan *et al.*, 1985). Using Northern blots, we have shown that stomach smooth muscle poly(A^+) RNA contains transcripts for Is and not for Ic. Cardiac muscle contains mainly transcripts for Ic, but also a small amount of Is transcripts. Finally, with the aid of Western blots using two antibodies, we have shown that the ratio of protein/mRNA present in cardiac muscle is approx. 10 times higher than in stomach smooth muscle. These findings are now discussed in terms of tissue homogeneity, possible pitfalls in the methods used, the role of alternate splicing in regulation of the abundance of the translation products, the possible physiological role of the Is isoform and comparison with results of other workers.

A major pitfall in using cDNA cloning and Northern or Western blots to determine the type of messages present in a given tissue is the heterogeneity of the tissue. In this work, in order to obtain stomach smooth muscle, we removed mucosa, submucosa, serosa, extraneous nerves and blood vessels, so that the tissue contained > 90% smooth muscle cells (Grover *et al.*, 1988). However, even after this careful dissection, the tissue contained myenteric plexus, and in addition two types of smooth muscles are present: circular and longitudinal (Grover *et al.*, 1988). Despite this, we obtained cDNA clones and poly(A^+) RNA containing only the Is and not the Ic isoform. This is consistent with the findings of Lytton *et al.* (1988) with rabbit myometrium. These findings are however inconsistent with those obtained by another group using cDNA libraries from the whole stomach, who found that isoforms Is and Ic were both present (Gunteski-Hamblin *et al.*, 1988). We also report that the cardiac muscle poly(A^+) RNA contains transcripts for Ic as well as for Is. This result confirms the findings of Lytton *et al.* (1988). It is possible that the presence of both Ic and Is reflects heterogeneity of the cardiac tissue. It is known that the physiology of atria, ventricles, the pacemaker cells and the conducting system is different and hence it would not be surprising if the isoforms Is and Ic were indeed distributed differently in the heart. The possibility that the Is transcript detected in heart is due to the presence of nerves and blood vessels also cannot be ignored. Further work on different regions of the heart, hybridization *in situ* and immunohistochemistry using isoform-specific antibodies may resolve these questions.

In order to understand the implications of the difference in the 3′-non-coding regions of these isoforms, it is important to know the secondary structure of the mRNA. There are no means available at present to rigorously calculate the most stable secondary structure for mRNA species as large as 4 kb. Therefore we devised the following analysis to examine the impact of the alternate splicing on the secondary structure of the remaining RNA. For each base position we calculated the energies for binding of 5 bases to all other parts of the molecule (Cedergren *et al.*, 1988) and added these together to get the energy at that particular base location, thus determining a plot of energy versus base number for the cardiac splice (Ic) and the alternate splice (Is). Then, for each base position in the conserved sequence, we subtracted the energy for the Ic isoform from that for the Is isoform to calculate the plot for energy difference versus base number shown in Fig. 4. Even though we did not obtain clones containing a large portion of the 5′-non-coding region of Is, in this calculation we assumed it was the same as in the cardiac muscle, since this has been shown to be so in every reported study on these isoforms (Gunteski-Hamblin *et al.*, 1988; Lytton & MacLennan, 1988; Lytton *et al.*, 1988; Eggermont *et al.*, 1989).

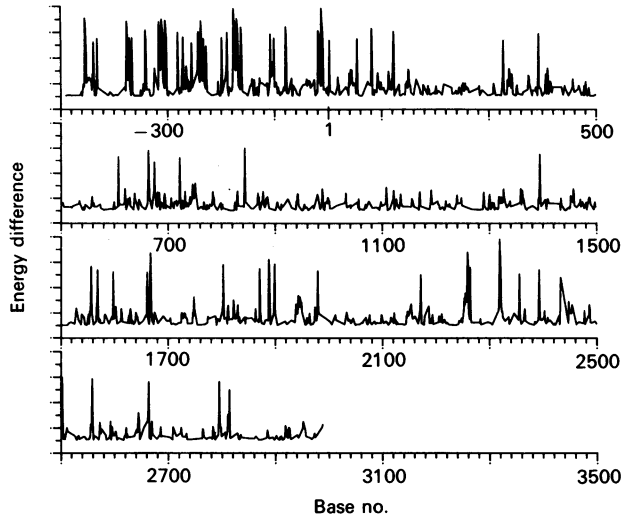

Fig. 4. Energy differences for binding between the splices Is and Ic

See the Discussion section for details.

The analysis showed that the energy difference was located mainly in the 5′-non-coding region, with smaller differences occurring at other base positions. This suggests that the alternate splice (Is) in the smooth muscle isoform increases the chances of interaction of the 5′-non-coding region. This increased chance of intramolecular binding could affect the translation of this mRNA. Alternative explanations of the effect of the 3′-non-coding region on translation are also possible. In several instances the 3′-non-coding region is known to influence translation, but the mechanism involved is not understood (Caput *et al.*, 1986; Kruys *et al.*, 1987; Casey *et al.*, 1988; Strickland *et al.*, 1988). The presence of the sequence TTATTTA has been proposed to be responsible for such a regulation (Caput *et al.*, 1986). Ic as well as Is contains this sequence only once in the 3′-non-coding region. From the model presented, we ascertain that the mRNA for the splice Is would be translated poorly. The ultimate test for the role of the 3′-non-coding region would be for pure full-length RNA for Ic and Is to be synthesized using cDNA clones and then translated by using lysates from cardiac and stomach smooth-muscle cells. This test would then indicate whether or not factors other than the predicted difference in the RNA structure are also involved in their regulation. However, at present, one should also consider post-translational events such as protein processing and turnover as possible mechanisms by which splicing could indirectly regulate the abundance of this protein.

It has been shown in several reports that cardiac muscle contains transcripts for the Ic isoform whereas smooth muscles and several non-muscle cells contain isoform Is. It has been suggested that the Is is a 'housekeeping' enzyme which the smooth muscle uses for contractility (Gunteski-Hamblin *et al.*, 1988; Lytton & MacLennan, 1988; Lytton *et al.*, 1988). Based on the following characteristics of the transport process carried out by the smooth muscle ER Ca^{2+} pump (Grover & Samson, 1986), we suggest an alternative interpretation. The smooth muscles contain two pumps: a 135 kDa pump in the PM and a 100 kDa pump in the ER. Both pumps are regulated by other proteins, the PM pump by calmodulin and the ER pump by phospholamban (Schatzmann, 1989). The Ca^{2+} concentration-dependence of the smooth muscle ER pump shows a higher degree of co-operativity than that of the smooth muscle PM Ca^{2+} pump, indicating that the activity of the ER pump may be more amenable to change during the excitation–contraction cycle. The pH and the $[MgATP_2]$-dependence of the ER pump are similarly consistent with the interpretation that the ER pump is more likely to respond to such changes. We have proposed in the past that due to these properties it is the 135 kDa pump which is more likely to play a housekeeping role. Indeed, all tissues tested so far and reported to contain the Is splice are involved in a stimulus–response coupling. Therefore we propose that the 100 kDa pump has a key role in stimulus–response coupling in tissues with slow response time. However, in cardiac and skeletal muscles, which have faster response times, this role is performed by more specialized isoforms of the Ca^{2+} pump. Whether the isoforms resulting from the alternate splicing differ in their catalytic and regulatory properties, or whether the alternate splicing itself is a mechanism to supply the cardiac cells with a higher level of SR Ca^{2+} pump activity, is not known. How this alternate splicing is related to the development of the SR in these tissues and the exact subcellular localization of Is are also unknown.

In this communication we have provided evidence for regulation of Ca^{2+}-pump expression at the level of transcription via alternate splicing, which in turn may also regulate translation due to differences in the secondary structure of mRNA. We anticipate that the discussion on these issues, the availability of various clones, antibodies, and advances in technology will aid in more focused answers to these points in the future.

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