Calsequestrin is a component of smooth muscles: the skeletal- and cardiac-muscle isoforms are both present, although in highly variable amounts and ratios

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Expression by smooth-muscle cells of calsequestrin (CS), the low-affinity/high-capacity Ca^{2+}-binding protein of striated-muscle sarcoplasmic reticulum (SR), has been investigated in recent years with conflicting results. Here we report the purification and characterization from rat vas deferens of two CS isoforms, the first deemed skeletal muscle, the second cardiac type, on account of their N-terminal amino acids and other relevant biochemical and molecular properties. Compared with vas deferens, the smooth muscles from aorta and stomach, in that order, were found to express lower amounts of CS, whereas in the uterus and bladder the protein was not detectable. The ratio between the two CS isoforms was also variable, with the stomach and aorta predominantly expressing the skeletal-muscle type and the vas deferens expressing the two CSs in roughly similar amount. Because of the property of CSs to localize within the skeletal-muscle SR lumen not uniformly, but according to the distribution of their anchorage membrane proteins, the expression of the protein suggests the existence in smooth-muscle cells of discrete endoplasmic-reticulum areas specialized in the rapidly exchanging Ca^{2+} storage and release, and thus in the control of a variety of functions, including smooth-muscle contraction.

INTRODUCTION

Intracellular rapidly exchanging Ca^{2+} stores play a key role in a variety of cell functions. The basic molecular components believed to be co-localized in these stores are Ca^{2+} channels, Ca^{2+} pumps and intralumenal Ca^{2+} binding proteins, i.e. the proteins competent for the release, uptake and storage of the cation, respectively (see [1–3]). Because of the relatively large amounts of Ca^{2+} accumulated by the pumps within the stores, the storage proteins are expected to be characterized, on the one hand, by large capacity, and on the other hand, by low affinity, a property that permits release to occur rapidly when channels are activated [1–3]. So far, two major families of such proteins have been described. The skeletal and cardiac isoforms of calsequestrin (CS) have been shown to be concentrated within the terminal and (in the heart) the corbular cisternae of the striated-muscle sarcoplasmic reticulum (SR) [1–5], whereas the calreticulins (CRs) are widely distributed within the endoplasmic reticulum (ER) of all cell types [1–3,6–8]; however, see [9,10].

Recent studies have demonstrated that CSs are not exclusive components of striated muscles, inasmuch as they have been revealed also in Purkinje neurons of birds [11–14] and in specialized myocytes of the heart conduction system [15,16]. In smooth-muscle cells, published results have been conflicting. In both the stomach and vas deferens, proteins immunologically indistinguishable from CSs [17,18], have been shown to be concentrated in specialized areas of the ER [18] which appear to play a key role in Ca^{2+} storage and release [19]. In the uterus, however, CS has been reported to be present only in trace amounts [20]. On the basis of the latter finding, the role of CS in smooth-muscle Ca^{2+} homoeostasis has been questioned [7,20].

In the present study, the question of CS expression in smooth-muscle cells has been reinvestigated by parallel analysis of five smooth-muscle-rich organs of the rat: the three previously investigated independently (stomach, vas deferens and uterus) plus the aorta and the bladder. The present data demonstrates that genuine CSs are indeed expressed in at least three of these organs, but with major differences in total amounts and in ratios between the two CS isoforms.

MATERIALS AND METHODS

CS purification

Male Wistar rats were killed by decapitation, and the vas deferens was rapidly collected, frozen in liquid N_2 and stored at −80 °C until use. Other smooth-muscle-rich organs (stomach, uterus, aorta and urinary bladder) were dissected out and processed in parallel.

For the purification of the CSs, the amounts of tissue (wet weight) used per experiment varied: about 20 g of vas deferens and bladder, 40 g of stomach, about 15 g of aorta and uterus. Homogenates were obtained in a Waring blender at high speed for 1 min in 0.1 M potassium phosphate buffer, pH 7.1, 2.66 M $(NH_4)_2SO_4$, 1 mM EGTA, 0.5 mM phenylmethanesulphonyl fluoride and 0.5 mM benzamidine. Putative CSs were purified from whole tissue homogenates by the procedure widely employed for the striated muscle proteins, i.e. $(NH_4)_2SO_4$ precipitation followed by DEAE-cellulose chromatography [21]. Fractions containing proteins reactive with specific anti-CS antibodies were pooled, applied to phenyl-Sepharose columns in the presence of 0.1 mM EGTA and then eluted with 10 mM $CaCl_2$ [22].

CSs standards from three skeletal muscles (chicken pectoralis major, rabbit fast-twitch adductor and rat slow-twitch soleus) were purified as described above. Protein concentration was determined as described by Lowry et al. [23].

Abbreviations used: CS, calsequestrin; CR, calreticulin; ER, endoplasmic reticulum; SR, sarcoplasmic reticulum.

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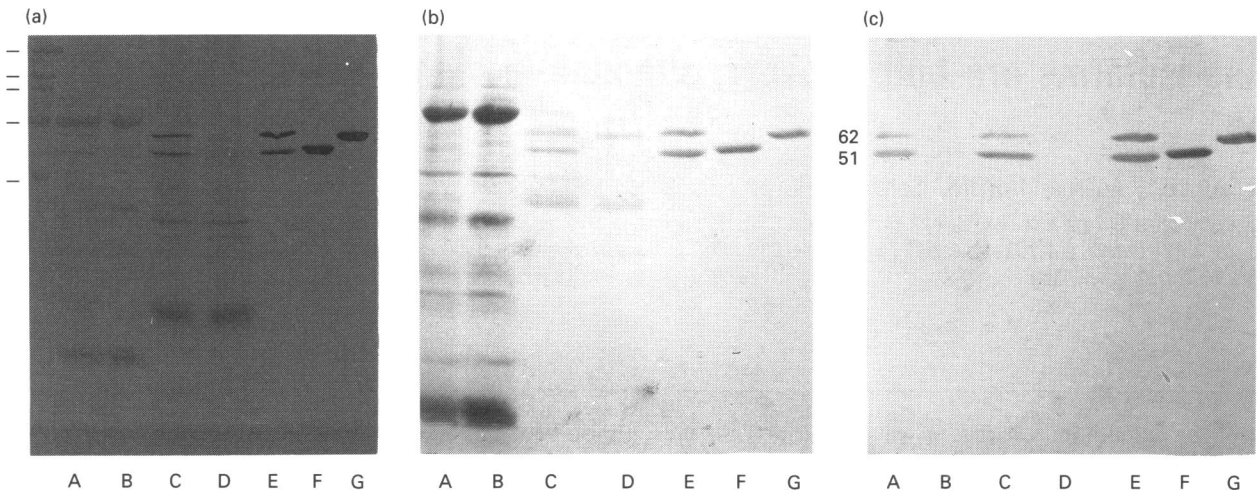

Figure 1 Purification of rat vas deferens smooth-muscle CSs by DEAE-cellulose and phenyl-Sepharose chromatography: Stains-All staining (a), Ponceau Red staining (b) and Western blot with anti-CS antibodies (c)

Key to lanes of each panel: A, $(NH_4)_2SO_4$ precipitate loaded on to DEAE-cellulose (20 μg of protein); B, DEAE-cellulose void volume (20 μg); C, pool of CS-positive DEAE fractions (collected between 0.4 and 0.6 M NaCl; about 7 μg); D, phenyl-Sepharose void volume (about 7 μg); E, fraction no. 3 eluted from phenyl-Sepharose on addition of 10 mM $CaCl_2$ (about 0.6 μg); F, CS purified from chicken skeletal muscle (about 1 μg); G, CS purified from rabbit fast-twitch skeletal muscle (about 1 μg). Apparent molecular masses of CSs from vas deferens, i.e. 62 and 51 kDa, were calculated from graphs of relative mobilities versus log of molecular mass of standards. Bio-Rad molecular-mass standards are indicated by short horizontal lines on the left-hand side and are (in kDa): myosin heavy chain, 200; β-galactosidase, 117; phosphorylase, 97; BSA, 66; ovalbumin, 45.

Analytical procedures

SDS/slab-gel electrophoresis was carried out on polyacrylamide gels [24]. Slab gels were stained by either the Coomassie Blue or the Stains-All (13) procedure.

Electrophoretic transfer to nitrocellulose, staining with Ponceau Red and immunodecoration with anti-(chicken skeletal-muscle CS) antibodies (Western blotting) were carried out as described [13]. The anti-CS antibodies employed recognized both isoforms of rat vas deferens CS [18]. Quantification of the immunolabelled bands was performed by microdensitometry, with reference to scale portions of authentic standards run in parallel. The chicken skeletal-muscle CS and the rabbit skeletal-muscle CS were used as standards for the cardiac and the skeletal CS isoforms respectively.

For N-terminal amino acid sequencing, the two CS-positive bands were electroblotted on to a Millipore Immobilon membrane [11,25], and then sequenced in a pulsed liquid Applied Biosystems model 477A amino acid analyser.

$^{45}Ca^{2+}$ ligand overlay

This was carried out on purified CS bands blotted on to nitrocellulose sheets. The assay medium contained 5 mM $MgCl_2$, 60 mM KCl, 5 mM imidazole, pH 7.4, and 10 μM $^{45}CaCl_2$ [13]. K_d and $B_{max.}$ values were calculated from the ^{45}Ca-overlay data obtained by incubating individual nitrocellulose lanes at $[Ca^{2+}]$ in the range 0.01–4 mM. After washing, the CS bands were revealed by autoradiography, excised and counted for radioactivity. For each lane, background was obtained by counting the radioactivity of a piece of nitrocellulose membrane processed in parallel and equal in area to the CS band [13].

RESULTS

The CSs from the various smooth muscles were purified by the procedure widely employed for the skeletal-muscle proteins [21,22]. At each step of the procedure [homogenization, $(NH_4)_2SO_4$ precipitation, DEAE-cellulose chromatography], samples were collected and analysed for the presence and purity of CS by SDS/slab-gel electrophoresis and Western blotting.

Figure 1 summarizes the results obtained on the rat vas deferens muscle. The three panels show the results obtained for identical samples either stained with Stains-All (SDS gel; Figure 1a) and Ponceau Red (nitrocellulose blot; Figure 1b), or immunodecorated with anti-CS antibodies (Figure 1c). Both the homogenate and the pool of the selected DEAE-cellulose fractions displayed two Stains-All metachromatically blue-stained bands (apparent molecular masses about 62 and 51 kDa) that were also decorated by anti-CS antibodies (lanes A and C in Figures 1a and 1c). No CS positivity was detected in the DEAE-cellulose and phenyl-Sepharose void volumes (lanes B and D in Figures 1a and 1c). Lane E in all panels represents one of the several fractions eluted from phenyl-Sepharose upon addition of Ca^{2+}, showing two virtually pure polypeptides of the above apparent molecular mass (Figure 1b), which were metachromatically blue-stained with the Stains-All procedure (Figure 1a), and were recognized by anti-CS antibodies (Figure 1c). Based on the phenyl-Sepharose elution profile [22], the 62 and 51 kDa polypeptides were tentatively identified as CSs.

Other rat smooth muscles, i.e. stomach, aorta, bladder and uterus, were investigated for the presence of CSs. Selected pooled fractions obtained by DEAE-cellulose chromatography were subjected to phenyl-Sepharose chromatography, and fractions eluted upon addition of Ca^{2+} are shown in Figure 2, after Stains-All staining (panel a) or Western blotting (panel b). The metachromatically blue-staining bands (Figure 2a), immunoreactive for anti-CS antibodies (Figure 2b) with apparent molecular masses of 51 and 62 kDa were clearly visible in the aorta and stomach (arrows in Figure 2) and not appreciable in the uterus and bladder.

The levels of CS expression in the three positive organs were established by microdensitometry, comparing the homogenate

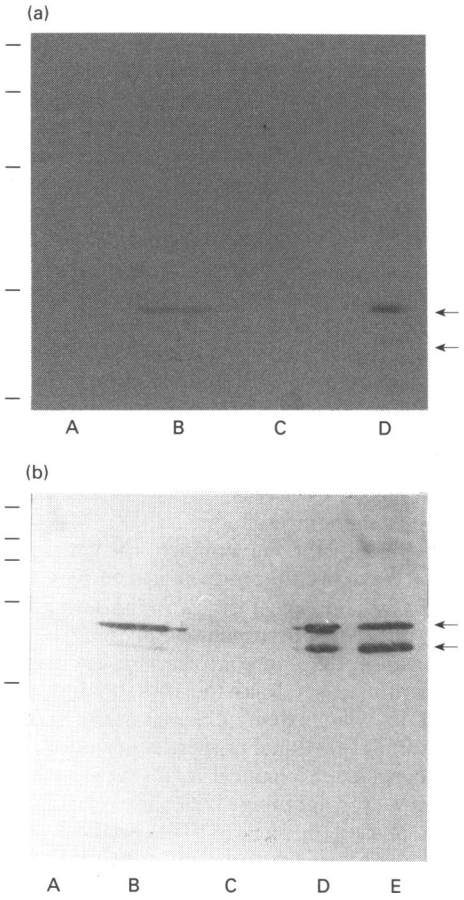

Figure 2 Purification of CSs from smooth muscle of stomach, aorta, uterus and bladder by DEAE-cellulose and phenyl-Sepharose chromatography: Stains-All staining (a) and Western blot with anti-CS antibodies (b)

Key to lanes (150 μl each well): individual and representative fractions eluted from phenyl-Sepharose on addition of 10 mM $CaCl_2$, obtained from bladder (A), aorta (B), uterus (C), stomach (D) and vas deferens (E). (**a**) 5–10%-polyacrylamide linear gradient gel; (**b**) 12% polyacrylamide gel. Arrows point to CSs. Molecular-mass standards were as in Figure 1.

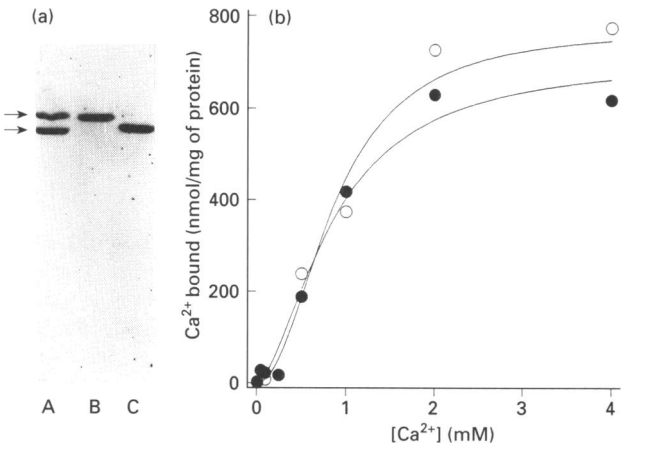

Figure 3 ^{45}Ca-ligand overlay (a) and $^{45}Ca^{2+}$-binding characteristics (b) of CSs from rat vas deferens smooth muscle purified by phenyl-Sepharose chromatography

(**a**) ^{45}Ca-ligand overlay was carried out as described in the Materials and methods section in the presence of 10 μM $^{45}CaCl_2$. About 1 μg of protein was loaded in each lane. For the purity of the samples, see lanes E–G of Figure 1. Key to lanes: A, fraction no. 3 eluted from phenyl-Sepharose on addition of 10 mM $CaCl_2$; B, CS purified from rabbit fast-twitch skeletal muscle; C, CS purified from chicken pectoralis major skeletal muscle. Arrows point to CSs. (**b**) $^{45}Ca^{2+}$-binding characteristics were determined by liquid-scintillation counting of electrobotted CSs incubated in assay media containing $^{45}CaCl_2$ ranging from 0.01 to 4 mM (see the Materials and methods section for details). Key to symbols: ○, smooth-muscle 62 kDa; ●, smooth-muscle 51 kDa. Computer fitting of the data was performed by using NFIT (Island Products, UTMB, Galveston, TX, U.S.A.).

Table 1 N-terminal amino acid sequence of CSs from rat vas deferens, aorta and stomach smooth muscles, and comparison with that of CS from striated muscles of different species (rat, rabbit, dog, chicken and frog)

The standard single letter code for amino acids is used. Underlined residues represent typical short motifs of either skeletal-muscle or cardiac CS.

Protein	Sequence	References
Rabbit	EEGLDFPEYDG	4
Dog	EEGLDFPEYDG	21
Frog	EDGLDFPEYDG	37,38
Rat	EDGLDFPEYDG	39
Rat vas deferens 62 kDa	EDGLDFPEY	Present work
Rat aorta 62 kDa	XXXLDFPEYDXV	Present work
Rat stomach 62 kDa	XEGLDFPEYDGV	Present work
Cardiac CS		
Rabbit	EEGLNFPTYDG	40
Dog	EEGLNFPTYDG	5
Frog	EEGLNFPTY	41
Rat	EEGLNFPTYD	39
Rat vas deferens 51 kDa	EEGLNFPTYXG	Present work
Chicken striated muscles*	EEGLNFPTYDG	42,43

* In chicken striated muscles, there is a single CS gene and one gene product [42].

immunodecorated bands of the Western blots with authentic CS standards run in parallel (see the Materials and methods section). This procedure permits correction for the specificity of the antibodies, i.e., they were more specific for the cardiac than for the skeletal CS isoform, and gave thus rise to stronger immunolabelling of the 51 kDa band than of the 62 kDa band. The estimated values were (μg of CS/g of tissue): vas deferens 36, contributed approximately equally by the two isoforms; aorta, 4; and stomach, 2, with over 80% present as the skeletal isoform.

Figure 3(a) shows that both CS-positive isoforms of vas deferens (lane A) bind Ca^{2+}, as judged by $^{45}Ca^{2+}$ overlay, and that binding is as intense as that of the fast-twitch skeletal-muscle CS from either rabbit or chicken (lanes B and C respectively). Quantification of $^{45}Ca^{2+}$ binding, performed by ^{45}Ca-ligand overlay at varying $^{45}CaCl_2$ concentrations (Figure 3b; see also [13]), revealed for both bands low affinity (K_d about 0.8 mM) and high capacity (700–800 nmol of Ca^{2+}/mg of protein).

The final demonstration that the smooth-muscle proteins having apparent molecular masses of 51 and 62 kDa are CS isoforms, not only by general biochemical and immunological, but also by molecular, criteria came from their N-terminal sequence analysis reported in Table 1. As can be seen from the comparison with known sequences of striated-muscle CSs, the higher-molecular-mass band exhibited a fast-twitch skeletal-muscle-type sequence in all the three smooth muscles, with the surprising occurrence of a glutamic acid in position 2 in the stomach CS. This residue corresponds to the glutamic acid

present in skeletal muscles of rabbit and dog, but differs from the aspartic acid of other rat CSs so far investigated. The 51 kDa band could be investigated only in the vas deferens, for reasons of quantity. The sequence obtained is identical with that of cardiac CSs of all the species investigated (Table 1).

DISCUSSION

The present results demonstrate beyond any doubt that the smooth-muscle cells of the rat vas deferens express authentic CSs, of both the cardiac and fast-twitch skeletal-muscle types. In fact, the proteins not only exhibit the appropriate migration rates on SDS/PAGE and are recognized by highly specific antibodies (Figures 1a and 1c; see [18]), but also share the biochemical properties of the striated-muscle CSs. At least one of these properties, the Ca^{2+}-dependent change in hydrophobicity revealed by phenyl-Sepharose chromatography, is shown by no other Ca^{2+}-binding protein so far identified, including CR. The definitive proof is, however, given by the N-terminal amino acid sequences of the smooth muscle CSs (Table 1), which yielded results identical with those of the corresponding striated-muscle isoforms. The CS identification stands true also for the other two smooth-muscle-rich organs investigated, aorta and stomach. In these organs, the levels were definitely lower than in the vas deferens. However, the N-terminal sequences (skeletal-muscle isoform) and combined biochemical properties were consistent with those of the vas deferens. In contrast with the smooth muscles of the three organs mentioned so far, those of the uterus and the bladder failed to express appreciable levels of CS.

The possibility that the lumenal Ca^{2+} binding typical of striated-muscle fibres is expressed also by smooth-muscle cells was first raised a few years ago by Wuytack et al. [17], working with pig stomach, on the basis of SDS-gel migration and immunolabelling experiments. More recently, Milner et al. [20], working on rat uterus by molecular and immunological techniques, found evidence for only trace amounts of CS, whereas Raeymaekers et al. [26] reported that the protein is expressed in some (stomach, ileum and trachea), but not in other (aorta, main pulmonary artery), smooth muscles of the pig. Together with these results, our previous [18] and present data lead to the conclusion that CS is a smooth-muscle component, but highly variable in amount in different organs, from only trace amounts to the values observed in the vas deferens (36 μg/g of tissue, i.e. one-tenth of average values of skeletal muscles).

An unexpected finding in the present data pertains to the expression of two CS isoforms in smooth muscles. In striated muscles precise criteria have been established, i.e., only the skeletal type in fast-twitch fibres, both the skeletal and the cardiac isoforms in slow-twitch fibres [27], only the cardiac isoform in the heart. In the smooth muscles, on the other hand, both isoforms are expressed, but with highly variable ratios. Thus in the stomach and aorta the skeletal isoform largely predominated, whereas in the vas deferens the ratio was roughly 1. The isoform co-expression can be explained in at least two different ways, which remain to be investigated. First, in the smooth muscle distinct cells could express either of the two isoforms. Alternatively, all cells could transcribe the genes of both isoforms, as previously demonstrated for slow-twitch skeletal muscles [27]. So far, the functional significance of CS heterogeneity has not been clarified.

In striated muscles, CS is widely believed to play a key role in the control of cellular Ca^{2+} homoeostasis. The general structure of the protein resembles that of other major endolumenal Ca^{2+}-binding proteins of the ER, CR, with the large acidic C-terminal domain accounting for the low-affinity interaction with the cation [1,2,4–6]. However, CR, which is ubiquitous among cells, expresses the tetrapeptide KDEL at its very C-terminus [6]. Its lumenal ER localization appears not to be stable, but to result from continuous recycling from the Golgi complex, mediated via specific KDEL-receptor binding [8,28]. In contrast, CS is KDEL-free [4], and its concentration within peculiar endomembrane areas of the SR, i.e. the terminal and corbular cisternae in the skeletal and heart muscles [29,30], or the calciosomes of Purkinje neurons [12,14,31], may depend on the stable anchorage to specific membrane proteins [1,31]. By analogy with other cell types, a plausible hypothesis is that specialized CS-containing areas of the ER system also exist in smooth muscles and that such areas may possess a higher potential for Ca^{2+} binding. This conclusion is supported by the results of at least two studies. In vas deferens smooth-muscle cells, we have previously shown that CS-rich areas are located superficially, below the plasma membrane, and are enriched also in the Ca^{2+}-specific channel, the Ins(1,4,5)P_3 receptor, whereas areas located around the nucleus are poor in both components [18]. Moreover, in the smooth-muscle cells of the pig stomach, the CS-rich superficial areas of the ER have been recently shown to play a key role in the control of Ca^{2+} homoeostasis, working co-ordinately with plasmalemma molecules of fundamental importance, such as channels and transporters, in contrast with the deep ER areas, which contain primarily CR as the Ca^{2+}-binding protein [19]. Whether, and to what extent, CS-containing specialized ER areas are relevant to functional properties of smooth muscles, for example the pharmaco-mechanical versus the electro-mechanical excitation–contraction coupling [32], or the type of contractility, i.e. tonic versus phasic [26], remains to be specifically investigated. It appears, however, suggestive that some correlation exists among the levels of expression of CS with those of the intracellular Ca^{2+} channels, the Ins(1,4,5)P_3 and the ryanodine receptors, in the various smooth muscles [33,34], and that variability exists also in the expression of a distinct SR/ER Ca^{2+}-ATPase [35,36]. It is therefore possible that what at present appears no more than a complex pleitropism could ultimately be attributed to specific physiological characteristics of the various smooth muscles.

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