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Expression of isoforms of internal Ca^{2+} pump in cardiac, smooth muscle and non-muscle tissues

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Smooth muscle and several non-muscle tissues contain mRNA for an alternative splice of the mRNA for the cardiac sarcoplasmic reticulum (SR) Ca,Mg-ATPase. Based on amino acid composition deduced from cDNA sequences the cardiac isoform (I_c) is 110 kDa while the smooth muscle and the non-muscle isoform (I_s) is 115 kDa. This prediction in their molecular masses was tested at the protein level in rabbit stomach, aorta, uterus and vas deferens smooth muscles; stomach mucosa, brain, liver, kidney and heart. The major species of the acylphosphates formed in the presence of Ca^{2+} and electrophoresed in acid SDS-acrylamide gels were 5 kDa smaller for the heart (I_c) than those for all the other tissues (I_s). The size difference was also confirmed in Western blots using a monoclonal antibody which binds to both I_s and I_c. Thus consistent with the mRNA splices for the internal Ca^{2+} pumps previously reported to be present in these tissues, rabbit heart expresses predominantly the Ca^{2+} pump protein I_c and the various smooth muscle, mucosa, brain, liver and kidney express mainly the isoform I_s.

Introduction

Based on physiological experiments and biochemical evidence it has been proposed that there are two types of Ca pump – one present in the plasma membrane (PM) and another in the endoplasmic reticulum (ER) or sarcoplasmic reticulum (SR) [5,8,9,23]; although in some recent studies it has been suggested that the internal Ca pump may be localized in calciosomes [20]. The PM Ca pump is structurally and immunologically distinct from the internal Ca pump [5,8,9,21,23,24]. The internal Ca pump in smooth muscle reacts very well with the anti-cardiac SR antibodies but not with several monoclonal antibodies against fast skeletal SR Ca pump [6,9]. Based on cDNA cloning and mRNA hybridization studies a number of isoforms of each of these pumps are predicted. Table I contains the subunit molecular masses of the predicted isoforms. The PM Ca pump isoforms are 127–135 kDa [7,17–19] while all the isoforms predicted for the internal Ca pumps are 109–115 kDa (Table I). Since antifast skeletal muscle SR Ca pump antibodies do not react with smooth muscle, the anticardiac antibody IID8 used here does not react with the fast skeletal muscle and the smooth and cardiac/slow twitch skeletal muscle internal Ca pumps are encoded by one gene and the fast twitch skeletal muscle SR Ca pump by a different gene, the fast skeletal muscle SR Ca pump is not considered here any further (Refs. 1–4, 6, 9, 11–16, Table I). Smooth muscles, and several non-muscle tissues contain transcripts for an alternative splice (I_s) of

TABLE I

Molecular mass of calcium pump proteins based on cDNA sequences

Membrane	Genes[a] and transcripts	Protein size (kDa)	References
Plasma membrane	4 genes × 4 splices	127–135	7,17,18,19
Internal membrane			
Slow-twitch/cardiac gene (SERCA2)			
	I_c	110	16
	I_s	115	1,6,11–15
SERCA3 gene	SERCA3	109	4
Fast-twitch skeletal gene (SERCA1)			
adult		110	2,3
neonatal		111	2

[a] The nomenclature of Ca pump genes is according to Ref. 4. I_c and I_s correspond to the gene products SERCA2a and SERCA2b of Ref. 4.

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the mRNA encoding the cardiac SR Ca pump (I_c) [1,6,11–15]. Based on the protein sequences deduced from cDNA the isoform I_c has 997 amino acids and I_s has 1042 – the N terminal 993 amino acids being identical. Thus the protein encoded by the I_s transcripts should have a molecular mass approx. 5 kDa higher than the protein encoded by the I_c mRNA (Table I). Here we use this size difference prediction to determine the isoform expression at the protein level.

Experimental Methods

Membrane preparations. Albino rabbits weighing 1–2 kg were euthanized and the various organs were removed immediately and placed in an ice-cold homogenization medium (20 mM morpholino-propane sulfonate-NaOH (pH 7.0), 8% sucrose, 0.5 mM PMSF, 1 mM trypsin inhibitor type I, 1 mM TLCK, 1 mM dithiothreitol and 0.5% ethanol). All further manipulations were carried out at 0–4°C. The stomachs were dissected to separate mucosa and a smooth muscle layer containing both circular and longitudinal muscles as described previously [9]. All the other tissues were dissected free of fat, connective tissue and superficial blood vessels. The tissues were homogenized using a Polytron PT20 for 2×5 s in 10 volumes of the homogenization medium and were centrifuged at $1000 \times g$ for 10 min to remove the cell debris and nuclei. To the supernatants KCl was added to 0.7 M and the samples stirred for 15 min and centrifuged at $140000 \times g$ for 60 min. The resulting pellet contained all the membranes and was designated crude microsomes which were used in all the experiments except where specified otherwise.

In some experiments, where specified further purified membranes were used. This purification procedure is based on differential and isopycnic centrifugation and yields a relatively pure plasma membrane fraction F1 and an ER-enriched F3 fraction. The method for its preparation and characterisitcs of the resulting fractions have been described previously [9].

Phosphorylation experiments. The acylphosphoenzyme intermediates were detected by a reaction at 0°C for 20 s in samples containing 100 mM KCl, 30 mM imidazole-HCl (pH 6.8) (pH at 20–23°C), 5 μM ATP, 67 μCi [γ-^{32}P]ATP (3000 Ci/mmol), 2 mg/ml of the membrane protein, and 50 μM $CaCl_2$ as described previously [9,10]. In initial control experiments in some samples $CaCl_2$ was replaced with 0.5 mM EGTA (pH 6.8). The reaction was stopped with an ice cold solution containing 10% trichloroacetic acid, 50 mM phosphoric acid and 1 mM ATP. The resulting protein precipitate was washed three times in this stopping solution and then suspended in a sample buffer containing 10 mM morpholinopropane sulfonate, 20 mM dithiothreitol, 1 mM ethylenediaminotetraacetate, 3% SDS, and 0.1% of the tracking dye methyl green at pH 5.5. The gel electrophoresis system consisted of 12 cm long separating gel at pH 4.0, 1.5 mm thick 10% polyacrylamide/0.5% bisacrylamide slab gels and 0.5 cm long 5% acrylamide/0.25% bisacrylamide stacking gel at pH 6.0. Electrophoresis was carried out at 4°C by passing a current of 50 mA/gel for 12 h. Under these conditions the dye methyl green reached the front in 5 h. The gels were dried and autoradiographed.

Western blotting. Electrophoresis was carried out in 1.5 mm thick 10% polyacrylamide/0.5% bisacrylamide separating gel slabs and 5% acrylamide/0.25% bisacrylamide stacking gel according to Laemmli except that the runs were carried out for 12 h at 30 mA/gel at 4°C. The gel lengths of the stacking and the separating gels were as described above. Under these conditions Bromophenol blue migrated to the front of the gel in 5 h. Electrotransfer of the proteins to nitrocellulose (Biotrace RP, Gelman) was carried out according to Ref. 9. The primary antibody used here was the antidog cardiac SR Ca pump mouse monoclonal antibody IID8 which was a gift from Dr. K.P. Campbell at University of Iowa. IID8 also binds the internal Ca pump in the stomach smooth muscle shown previously [9]. The blots were treated with IID8 and then with the rabbit antimouse ^{125}I-labelled antimouse IgG followed by autoradiography.

Results

Size separation of acylphosphoenzyme intermediates

Fig. 1 shows the results of an initial experiment on acylphosphoenzyme SDS-acid gel electrophoresis. Under these conditions the acylphosphate-enzyme intermeidates were formed only in the presence of Ca^{2+} and not when Ca^{2+} had been depleted to very low levels by using EGTA (Fig. 1). When the plasma membrane-enriched fraction F1 from rabbit stomach smooth muscle was used, a very faint single band at approx. 130 kDa appeared but when crude microsomes from stomach smooth muscle were used a very intense band below the 130 kD band appeared. When the gels were exposed for shorter periods of time the 130 kDa band was not observed, the lower band was less diffuse and it migrated at a rate similar to the 116 kDa molecular mass marker β-galactosidase. This band has previously been shown to be the ER Ca pump in smooth muscle. When crude microsomes from heart were used, a single band was observed. Exposing the gel for a shorter time period showed that this band may have migrated slightly faster than the stomach microsome band.

The difference in migration between the 110–115 kDa acylphosphates of heart and the stomach smooth muscle microsomes in Fig. 1 was insufficient for identification of isoforms predicted to be 115 kDa for I_s and 110 kDa for I_c. To improve this resolution a number of experiments including those involving gradient gels were

carried out. The size difference between the main species of acylphosphates in the stomach smooth muscle and the heart microsomes was observed best under the following conditions: (a) the gels were exposed for short periods so that single sharp band corresponding to the internal Ca pump appeared, (b) bands were of equal intensity for heart and the other tissues, (c) to ensure that the observed differences were not due to artifacts in gel preparation and/or electrophoresis the samples of heart were alternated with the other samples, and (d) the electrophoresis was carried out for 12–16 h at an optimal current at 4°C as described in the Experimental Methods. The conditions established above were used for the isoform identification in Fig. 2. Under these conditions the acylphosphoenzyme intermediates formed from heart microsomes (I_c) consistently migrated faster than the similar intermediates from stomach smooth muscle (I_s) as shown in Fig. 2. This size difference between the stomach smooth muscle and the heart microsomes was observed routinely by using the molecular mass markers which were stained with India Ink and hence not shown in the autoradiograms in Fig. 2. The stomach smooth muscle band was observed to travel at a rate similar to β-galactosidase and the heart muscle band moved slightly faster than β-galactosidase (116 kDa) but slower than phosphorylase *b* (97.5 kDa). Thus the sizes of these bands could be identified with respect to the PM Ca pump band (130 kDa), molecular mass markers used and with respect to each other ($I_s > I_c$).

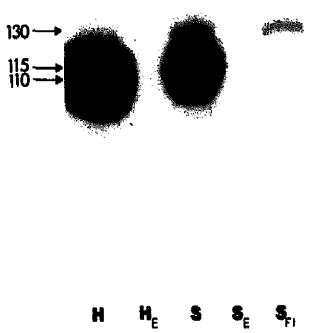

Fig. 1. A preliminary experiment on SDS gel electrophoresis of acylphosphate intermediates of Ca pump. The phosphorylation was carried out on crude microsomes from heart (H and H_E) or stomach smooth muscle (S and S_E) or the stomach smooth muscle plasma membrane enriched fraction F1 (S_{F1}) in the presence of 50 μM $CaCl_2$ or 0.5 mM EGTA (H_E and S_E). The band for the PM Ca pump at 130 kDa was observed only on very long exposures of the autoradiographs as shown. Shorter exposures led to disappearance of the PM Ca pump band and to much sharper internal Ca pump band at 110–115 kDa position. The microsomal proteins applied in these lanes were 6.0 μg for heart and 50 μg for stomach smooth muscle. See Experimental Methods for details. β-Galactosidase (116 kDa) and phosphorylase *b* (97 kDa) were used as molecular weight markers.

Using the method established above the isoform type of the internal Ca pump present in the various tissues was examined and the results are presented in Fig. 2. The crude microosmes from rabbit stomach smooth muscle, uterus, aorta, vas deferens, stomach mucosa and liver gave single bands corresponding to the higher molecular weight isoform (I_s). Crude microsomes from brain also gave a band corresponding to the I_s isoform at 115 kDa but it also gave a very intense band at 130 kDa corresponding to the plasma membrane Ca pump. Thus the brain tissue expressed much higher levels of the PM Ca pump than the others. Kidney microsomes gave a band at 115 kDa corresponding to I_s but these also showed bands of lower molecular weights. When the phosphorylation was carried out in presence of EGTA instead of Ca^{2+} the lower bands persisted even though the 115 kDa band was abolished. Thus the lower bands may be due to a very high level of Ca-independent phosphorylation and not degradation products of the Ca pump.

It is emphasized that due to the nature of this method only the predominant species can be detected. Hence the presence of the isoform in a much smaller proportion can not be ruled out.

Subunit size determination using IID8

IID8 is a monoclonal antibody produced against dog heart SR Ca pump but it also reacts very well with the rabbit cardiac muscle and stomach smooth muscle [9] but not with the skeletal muscle Ca pump. This antibody was used for confirming the size difference of the internal Ca pump isoforms present in the rabbit stomach (I_s) and heart (I_c). Again this size difference could be observed only under the optimal experimental conditions as described in the Experimental Methods. As a further confirmation both the crude microsomes and the ER-enriched membrane fraction F3 [9] were used for identification of the isoforms. Fig. 3 shows that the reactivity with the crude microsomes or the ER-enriched fraction F3 from the stomach smooth muscle occurred to a larger subunit species than in cardiac membranes. Also much smaller amounts of the cardiac protein were needed to obtain bands of similar intensities to those observed with stomach smooth muscle microsomes. Since the amount of protein loaded for detecting I_s and I_c was different, a control experiment was conducted to test if the amount of protein electrophoresed affected the rate of migration of I_c. Increasing the amount of protein loaded by adding different

amount of bovine serum albumin to a constant amount of cardiac microsomes did not alter the mobility of I_c (not shown).

These results are therefore consistent with those obtained using the acylphosphate intermediate detection system. Once again with this method only the dominant species was observed.

Discussion

In this study we have demonstrated that consistent with the studies at mRNA level the smooth muscle, stomach mucosa, brain, liver and kidney express an internal Ca pump protein which is 5 kDa or so larger than the cardiac/slow twitch muscle SR isoform (I_c).

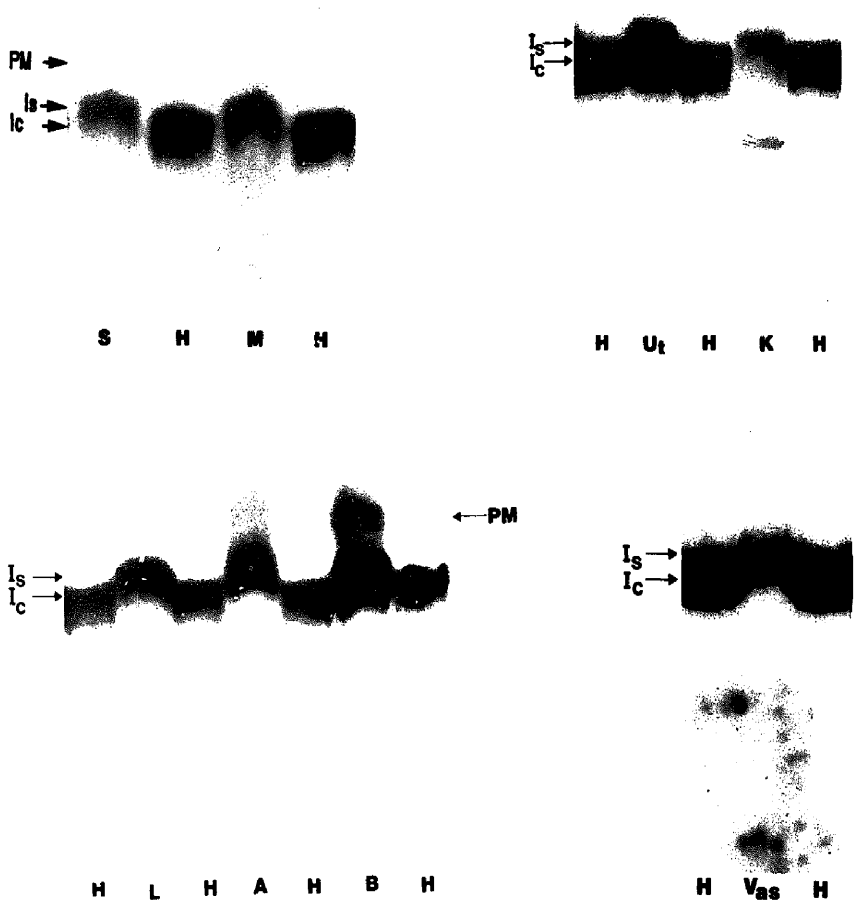

Fig. 2. Isoform identification by SDS gel electrophoresis of acylphosphate intermediates of Ca pump. The phosphorylation was carried out on crude microsomes and followed by a preliminary electrophoresis experiment. Based on the preliminary experiment, the longer electrophoresis run was carried out as described in the Experimental Methods using amounts of samples which would give some what similar intensities. A = aorta, B = brain, H = heart, K = kidney, L = liver, M = stomach mucosa, S = stomach smooth muscle, Ut = uterus, and Vas = vas deferens. The expected position of the I_s and I_c isoforms based on the molecular weight markers β-galactosidase (116 kDa) and phosphorylase *b* (97 kDa) are shown. The figure is a composite from samples run on different days from various membrane preparations. Even though only one sample per tissue is shown here, samples from each tissue were tested using more than three preparations and similar results were obtained.

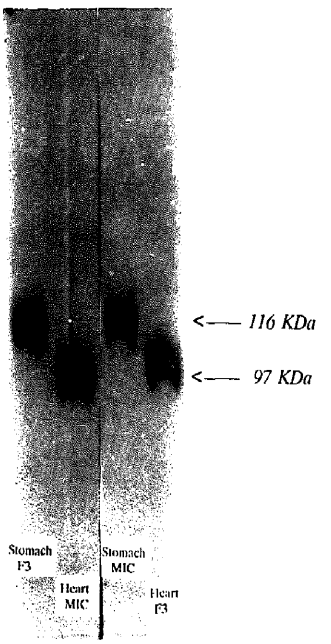

Fig. 3. Western blotting of heart and stomach smooth muscle membranes using IID8. The membranes used were crude microsomes (MIC) or the ER enriched fraction (F3). The positions of the molecular weight markers β-galactosidase (116 kDa) and phosphorylase *b* (97 kDa) are also shown. The amount of protein applied was 10 μg for heart, 200 μg for stomach MIC and 100 μg for stomach F3. See Experimental Methods for details.

This size difference can also be detected using the monoclonal antibody (IID8) which has previously been reported to react with the Ca pumps from the rabbit heart/slow twitch and stomach smooth muscle. The Discussion will encompass advantages and disadvantages of the techniques employed and implications of the reported findings.

The method for distinction between the internal Ca pump isoforms I_s and I_c using acylphosphoenzyme intermediates which are formed in the first step of the reaction cycle of the Ca pump is very sensitive. It can be used very easily and does not require the preparation of any antibodies. A major disadvantage of this method is that the isoform present in smaller proportions is not detected. It has been reported that several tissues containing mRNA for the isoform I_s may also contain smaller amounts of mRNA for I_c and vice-versa [4,6,11–15]. The extent of expression of the lower abundance mRNA can not be tested using this technique. Any methods short of complete protein sequencing will face the criticism that the observed differences may result from some unknown post-translational modifications. For instance in a recent study [22] sequence specific antibodies were used for identification of isoforms of the internal Ca^{2+} pump. It is pointed out that there are two potential glycosylation sites in the C-terminus of I_s and thus a tissue specific post-translation modification could produce the results reported therein. However, argument in favour of the study in Ref. 22 and our study is that the results observed at the protein level are in accordance with the prediction from the nucleic acid sequences.

The intensity ratio of the acylphosphate bands for the PM and the ER Ca pump proteins was higher in brain microsomes than in microsomes from heart, smooth muscle, liver or kidney. These results are consistent with the report that the brain contains a higher level of transcripts for the PM Ca pump than any other tissue studied so far [7]. In a preliminary experiment using a PM Ca pump selective antibody it was shown that the brain tissue does indeed express much higher levels of the PM Ca pump protein.

In some cDNA cloning studies the libraries were prepared from whole stomachs [11] which also contained mucosa. Therefore it was interesting to determine if and what isoform of the internal Ca pumps was expressed by the rabbit stomach mucosa. The isoform expressed was I_s. All the clones reported from rabbit stomach smooth muscle corresponded to I_s and only the transcripts for I_s were observed in Northern blots as well [12]. The presence of clones for I_c as well as I_s has been reported in rat and pig stomachs [6,11] and transcripts for another isoform internal Ca pump transcribed from a different gene have been reported in a number of tissues [4]. However, in all these studies the level of transcripts or the number of clones obtained for the other isoforms is very small. In the method described here if two isoforms are expressed the lower abundance isoforms will be undetectable. Furthermore, since mucosa as well as smooth muscle express mainly the I_s isoform the origin of I_c transcripts in the stomach libraries remains unknown.

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