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Altered properties of calsequestrin and the ryanodine receptor in the cardiac sarcoplasmic reticulum of hibernating mammals

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A novel isoform of calsequestrin was identified in sarcoplasmic reticulum vesicles from myocardial tissue of two species of hibernating ground squirrel. The protein was identified as calsequestrin by its cross-reactivity with antibodies raised against bovine cardiac calsequestrin, its pH-sensitive mobility in sodium dodecylsulphate-polyacrylamide gels, staining blue with the cationic carbocyanine dye 'Stains-All', binding peroxidase-conjugated concanavalin A, its endoglycosidase F sensitivity. Its NH_2-terminal amino acid sequence is similar, but not identical, to that already determined for cardiac calsequestrin. Some of the biochemical properties of this protein distinguish it from the other mammalian isoforms. It has a unique electrophoretic mobility in both alkaline and neutral sodium dodecylsulphate-polyacrylamide gel electrophoresis, it appears to have a molecular weight approximately 7% greater than that of cardiac calsequestrin from other mammalian species, and its glycosylation pattern differs. This novel form of calsequestrin is expressed in cardiac SR vesicles which possess an abnormally high number of Ca^{2+}-release channel/ryanodine receptor molecules. This ryanodine receptor also shows an altered Ca^{2+}-sensitivity of ryanodine binding. The divergent biophysical properties of this novel form of cardiac calsequestrin, together with the apparently atypical ryanodine receptors in the cardiac sarcoplasmic reticulum membranes may have some functional significance in the adaptive mechanisms which allow the heart to function despite the severely reduced body temperatures (to approx. 0°C) encountered during hibernation.

Introduction

Calsequestrin is the major Ca^{2+} binding protein in the sarcoplasmic reticulum (SR) of both skeletal and cardiac muscle [1]. In both tissues its primary physiological function is to sequester large amounts of Ca^{2+} in the lumen of the SR, thereby reducing lumenal concentrations of free Ca^{2+} and facilitating further uptake by the Ca^{2+}-ATPase. Under physiological conditions calsequestrin has been estimated to bind up to 40–50 moles of Ca^{2+} per mole of protein, with a dissociation constant of 1 mM [1–5]. Two isoforms of mammalian calsequestrin have been identified and characterized; a skeletal muscle isoform and a cardiac muscle isoform [1]. The complete primary structures of both the skeletal muscle [6] and cardiac muscle [7] forms of calsequestrin have recently been reported. The protein is a glycoprotein which contains N-linked high mannose sugars [8] and binds Con A [9]. Both isoforms of calsequestrin can be identified by a number of common biochemical properties, i.e., they have a high binding capacity for Ca^{2+}, they stain blue with the cationic carbocyanine dye 'Stains-All' [9–12] and they have a pH-sensitive electrophoretic mobility on SDS-PAGE [13].

Calsequestrin is localized in the lumen of the junctional SR [14,15] where it has been shown to bind to some protein constituents of the junctional face membrane, for example a 26-kDa protein [5] and the 400-kDa 'foot' protein [16]. It may also be linked to the junctional face membrane by connecting elements which are discernible by electron microscopy [17,18]. The 400-kDa

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Abbreviations: SR, sarcoplasmic reticulum; SDS-PAGE, sodium dodecylsulphate-polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; PVDF, polyvinylidene difluoride; Con A, concanavalin A; Endo F, endoglycosidase F; Endo H, endoglycosidase H; PMSF, phenylmethylsulphonyl fluoride.

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'foot' protein, or ryanodine receptor, has recently been isolated and cloned, and when incorporated into a planar lipid bilayer it behaves as a Ca^{2+} channel [19–25]. Ikemoto et al. [26] showed that Ca^{2+} binding to calsequestrin may exert some effect on the Ca^{2+} channel and it has been suggested that calsequestrin may, therefore, be able to regulate Ca^{2+} channel functions, either directly or indirectly, via its interactions with both the 26-kDa junctional face membrane protein and with the ryanodine receptor [26,27]. Size analysis of the ryanodine receptor/Ca^{2+} channel polypeptide by SDS-PAGE indicates that the skeletal muscle form (approx. 360000–450000)[19,20] differs from the cardiac muscle form (approx. 340000) [21].

Some mammals, termed hibernators, have evolved the ability to exhibit prolonged periods of torpor characterized by a marked depression of body temperature, sometimes to as low as 0°C, with minimum respiration rate and heart rate. The torpid animal can, however, readily revert to the active state, relying totally on endogenously produced heat [28]. During deep hibernation the functional status of the cardiovascular system is significantly modified (for review, see Refs. 28 and 29). Myocardial contraction during hibernation shows an increased force, yet a decreased reliance on extracellular Ca^{2+}. Therefore, hibernating mammals appear to have an increased dependence on the recycling of intracellular Ca^{2+} [30,31]. These changes in excitation-contraction coupling have been interpreted as being adaptive for the maintenance of circulation despite a very slow heart rate (5–10 beats per min) and a low body temperature (near 0°C). Recent evidence indicates that these adaptations are associated with changes in Ca^{2+} metabolism by the cardiac SR [32].

We have investigated some of the biochemical mechanisms which might be responsible for the modified SR function observed in hibernating mammals. In this paper we report the identification, isolation and characterization of a novel isoform of mammalian calsequestrin, which is present in the cardiac SR of the two species of hibernating ground squirrel studied. We also show that this isoform of calsequestrin is expressed in SR vesicles which exhibit unusual properties of the ryanodine receptor/Ca^{2+} channel molecule. We propose that the novel calsequestrin and ryanodine receptor in the hibernators may be important components of adaptive changes associated with hibernation.

Experimental Procedures

Materials

Gel electrophoresis reagents and molecular weight standards were obtained from Bio-Rad. DEAE-Sephadex (A-25) was purchased from Pharmacia. Nitrocellulose and PVDF membranes were purchased from Millipore. Horseradish peroxidase-linked rabbit anti-goat and goat anti-rabbit antibodies, Endo F, DMSO, benzamidine and PMSF were obtained from Boehringer Mannheim. Horseradish peroxidase-linked Con A, and 'Stains-All' were purchased from Sigma. $^{45}Ca^{2+}$ was from Amersham. [^{3}H]Ryanodine was from New England Nuclear. Ryanodine was from AgriSystems Int., St. Wind Gap, PA. Richardson's (*Spermophilus richardsonii*) and Columbian (*Spermophilus columbianus*) ground squirrels were trapped in Alberta and were maintained in the Animal Facility of the Department of Zoology, University of Alberta.

Preparation of SR vesicles

Skeletal muscle SR vesicles from the ground squirrels were isolated and fractionated according to the method of Meissner [33]. Cardiac muscle SR vesicles were isolated as described by Chamberlain et al. [34], except that the gradient purification steps were omitted. Protein concentrations were determined by the method of Lowry et al. [35] using bovine serum albumin as a standard.

Ryanodine binding

[^{3}H]Ryanodine binding to SR vesicles was carried out at 37°C for 120 min in a solution containing 0.5–200 nM [^{3}H]ryanodine, as described previously [36]. The Ca^{2+}-dependence of ryanodine binding to SR vesicles was measured as described by Michalak et al. [36].

Purification of calsequestrin

Calsequestrin was isolated by selective ammonium sulphate precipitation in the presence of 0.5 mM benzamidine and 0.5 mM PMSF, followed by fractionation on DEAE-Sephadex [37]. Fractions from the DEAE-Sephadex column containing calsequestrin were pooled and the protein further purified by Phenyl-Sepharose chromatography [11].

SDS-PAGE and transfer of proteins to nitrocellulose

SDS-PAGE was generally carried out on 10% acrylamide gels according to the method of Laemmli [38]. Where specified a 10–20% gradient of acrylamide (Bio-Rad Model 385 gradient former) was used. SDS-PAGE at neutral pH was performed as described by Weber and Osborn [39], and two-dimensional gel electrophoresis was conducted as described by Michalak et al. [13]. Standards were Bio-Rad low-range molecular weight proteins; phosphorylase *b* (97400), bovine serum albumin (66200), ovalbumin (42700), bovine carbonic anhydrase (31000), soybean trypsin inhibitor (21500) and lysozyme (14400), or Bio-Rad prestained markers; phosphorylase *b* (135000), bovine serum albumin (75000), ovalbumin (50000), carbonic anhydrase (39000), soybean trypsin inhibitor (27000) and lysozyme (17000). Gels were stained with Silver, Coomassie

blue or with the carbocyanine dye 'Stains-All', or proteins were electrophoretically transferred to nitrocellulose according to the method of Towbin et al. [40].

Preparation of antibodies and immunostaining of Western blots

Goat anti-(rabbit) skeletal calsequestrin and rabbit anti-(bovine) cardiac calsequestrin antibodies were raised against ammonium sulphate-purified proteins separated on SDS-PAGE, as described previously [13,41]. Nitrocellulose membranes were blocked with 5% milk powder in phosphate-buffered saline, and then incubated with antisera as described by Michalak et al. [41]. Antibody binding was detected with appropriate peroxidase-conjugated second antibodies and a standard peroxidase colour development reaction.

$^{45}Ca^{2+}$ and Con A binding

SR proteins and purified calsequestrin were subject to SDS-PAGE and electroblotted to nitrocellulose filters. Ca^{2+} binding to proteins was determined using a $^{45}Ca^{2+}$ overlay technique described by Maruyama et al. [42]. $^{45}Ca^{2+}$ bound was detected by autoradiography. In some experiments 0.5, 1.0 and 1.5 μg of purified calsequestrin were 'dot blotted' directly onto nitrocellulose, the Ca^{2+} overlay repeated, and the amount of $^{45}Ca^{2+}$ bound was determined by liquid scintillation counting. Incubation with Con A was carried out as described by Michalak et al. [41].

Endo F removal of carbohydrate chains

Purified calsequestrin (75 μg/ml) was incubated at 37°C for 20 h with 1.25 units of Endo F/ml as described by Michalak et al. [41]. The reaction was terminated by the addition of 25% Ficoll 400 (Pharmacia), 25 mM EDTA, 1% SDS and 0.05% Bromophenol blue. The apparent M_r of the proteins was determined by SDS-PAGE [38].

V8 Proteinase digestion of proteins

Staphylococus aureus proteinase V8 digestion of proteins was carried out essentially as described by Cleveland et al. [43]. Briefly, purified protein was subject to SDS-PAGE [38] and the gels stained with Coomassie blue. Bands were cut from the gel and equilibrated with stacking buffer. The protein (approx. 2 μg) was then electrophoretically transferred, with 0.1 μg V8 proteinase, into an acrylamide stacking gel (5%). The proteolytic fragments were resolved on a 20% acrylamide gel.

Protein sequencing

NH_2-terminal sequence analysis of ground squirrel cardiac calsequestrin was carried out on purified protein electroblotted to PVDF membrane [44]. Automated sequence analyses [45] were performed with an Applied Biosystems Model 470A gas-liquid phase protein sequencer connected on-line to an Applied Biosystems Model 120A HPLC, using current protocols of Applied Biosystems. All sequencer chemicals were from Applied Biosystems, Foster City, CA.

Results

Identification of calsequestrin in ground squirrel cardiac SR

Fig. 1A shows that the protein composition of ground squirrel cardiac SR is similar to that of bovine cardiac SR. However, the mobility of proteins in the 55 to 60-kDa region differs. In particular, a prominent band is observed (Fig. 1A, arrow), with an apparent M_r of approx. 58 000, which is evident in neither bovine cardiac SR nor rabbit skeletal SR. The immunological charac-

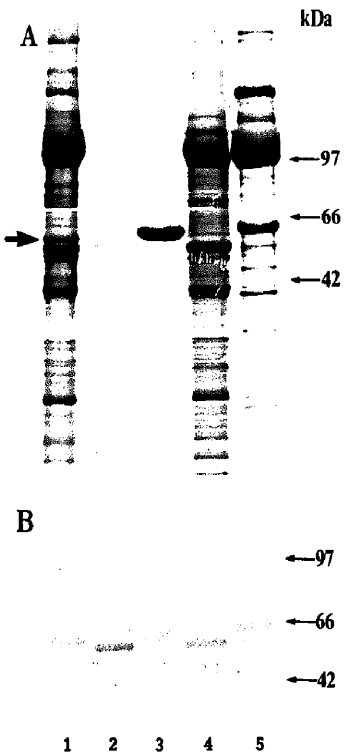

Fig. 1. Immunological identification of calsequestrin in cardiac SR from Richardson's ground squirrel. SR proteins were separated by SDS-PAGE (10–20% acrylamide) and immunoblotting carried out as described in 'Experimental Procedures'. (A) Coomassie blue staining; (B) Immunostaining with anti-(bovine) cardiac calsequestrin. Lane 1, ground squirrel SR; lane 2, purified canine cardiac calsequestrin; lane 3, purified rabbit skeletal muscle calsequestrin; lane 4, bovine cardiac SR; lane 5, rabbit skeletal muscle SR. The thick arrow indicates ground squirrel calsequestrin. The thin arrows indicate the position of Bio-Rad low-range molecular weight marker proteins.

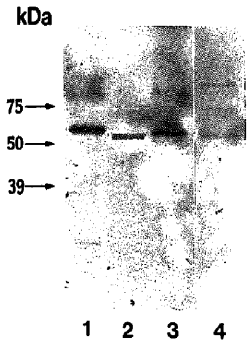

Fig. 2. Detection of Ca^{2+} binding proteins in SR vesicles from skeletal and cardiac muscle. SR proteins were separated by SDS-PAGE (10% acrylamide), transferred to nitrocellulose filters and incubated with $^{45}Ca^{2+}$. $^{45}Ca^{2+}$ binding proteins were detected by autoradiography. All protocols are described in Experimental Procedures. Lane 1, rabbit skeletal muscle SR; lane 2, purified canine cardiac calsequestrin; lane 3, ground squirrel cardiac SR; lane 4, bovine cardiac SR. The arrows indicate the positions of Bio-Rad prestained molecular weight marker proteins.

teristics of this protein were investigated by immunostaining with antisera against (bovine) cardiac calsequestrin and (rabbit) skeletal calsequestrin. As shown in Fig. 1B, the 58-kDa protein in ground squirrel cardiac SR vesicles crossreacted with antibodies against (bovine) cardiac calsequestrin; however, it failed to react with antisera to (rabbit) skeletal calsequestrin (results not shown). It has previously been observed that the goat anti-skeletal muscle calsequestrin antibody used in this study does not crossreact with the cardiac form of calsequestrin [46]. Based on these experiments we conclude that the 58-kDa protein, which is immunoreactive with anti-cardiac calsequestrin, corresponds to ground squirrel cardiac calsequestrin (see also below). This protein has a mobility in Laemmli SDS-PAGE which suggests an apparent molecular weight higher than cardiac and lower than skeletal muscle calsequestrin (Fig. 1A and B).

Calsequestrin is the major Ca^{2+} binding protein in SR membranes and can be identified by a Ca^{2+} overlay technique (Fig. 2) [42]. The 58-kDa protein identified as calsequestrin using anti-(bovine) cardiac calsequestrin antisera was the major Ca^{2+} binding protein in cardiac SR from Richardson's ground squirrel (Fig. 2, lane 3). Additional, high molecular weight Ca^{2+} binding proteins were observed in skeletal and cardiac muscle SR by the Ca^{2+} overlay technique (Fig. 2, lanes 1, 3 and 4). They probably correspond to the high molecular weight Ca^{2+} binding glycoproteins (130- to 165-kDa) previously detected in skeletal and cardiac muscle SR membranes [47]. The ground squirrel cardiac calsequestrin also stained blue with the cationic carbocyanine dye 'Stains-All' (data not shown), in a similar manner to that observed for skeletal and cardiac muscle isoforms of the protein [10,12,48]. The presence of a new isoform of calsequestrin was independent of the physiological state of the animal, i.e., hibernating vs. active (data not shown).

The new form of calsequestrin is specific for cardiac SR

In order to confirm that this new form of calsequestrin is specific for cardiac muscle we examined calsequestrin in skeletal muscle SR vesicles from two different species of hibernating ground squirrel (Richardson's and Columbian). SR vesicles from rabbit skeletal muscle and bovine cardiac muscle were used for comparison. Immunoblots incubated with the anti-cardiac calsequestrin antiserum showed that the mobility of cardiac calsequestrin from Columbian ground squirrels is identical to that detected in the Richardson's ground squirrel (Fig. 3A, lanes 1 and 2) but is different from that of canine cardiac calsequestrin (Fig. 3A, lane 3). In contrast, the mobility of skeletal muscle form of

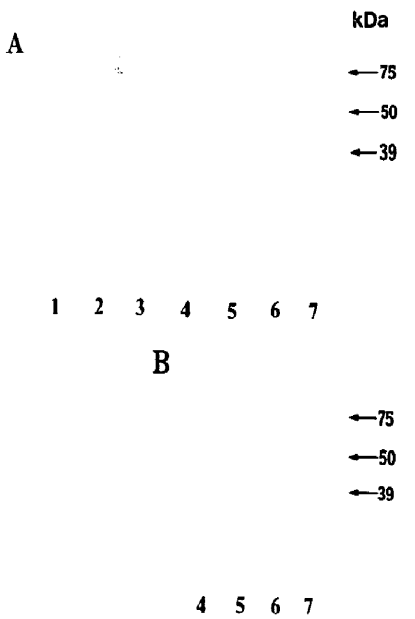

Fig. 3. Immunoblot analysis of SR proteins from skeletal and cardiac muscle of two species of ground squirrel. SR proteins were separated by SDS-PAGE (10% acrylamide) and transferred to nitrocellulose membranes. Immunostaining with (A) anti-cardiac and (B) anti-skeletal calsequestrin antisera was carried out as described in Experimental Procedures. Lane 1, Columbian ground squirrel cardiac SR; lane 2, Richardson's ground squirrel cardiac SR; lane 3, canine cardiac SR; lane 4, rabbit skeletal muscle SR; lane 5, Richardson's ground squirrel skeletal muscle SR; lane 6, Columbian ground squirrel skeletal muscle SR; lane 7, purified rabbit skeletal muscle calsequestrin. The arrows indicate the positions of Bio-Rad prestained molecular weight marker proteins.

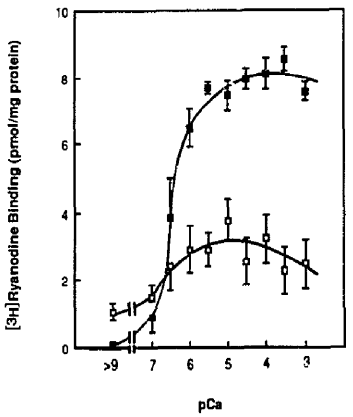

Fig. 4. Ca^{2+} dependence of ryanodine binding to cardiac SR. Measurement of specific binding of ryanodine to SR vesicles and determination of free Ca^{2+} concentration were carried out as described under Experimental Procedures. ■, ground squirrel cardiac SR; □, sheep cardiac SR. Data points represent mean values (± S.E.) for 3–6 different SR preparations. Duplicate determinations were made at each Ca^{2+} concentration.

calsequestrin was identical in all SR preparations screened (Fig. 3A and B, lanes 4–7). The altered calsequestrin and the ryanodine receptor (see below) were expressed in cardiac SR irrespective of the physiological state of the animal (data not shown).

The ryanodine receptor in ground squirrel cardiac SR vesicles

Recent evidence indicates that calsequestrin may interact with the Ca^{2+} channel/ryanodine receptor in SR membranes [26]. In order to determine whether the expression of a novel isoform of calsequestrin is associated with any differences in the ryanodine receptor/Ca^{2+} channel, we also measured some of the properties of this protein in cardiac SR from ground squirrels. Ryanodine binding was measured in SR preparations from the hibernators and compared with binding to SR from sheep cardiac muscle. The values obtained ($n = 6$ for each group ± S.E.) were 12.27 ± 1.89 pmol/mg of protein and 2.18 ± 0.48 pmol/mg of protein, respectively, the binding to SR vesicles prepared from the hibernators being nearly 6-fold greater than binding to the sheep cardiac SR. In order to determine whether this difference was related to differences in the maximum number of binding sites or to a change in the affinity of the binding sites, Scatchard analysis was carried out and revealed that the ground squirrels cardiac SR vesicles bind ryanodine with K_d of 11.3 nM and B_{max} of 19.6 pmol/mg of protein. The results show that ground squirrel cardiac SR exhibits an increased number of ryanodine binding sites compared with the bovine and sheep cardiac SR ($K_d = 3.6$ nM and $B_{max} = 0.82$ pmol/mg of protein; [36], but that the affinity of the ryanodine receptor/Ca^{2+} channel for the ligand is lower in cardiac SR from the hibernators. Interestingly,

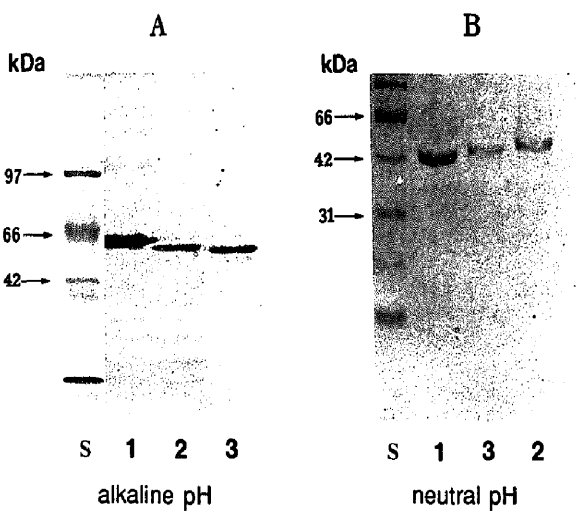

Fig. 5. SDS-PAGE of purified calsequestrin. Calsequestrin was purified from cardiac and skeletal muscle and its mobility determined on SDS-PAGE as described in Experimental Procedures. (A) SDS-PAGE at alkaline pH [38]. (B) SDS-PAGE at neutral pH [39]. 1, rabbit skeletal muscle calsequestrin; 2, ground squirrel cardiac calsequestrin; 3, canine cardiac calsequestrin. The arrows indicate the mobilities of Bio-Rad low-range molecular weight marker proteins (S).

the K_d value for ryanodine binding in the ground squirrel cardiac SR is close to the value previously reported for rabbit skeletal muscle SR (K_d = 11.3 nM and B_{max} = 10.2 pmol/mg of protein; [36]).

Ryanodine binding to SR vesicles was also measured and plotted as a function of the free Ca^{2+} concentration (Fig. 4). At the lowest Ca^{2+} concentration, binding to ground squirrel cardiac SR was significantly lower than that to the sheep cardiac SR. However, binding was strongly dependent on Ca^{2+} concentration in the ground squirrels, increasing 7-fold (compared with only 2-fold for the sheep cardiac SR) as the result of a 10-fold increase in concentration of Ca^{2+} (10^{-7}–10^{-6} M). This rapid increase in binding is similar to the Ca^{2+} sensitivity previously reported for rabbit skeletal muscle SR [36].

Characteristics of purified calsequestrin

In order to further characterize the ground squirrel form of cardiac calsequestrin we purified the protein from cardiac homogenates as described under 'Experimental Procedures'. Fig. 5 shows SDS-PAGE of calsequestrin purified from three different sources: rabbit skeletal muscle (Fig. 5A, lane 1), ground squirrel cardiac muscle (Fig. 5A, lane 2), and canine cardiac muscle (Fig. 5A, lane 3). Under the conditions of the Laemmli SDS-PAGE (alkaline pH) the apparent molecular weights of the three isoforms were estimated to be approximately 61-, 58- and 55-kDa, respectively. The estimated M_r values for the skeletal muscle isoform and for the cardiac muscle isoform of calsequestrin are consistent with previous reports [1,9,11], and the value for the ground squirrel calsequestrin is consistent with the results obtained with isolated SR vesicles (see above).

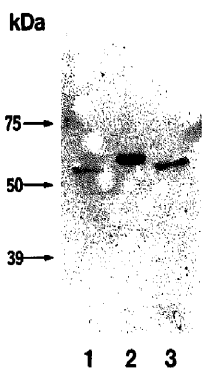

Fig. 6. $^{45}Ca^{2+}$ binding to purified calsequestrin. 1.5 μg of purified calsequestrin was subject to SDS-PAGE, electrophoretically transferred to nitrocellulose and incubated with $^{45}Ca^{2+}$ as described in Experimental Procedures. Lane 1, canine cardiac calsequestrin; lane 2, rabbit skeletal muscle calsequestrin; lane 3, ground squirrel cardiac calsequestrin. The arrows indicate positions of Bio-Rad prestained marker proteins.

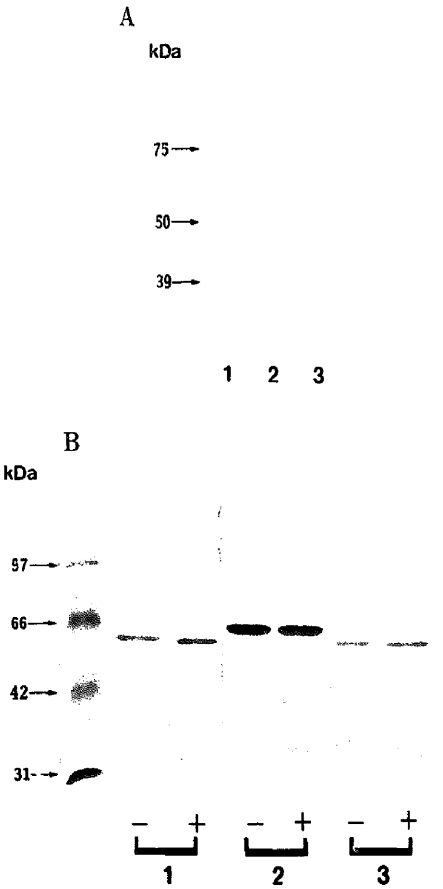

Fig. 7. Carbohydrate analysis of calsequestrin. (A) Con A binding to purified calsequestrin. Purified protein (2.5 μg) was subject to SDS-PAGE, transferred to nitrocellulose and incubated with Con A as described in Experimental Procedures. Lane 1, rabbit skeletal muscle calsequestrin; lane 2, ground squirrel cardiac calsequestrin; lane 3, canine cardiac calsequestrin. (B) Endo F treatment of purified calsequestrin. Purified calsequestrin was incubated with (+) and without (−) Endo F and the apparent mobilities analyzed on Coomassie blue-stained SDS-PAGE (10–20% gradient acrylamide) as described in Experimental Procedures. 1, ground squirrel cardiac calsequestrin; 2: rabbit skeletal muscle calsequestrin; 3, canine cardiac calsequestrin. The arrows indicate the positions of Bio-Rad prestained molecular weight marker proteins and low-range molecular weight marker proteins and in A and B, respectively.

The mobility of calsequestrin on SDS-PAGE gels is pH sensitive [13]. Under neutral pH conditions the mobility of the skeletal and (canine) cardiac forms of calsequestrin indicate M_r values of 42000 and 45500 respectively (Fig. 5B, lanes 1 and 3). In this system, the relative mobility of ground squirrel cardiac calsequestrin again differs from that observed for the (canine) cardiac and (rabbit) skeletal protein (compare Fig. 5A and B).

The apparent M_r of the ground squirrel isoform of cardiac calsequestrin was 48 500 (Fig. 5B, lane 2).

All three isoforms of calsequestrin bound Ca^{2+} as shown by the $^{45}Ca^{2+}$ overlay technique (Fig. 6). Although $^{45}Ca^{2+}$ binding to calsequestrin cannot be quantitated using the overlay technique, we consistently observed greater apparent radiolabelling of ground squirrel cardiac calsequestrin compared with the canine cardiac protein (Fig. 6). This apparent difference in radiolabelling of calsequestrin was also detected, both by autoradiography and by liquid scintillation counting, when different amounts (0.5, 1.0 and 1.5 μg) of each isoform of purified cardiac calsequestrin were 'dot blotted' directly onto nitrocellulose and the incubation with $^{45}Ca^{2+}$ repeated (results not shown).

Carbohydrate analysis of purified calsequestrin

Fig. 7A shows the binding of peroxidase-labelled Con A to purified calsequestrin electrophoretically transferred to nitrocellulose membrane. In agreement with earlier observations [9] binding of Con A to the skeletal isoform of calsequestrin is barely detectable (Fig. 7A, lane 1). Both cardiac isoforms (canine and ground squirrel) bind the lectin more clearly (Fig. 7A, lanes 2 and 3). Under the conditions of the incubation, ground squirrel cardiac calsequestrin appeared to bind Con A more avidly than did the (canine) cardiac isoform. This difference was also observed when 0.5, 1.0 and 1.5 μg of purified calsequestrin were directly 'dot blotted' onto nitrocellulose and the membrane incubated with Con A (data not shown). These results indicate that both cardiac forms of calsequestrin contain N-linked high mannose carbohydrate moieties and that the ground squirrel cardiac calsequestrin might contain an additional carbohydrate chain(s).

In order to confirm the presence of N-linked carbohydrate the three isoforms of calsequestrin were incubated with Endo F. The apparent M_r of both ground squirrel and (canine) cardiac calsequestrin was reduced by incubation with Endo F, whilst the skeletal isoform of the protein was unaffected (Fig. 7B). The reduction in apparent M_r for canine cardiac calsequestrin and ground squirrel cardiac calsequestrin was, respectively, approx. 1 kDa and approx. 2 kDa. These findings confirm the suggestion that the ground squirrel cardiac calsequestrin, in addition to its differing mobility on SDS-PAGE, might also contain an additional carbohydrate chain(s).

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Ground squirrels  NH2 - EEGLNFPTYD GKDRVVSL?E KNFKQIL
Rabbit heart      NH2 - .......... ........S. .......
Canine heart      NH2 - .......... ........T. .......
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Fig. 8. NH_2-terminal amino acid sequences of cardiac calsequestrin. Rabbit and canine calsequestrin NH_2-terminal sequences are taken from Ref. 37. Residue 19 in ground squirrels calsequestrin could not be determined (?). Regions of identity are noted by a period (.) and differing amino acids are shown.

NH_2-terminal amino acid sequence of ground squirrel cardiac calsequestrin

NH_2-terminal amino acid sequencing was carried out on purified ground squirrel cardiac calsequestrin after electrophoretic transfer to PVDF membranes (Fig. 8). The sequencing data indicate that the novel isoform of cardiac calsequestrin has an NH_2-terminal sequence almost identical to that of the previously identified cardiac isoform [37]. The most notable difference is that residue 19 of mature ground squirrel calsequestrin could not be determined. A tyrosine or serine residue is normally observed at this position in mature canine and rabbit cardiac calsequestrin.

Discussion

In this report we have identified, purified and characterized a new isoform of mammalian calsequestrin present in the myocardial tissue of two species of hibernating ground squirrels. The protein was identified as cardiac calsequestrin by its cross-reactivity with anti-(bovine) cardiac calsequestrin antisera, by $^{45}Ca^{2+}$ overlay technique, by NH_2-terminal amino acid sequence analysis. This novel calsequestrin has an apparent M_r of 58 000 and 48 500 in alkaline and neutral pH SDS-PAGE systems, respectively. This is different from that described for either mammalian skeletal muscle (63 kDa vs. 42 kDa) or cardiac muscle (55 kDa vs. 46 kDa) calsequestrins [1]. Our results suggest that ground squirrel cardiac calsequestrin has a molecular weight some 7% greater than that of the previously identified cardiac isoform. This new form of calsequestrin appears to be unique to these hibernating species. This new isoform of calsequestrin is also associated with SR vesicles exhibiting unusual properties of the ryanodine receptor.

Skeletal and cardiac calsequestrin are both glycoproteins [8] and therefore bind the lectin Con A [9,11]. The skeletal isoform, however, binds Con A only very weakly compared with the cardiac form [9,11], and this is at least partly due to the presence of a second glycosylation site at the carboxyl terminal of the cardiac isoform [7]. As a result of differences in glycosylation sites, cardiac calsequestrin is known to be Endo H sensitive, whereas the skeletal isoform of the protein is not [9]. The ground squirrel isoform of calsequestrin binds Con A more avidly than both other isoforms of the protein, indicating that its glycosylation pattern may be unique. This is also supported by Endo F digestion of purified calsequestrin. The increase in mobility resulting from Endo F digestion was greater in the novel isoform of calsequestrin than in the canine cardiac calsequestrin, supporting our conclusion that the glycosylation pattern of the ground squirrel isoform differs from that of the other cardiac isoform. However, it was apparent that even after digestion with Endo F the two isoforms of cardiac calsequestrin have differing mobili-

ties in SDS-PAGE. The electrophoretic mobilities observed in both neutral and alkaline SDS-PAGE therefore indicate that there is a true difference in the size of the ground squirrel cardiac calsequestrin when compared with the other forms.

Despite the evidence that the novel isoform of cardiac calsequestrin is structurally distinct, sequencing data indicate that it has an NH_2-terminal sequence almost identical to that of the previously identified cardiac isoform [37]. The most notable difference is a tyrosine or serine residue which is normally observed at position 19 of mature canine and rabbit cardiac calsequestrin and which could not be detected in the ground squirrel protein. The failure to detect this residue could be related either to phosphorylation or to glycosylation of this amino acid residue. Further studies are required to clarify this important observation.

Where the biochemical and ultrastructural features of junctional SR are conserved it has been observed that, in general, the properties of calsequestrin are also conserved; for example, in the skeletal muscle of a wide variety of mammals [49]. Since recent evidence indicates that calsequestrin may have some active role in the regulation of Ca^{2+} release from the terminal vesicles of the SR [5,29] it is reasonable to speculate that a novel isoform of calsequestrin might function differently in the particular tissue in which it is expressed. Of particular significance is the observation that calsequestrin interacts with the ryanodine receptor, or Ca^{2+}-release protein, of the junctional SR [16]. Our results show that the novel isoform of cardiac calsequestrin is expressed in SR vesicles which exhibit an abnormally high binding capacity for ryanodine, and which also have an unusual Ca^{2+} sensitivity of ryanodine binding. In SR vesicles isolated from the hibernators, ryanodine binding at very low Ca^{2+} concentrations was significantly lower than that in ovine cardiac SR, but increased rapidly in a manner similar to that reported previously for skeletal muscle SR [36]. However, as observed for the sheep cardiac muscle SR, ryanodine binding was not Ca^{2+} dependent at high Ca^{2+} concentrations. These apparently atypical properties of the ryanodine receptor in ground squirrel cardiac SR are suggestive that Ca^{2+} release mechanisms may differ in the cardiac muscle of these animals. This suggestion is supported by the observation that the Ca^{2+}-induced Ca^{2+} release from ground squirrel cardiac SR vesicles follows a profile identical to that of the Ca^{2+} sensitivity of ryanodine binding (Belke, D., unpublished data). Airey et al. [50] reported recently that avian skeletal muscle contains an altered ryanodine receptor/Ca^{2+} channel ('foot' protein). As compared to the rabbit skeletal muscle ryanodine receptor the avian receptor is composed of two (α and β) polypeptides, exhibits different mobility on SDS-PAGE, and has a unique antibody reactivity and limited proteolysis pattern [50]. These observations suggest that altered proteins of the junctional SR such as calsequestrin and/or the ryanodine receptor observed by Airey et al. [50] in chicken pectoralis muscle and by us in hibernating animals may make a different functional contribution to the muscle cell.

Our findings may be directly related to the maintenance of cardiac function during hibernation. During hibernation, cardiac muscle shows a significant increase in the force of contraction elicited by electrical stimulation compared with that observed in the non-hibernating state. Cardiac excitation-contraction coupling has also been shown to become more dependent on recycling of intracellular Ca^{2+} [30,31], and this has been related to changes in Ca^{2+} metabolism by the SR [32]. We propose that the novel calsequestrin and ryanodine receptor proteins in the hibernators may be important components of the mechanisms by which these animals are able to regulate Ca^{2+} release from and storage in the cardiac SR. For example, it is possible that the very steep slope of Ca^{2+} sensitivity of ryanodine binding (Fig. 4), coupled with the very high maximum binding capacity could effect a significantly greater Ca^{2+}-induced Ca^{2+} release, resulting in the enhanced cardiac contractility observed during hibernation.

In conclusion we have identified a novel isoform of cardiac calsequestrin which is expressed in at least two hibernating species, Richardson's ground squirrel and the Columbian ground squirrel. This new form of calsequestrin is co-expressed in the heart of these animals together with an altered ryanodine receptor/Ca^{2+} channel molecule. The combination of a new isoform of calsequestrin and of the ryanodine receptor are independent of the physiological state of the animal (hibernating vs. active). However, they may have some important functional significance in adaptive SR Ca^{2+} metabolism which occurs during hibernation. More detailed study of the functional properties of these atypical proteins may provide important insight into the precise role of calsequestrin in the regulation of Ca^{2+} release from the SR. In addition, further studies will provide valuable information concerning the adaptive molecular mechanisms which allow the heart to function at the severely depressed body temperatures experienced during hibernation.

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