Identification and localization of caldesmon in cardiac muscle

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Caldesmon has been detected in smooth muscle and in a number of non-muscle cells. It binds both actin and myosin and may act as a regulator of contraction or a structural element in smooth muscle. The presence of caldesmon in striated muscle has not been well established. To address this issue, polyclonal antibodies and a panel of monoclonal antibodies were raised against chicken gizzard smooth muscle caldesmon and used to demonstrate that caldesmon is present in adult cardiac muscle of a variety of mammalian species. Western-blot analysis revealed the presence of caldesmon in ventricular myocytes isolated from rat heart. The epitopes for the individual monoclonal antibodies were mapped to the caldesmon primary structure using chymotryptic

and 2-nitro-5-thiocyanatobenzoic acid fragments. Bovine and rat cardiac caldesmons were recognized only by a subset of these monoclonal antibodies, indicating primary sequence differences from the chicken smooth muscle protein. Immunofluorescence labelling of isolated myocytes from rat, rabbit and guinea pig cardiac muscle revealed a striated pattern of fluorescence labelling. Dual labelling of caldesmon and myosin or caldesmon and α -actinin demonstrated that caldesmon was present at the centre of the I-band rather than in the A-band, as might have been expected from the myosin binding properties of the smooth muscle protein. These results suggest a structural role for caldesmon in cardiac muscle cells.

INTRODUCTION

Caldesmon is a heat-stable, actin-binding protein originally isolated from chicken gizzard smooth muscle by Sobue et al. [1]. It was subsequently detected in a number of other smooth muscle cell types, in smooth muscle cells in culture and in non-muscle cells (for reviews, see [2–4]). Two size classes of caldesmon have been distinguished: *h*-caldesmon is predominantly expressed in smooth muscle tissue and *l*-caldesmon in cultured cells and non-muscle tissues [5]. They differ in that *l*-caldesmon lacks the central repeat domain found in *h*-caldesmon [6]. Although the function of caldesmon in non-smooth muscle cells remains largely undetermined, it has been suggested that it may be involved in regulating the interaction of actin and myosin, in the assembly of microfilaments or in organizing contractile structures (see the Discussion section in [3,7,8]). In smooth muscle, caldesmon has been shown *in vitro* to bind to actin [9–12], tropomyosin [13–16], myosin [17–19] and Ca^{2+} /calmodulin [1], and to inhibit smooth muscle actomyosin MgATPase activity [20,21]. The high abundance of caldesmon in smooth muscle (up to 1 mol caldesmon/ $\approx$ 16 mol actin) [22], its ability to inhibit smooth muscle actomyosin MgATPase activity, and the control of this inhibitory activity by phosphorylation and dephosphorylation (reviewed in [4]), have led to the hypothesis that caldesmon is involved in the regulation of smooth muscle contraction. It has also been suggested, however, that caldesmon in smooth muscle is primarily an organizational protein and that its binding to both actin and myosin may keep thick and thin filaments in register for contraction [2,23].

The possibility of using information about the localization of caldesmon in smooth muscle cells relative to that of actin/tropomyosin or myosin to help determine its function is precluded by the lack of an apparent structural order in these cells when studied with the light or electron microscope. Striated muscle cells, by comparison, have a very well defined sarcomere structure

and study of the localization of specific proteins in these cells can provide clues about their cellular functions. This approach has not been applied to the study of caldesmon, however, since the presence of caldesmon in cardiac and skeletal muscle has not yet been firmly established. Using immunofluorescence microscopy, Ban et al. [24] and Lu et al. [25] failed to demonstrate the presence of caldesmon in rat cardiac tissue and cardiac myocytes in culture respectively. On the other hand, Bartegi et al. [26] found that an antibody to caldesmon labelled rabbit ventricular tissue. The labelling was chiefly cytoplasmic although some association with striated structures was noted. Caldesmon was detected on Western blots of chicken [27] and bovine [12] cardiac and skeletal muscle tissues and Sharma [28] detected its presence in the particulate fraction of cardiac muscle extracts. These conflicting results motivated us to use both biochemical and immunological approaches to investigate the presence of caldesmon in the heart more thoroughly. We report here that caldesmon is found in both atrial and ventricular myocytes isolated from adult hearts. Immunofluorescently labelled antibodies to caldesmon revealed a striated pattern of staining in isolated cardiac myocytes permeabilized with Triton X-100. Labelling was also seen at the cell periphery and around the nucleus. Dual labelling of caldesmon and α -actinin or caldesmon and myosin indicated that caldesmon was localized at the centre of the I-band rather than in the A-band region of the isolated myocytes.

MATERIALS AND METHODS

Materials

Freund's adjuvant, hypoxanthine-aminopterin-thymidine, hypoxanthine-thymidine, 2-nitro-5-thiocyanatobenzoic acid (NTCB), α -chymotrypsin, diisopropyl fluorophosphate, pristane (2,6,10,14-tetramethylpentadecane), glycine, Trizma base, β -mercapto-

Abbreviations used: NTCB, 2-nitro-5-thiocyanatobenzoic acid; TRITC, tetramethylrhodamine isothiocyanate isomer R; MM21, monoclonal antibody to myosin heavy chain; CDg-1–CDg-13, monoclonal antibodies to caldesmon; 11E10, monoclonal antibody to residues 23–39 of cardiac myosin.

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ethanol, Coomassie Brilliant Blue R250, 4-chloro-1-naphthol, EGTA, 5-bromo-4-chloro-3-indolyl phosphate, Nitroblue Tetrazolium and secondary antibodies against mouse and rabbit IgG conjugated to tetramethylrhodamine isothiocyanate isomer R (TRITC), FITC or alkaline phosphatase were purchased from Sigma. RPMI-1640 cell culture medium and fetal-calf serum were purchased from Gibco. Tissue-culture plasticware was purchased from Falcon. Protein G Fast Flow Sepharose was from Pharmacia. Dithiothreitol, ATP and acrylamide were obtained from Amresco, Bio-Rad and Sigma. Nitrocellulose membranes were from Schleicher and Schuell and Bio-Rad. Reagents and secondary antibodies for the chemiluminescent assay were purchased from Boehringer-Mannheim. Caldesmon and calponin were isolated from chicken gizzard as described by Scott-Woo and Walsh [29] and Winder and Walsh [30] respectively. Polyclonal antibodies against purified chicken gizzard caldesmon and calponin were raised in rabbits and the respective IgG fractions were purified by adsorption to Protein A-Sepharose [31].

Monoclonal antibody production

Mice (BALB/c) were immunized by injecting 20–50 µg of chicken gizzard caldesmon with Freund's complete adjuvant subcutaneously every 7 days for 4 weeks. A final injection of 100 µg caldesmon without adjuvant was given intraperitoneally four days before fusion. A mouse which demonstrated a titre towards caldesmon was killed, the spleen was removed and spleen cells were fused with mouse myeloma cells (SP2/o AG14) [32] according to standard methods [33], but with the modifications described by Araki et al. [34] and Higashihara et al. [35]. Cells were cultured in hypoxanthine-aminopterin-thymidine-supplemented medium for two weeks, followed by culture in hypoxanthine-thymidine-supplemented medium for two weeks and then cells were maintained in RPMI-1640 containing 10% (v/v) fetal-calf serum. Ten days after fusion, the caldesmon antibody titre was assessed by ELISA. Fifty-six positive cultures were expanded to 24-well plates and supernatant antibodies were analysed by immunoblot of α -chymotrypsin- and NTCB-digested caldesmon. Twenty cultures were selected for cloning by limiting dilution using non-immunized mouse spleen cells as a feeder layer. After dilution, cells were incubated for 5–7 days, without moving the plate, to allow colonies to become visible. Wells with only single visible colonies, from the 1 or 0.5 cells/well section of the plate, were tested by ELISA for caldesmon antibody titre. Positive colonies were expanded to 24-well plates and antibody supernatant was assessed by immunoblot of α -chymotrypsin-digested caldesmon. Fourteen clones were selected and recloned by a second limiting dilution. Clonal cell lines were cultured and 1×10^6 to 5×10^6 cells were injected intraperitoneally into pristane-primed mice (0.5 ml injected intraperitoneally 2 weeks before and 4 days before hybridoma injection) for ascites production. The mice were killed 8–10 days later, ascites fluid was removed from the intraperitoneal cavity, clarified by centrifugation and the supernatant was stored at -80°C to await antibody purification. Antibodies were purified from the ascites fluid by ammonium sulphate fractionation [35] followed by affinity chromatography on Protein G-Fast Flow Sepharose. Antibodies were eluted with 0.1 M glycine (pH 2.7) and immediately neutralized with 50 µg/ml of Trizma base. These antibodies were designated CDg-1 to CDg-13.

Anti-myosin-heavy-chain monoclonal antibody (MM-21) was produced as described previously [35] using turkey gizzard myosin as an antigen. Anti-myosin monoclonal antibody to residues

23–39 of cardiac myosin (11E10) [36] was kindly provided by Dr. Dominique Mornet, INSERM U300, Montpellier, France.

Digestion of caldesmon

Caldesmon was digested with α -chymotrypsin (1:100, w/w) in 20 mM Tris/HCl (pH 7.5)/50 mM KCl/1 mM EGTA/0.5 mM dithiothreitol at 25°C . Samples were removed at selected times and the reaction was stopped by the addition of 1 mM diisopropyl fluorophosphate. An equal volume of SDS-gel sample buffer [0.05 M Tris/HCl (pH 6.8)/1% (w/v) SDS/0.01% (w/v) Bromophenol Blue/30% (v/v) glycerol/2% (v/v) 2-mercaptoethanol] was added, the sample boiled for 2–5 min and subjected to SDS/PAGE as described below.

Caldesmon was digested with NTCB (1 mM) in 20 mM Tris/HCl (pH 8.0)/1 mM EGTA at 25°C for 1 h. Digestion was initiated by the addition of 16 µl/ml of 1 M Trizma base/1 M NaOH (1:1, v/v) to bring the pH to 9.0. The reaction was stopped at selected times by the addition of an equal volume of SDS-gel sample buffer.

Gel electrophoresis and immunoblot analysis

SDS/PAGE was carried out in 7.5–20% polyacrylamide gradient slab gels (1.5- or 0.75-mm thick) with 5% acrylamide stacking gels in the presence of 0.1% SDS at 8 mA (large gel) or 20 mA (minigel), using the discontinuous buffer system of Laemmli [37]. Gels were stained in 45% ethanol/10% acetic acid containing 0.1% (w/v) Coomassie Brilliant Blue R-250 and diffusion destained in 10% acetic acid. For Western blotting, the peptides were blotted on to nitrocellulose as described by Higashihara et al. [35]. The nitrocellulose was cut into strips and each strip was incubated individually with hybridoma culture medium supernatant for 30–60 min at room temperature. The strip was washed with PBS/0.5% (v/v) Tween-20 and incubated for 30–60 min with horseradish-peroxidase-conjugated anti-mouse IgG [F(ab')₂]. The strips were washed and antibodies were visualized by incubating in 4-chloro-1-naphthol substrate solution [0.05% (w/v) 4-chloro-1-naphthol/1.6% (v/v) methanol/50 mM Tris/HCl (pH 7.5)/200 mM NaCl containing 33 µl/100 ml of H₂O₂]. For Western blotting, the proteins were separated by SDS/PAGE, blotted on to nitrocellulose in buffer containing 25 mM Tris, 192 mM glycine, 20% methanol, and 0.1% SDS. Antibody labelling was visualized by the alkaline phosphatase, 5-bromo-4-chloro-3-indolylphosphate/Nitroblue Tetrazolium reaction or by the horseradish peroxidase chemiluminescent method using substrates from Boehringer-Mannheim as recommended by the manufacturer.

Permeabilization of cardiac myocytes

Isolated cardiac myocytes from adult rat ventricle were prepared as described by Rodrigues et al. [38]. Isolated cardiac myocytes from adult rabbit hearts were provided by Dr. Andrew P. Braun and Dr. Wayne Giles and myocytes from guinea pig hearts by Dr. William Cole, all of the University of Calgary, Calgary, Alberta, Canada. The myocytes were permeabilized with Triton X-100 using the methods of Kargacin and Fay [39] for isolated smooth muscle cells. EGTA (final concentration 2 mM) was added to suspensions of isolated cells in PBS. The suspension was allowed to equilibrate for 5 min and then centrifuged at low speed (10 g) in a bench-top centrifuge. The supernatant was removed with a pipette and the cells were resuspended in Ca²⁺-free solution (150 mM potassium methanesulphonate, 5 mM EGTA, 3 mM magnesium methanesulphonate, 10 mM piperazine-N,N'-bis(2-ethanesulphonic acid), pH 7.0). After equili-

bration for 5 min in Ca^{2+} -free solution, Triton X-100 was added to a final concentration of 0.1 % (v/v), the cells were incubated for 7 min and then centrifuged for 5 min at 10 *g*. The cells were washed for 5 min in Ca^{2+} -free solution, centrifuged for 5 min and resuspended in the appropriate solution, depending on the experiment (see below).

Fixation of cells with paraformaldehyde

Freshly isolated cardiac myocytes in suspension were washed once by centrifugation (10 *g*) for 5 min and resuspended in PBS. After a second 5-min centrifugation step, the cells were resuspended in a filtered PBS solution containing 1 % paraformaldehyde and 0.1 % Triton X-100 for 15 min. The suspension was then centrifuged for 5 min as above and resuspended in PBS. After two 5-min wash steps, the cells were finally resuspended in PBS containing 2 % (w/v) BSA and labelled with antibodies as described below.

Antibody labelling of fixed and Triton-X-100-permeabilized cardiac myocytes for light microscopy

For labelling permeabilized cardiac myocytes with antibodies for light microscopy, the washed cells (prepared as described above) were resuspended in a small volume (0.5–1 ml) of Ca^{2+} -free solution containing 2 % (w/v) BSA and divided into individual samples (50–100 μl each) in microcentrifuge tubes. Primary antibodies 11E10, MM-21 and the antibodies to caldesmon were added to the tubes at a final dilution of 1:100 for immunofluorescence labelling; the primary antibody to α -actinin (anti-sarcomeric α -actinin, clone EA-53; Sigma) was used at a final dilution of 1:200. The cell suspensions were then incubated at 4 °C either overnight or for shorter times, centrifuged for 5 min at 10 *g* and washed twice using the 5-min incubation and centrifugation steps described above. After the second wash, the cells were resuspended in 100 μl of Ca^{2+} -free solution with BSA and secondary antibodies, conjugated to either FITC or TRITC, were added to a final antibody concentration of 1:100 and 1:150. The cells were incubated at room temperature for 30–60 min, centrifuged and washed twice in Ca^{2+} -free solution. For experiments in which the co-localization of caldesmon and other proteins was studied, primary antibodies to the proteins of interest were added, in some cases simultaneously and, in other cases, the primary antibodies were added sequentially (with a wash step in between). Similar simultaneous or sequential additions were used for incubations with TRITC- or FITC-conjugated secondary antibodies. Cells were incubated with secondary antibodies at room temperature for 30–60 min, centrifuged and washed twice in Ca^{2+} -free solution as described above. Fluorescent labelling of wet mounts of cells (6 μl of cells in 95 % glycerol) was observed using an Olympus BHT2 epifluorescence microscope and a Nikon 100 $\times$ oil immersion objective (NA 1.25). For dual-label experiments, dichroic mirrors and appropriate band pass filters in both the excitation and emission light paths of the microscope were used to prevent cross-over of the TRITC and FITC fluorescence signals.

Fixed cells were immunofluorescently labelled as described above except that PBS was substituted for Ca^{2+} -free solution in all steps.

RESULTS

Identification of caldesmon in atrial and ventricular myocytes

To determine if caldesmon is expressed in adult cardiac tissue, enzymically-dissociated isolated myocytes derived from rat ven-

Figure 1 Caldesmon expression in ventricular myocytes from rat heart

Adult rat ventricular myocytes (approx. 4×10^6 cells) were suspended in PBS, diluted 1:1 with SDS-gel sample buffer and boiled before SDS/PAGE and Western blotting. The final protein concentration was 3.3 mg/ml. Western blot, using a polyclonal antibody to caldesmon, of (lane 1) purified chicken gizzard caldesmon (10 ng) and (lane 2) rat ventricular myocytes (165 μg total protein). Lanes 3 and 4, as in lanes 1 and 2, respectively, but with purified chicken gizzard caldesmon (2 μg) added to the antibody buffer (containing 0.25 μg IgG). Note the reduced labelling in lanes 3 and 4 when purified caldesmon was present in the antibody buffer to compete with binding of the antibody to the trans-blotted protein. Molecular masses (kDa) are indicated on the left; the molecular-mass markers were: myosin (204), β -galactosidase (121), BSA (82), ovalbumin (50.2), carbonic anhydrase (34.2), soybean trypsin inhibitor (28.1), lysozyme (19.4) and aprotinin (7.3). h-CaD, high-molecular mass caldesmon.

Figure 2 Western blots showing the absence of calponin from cardiac myocyte extracts

Myocytes were prepared for SDS/PAGE and Western blotting as described in the legend to Figure 1. Western blots, using a polyclonal antibody to calponin (CaP) of (lane 1) purified chicken gizzard calponin (43 ng), (lane 2) rat uterine smooth muscle (30 μl of tissue homogenate) and (lane 3) rat ventricular myocytes (165 μg total protein). Lanes 4–6 as lanes 1–3, respectively, but with purified chicken gizzard calponin (140 μg , + CaP) present in the antibody buffer (containing 124 μg IgG). Note the absence of labelling by the antibody in the cardiac myocytes (lanes 3 and 6). Also note the reduced labelling of purified calponin when calponin, present in the antibody buffer, competed with binding to the trans-blotted protein (lanes 4 and 5). Lanes 2 and 5 verify cross-reactivity of anti-chicken gizzard calponin with the rat protein. Molecular masses (kDa) are indicated on the left; molecular-mass markers were: carbonic anhydrase (34.2, upper bar), soybean trypsin inhibitor (28.1, middle bar) and lysozyme (19.4, bottom bar).

Figure 3 Digestion map of caldesmon

(A) Native (141 kDa) caldesmon (0.3 mg/ml) was digested with α -chymotrypsin. Coomassie-Blue-stained SDS/polyacrylamide gels were analysed as described in the Materials and methods section. Lanes 1–9 (numbered from the left) show caldesmon digested for 0, 2, 4, 6, 8, 10, 15, 20 and 30 min respectively. (B) The 93 kDa natural proteolytic fragment of caldesmon was digested with α -chymotrypsin for similar times and separated by SDS/PAGE. (C) Native (141 kDa) caldesmon (0.3 mg/ml) was digested with NTCB and separated by SDS/PAGE. Lanes 1–13 (numbered from the left) show caldesmon digested for 0, 1, 2, 3, 5, 10, 17, 30, 45, 60, 120, 180 min and 24 h respectively. (D) The 93 kDa natural proteolytic fragment of caldesmon was similarly digested with NTCB and subjected to SDS/PAGE. Molecular masses (kDa) are shown on the left of each panel; molecular-mass markers were: smooth muscle myosin heavy chain (205), β -galactosidase (116), phosphorylase b (97), BSA (66), ovalbumin (45), carbonic anhydrase (29) and smooth muscle myosin light chains (20 and 17). The positions of the major digestion products are shown on the right of (A) and (C); the numbers refer to approximate molecular masses of the products (see Results section). (E) Composite map of caldesmon primary structure derived from the digestion of caldesmon. The molecular mass of each fragment is indicated.

tricular or rabbit atrial or ventricular tissue were homogenized and proteins in the homogenate were separated by SDS/PAGE and transferred to nitrocellulose. Western blots of the rat ventricular homogenate labelled with a polyclonal antibody to caldesmon are shown in Figure 1. Purified chicken gizzard caldesmon (lane 1) and bands at approx. 150 and 70 kDa in the rat ventricular extracts (lane 2) were labelled with the antibody. The approx. 70 kDa protein was not seen in gizzard but it may be similar to the lower molecular-mass caldesmon isoform (*I*-caldesmon) found in other tissues [4]. When purified gizzard caldesmon was added to the antibody before Western blotting, labelling of the purified caldesmon sample was greatly reduced

(lane 3) and the proteins from the cardiac myocytes were not labelled (lane 4).

Since caldesmon is present at relatively high concentration in some smooth muscle cells [22], the caldesmon identified by the antibody (Figure 1) might be due to the presence of vascular smooth muscle cells in the cardiac myocyte preparations. To rule out possible contamination of the myocyte preparations with smooth muscle cells, an antibody to calponin, a protein specifically found in smooth muscle but not in cardiac muscle [40,41] was used. As shown in Figure 2, the antibody labelled purified chicken gizzard calponin (lane 1) and rat uterine smooth muscle calponin (lane 2) but did not label proteins in the ventricular

Figure 4 Immunoblot pattern of anti-caldesmon monoclonal antibodies (CDg1-CDg13)

A: Native (141 kDa) caldesmon was digested with α -chymotrypsin. The peptides were separated by SDS/PAGE and blotted onto nitrocellulose. The nitrocellulose was cut into strips, each strip was incubated individually with hybridoma culture medium supernatant and peptides were visualized as described in the Materials and methods section. The primary antibody used for each strip is indicated below each lane. Labelling with CDg-6 was a separate experiment and is shown to the far right of (A). **(B)** Native (141 kDa) caldesmon was digested with NTCB, the reaction was stopped after 3 h and peptides were separated, blotted and detected as described in (A). The primary antibody used for each strip is indicated below each lane. The positions of the molecular-mass markers (kDa) are shown on the left of (A) and (B) and on the right of lane 6. The molecular mass markers used were as for Figure 3. The positions and molecular masses of the major proteolytic fragments described in Figure 3 are shown to the right of the main panel in (A) and to the right in (B).

myocyte preparation (lane 3). The labelling of gizzard and uterine smooth muscle calponin was inhibited when purified chicken gizzard calponin was added to the antibody. The results shown in Figure 2 (lanes 2 and 5) also verify cross-reactivity of anti-calponin with rat uterine calponin which has a slightly higher molecular mass than the chicken gizzard protein (33 kDa, lanes 1 and 4). This is consistent with the high degree of sequence similarity between chicken gizzard and mammalian (rat, mouse and pig) calponins (87% sequence identity in the first 273 residues) [42,43]. Since calponin is present in smooth muscle tissue at approximately the same concentration as tropomyosin (1 calponin: 7 actin monomers) [44] and is, therefore, more abundant than caldesmon, our failure to detect calponin in the cardiac preparations indicates that there was no detectable smooth muscle contamination of the cardiac myocytes.

Characterization of monoclonal antibodies to caldesmon

A composite map of caldesmon primary structure derived from the digestion of caldesmon by chymotrypsin (Figures 3A and 3B) and NTCB (Figures 3C and 3D) is shown in Figure 3(E). Approximate molecular masses were determined for each fragment and are in agreement with previously published proteolytic digestion maps [45]. Where small discrepancies existed between

our fragment-size calculation and the previously published fragment size, the latter is indicated on the right of Figures 3(A) and 3(C).

An array of monoclonal antibodies to caldesmon (CDg-1-CDg-13) was generated as described in the Materials and methods section. These antibodies were mapped to the primary structure of caldesmon (Figure 3) by their reaction in immunoblot analysis to chymotryptic and NTCB-generated caldesmon fragments (Figure 4). These results are summarized in Table 1. A few antibodies had very similar cross-reactivities but these could be distinguished as described below. The CDg-7 epitope was very close to CDg-5 but could be distinguished by its cross-reactivity with a 20 kDa chymotryptic peptide that was not recognized by CDg-5. Also, CDg-7 did not recognize a 66 kDa chymotryptic peptide that was detected by CDg-5, 8, and 9. These minor peptides were probably derived from the 110 or 93 kDa naturally proteolysed caldesmon that contaminated the native caldesmon preparation. The epitopes for CDg-1 and CDg-2 were very similar but CDg-2 weakly reacted with a 50 kDa peptide in the NTCB digest that was not recognized by CDg-1. The epitope for CDg-2, therefore, was in the 25 kDa N-terminal peptide but was C-terminal to CDg-1, since it reacted with a larger peptide (50 kDa) that was probably a truncated form of the 110 kDa NTCB fragment that was generated from the 110 kDa natural proteolytic fragment. CDg-8 could be distinguished from CDg-9 by weak cross-reactivity with a 24 kDa peptide in the NTCB digest. This was confirmed in a separate experiment (results not shown). These minor peptides were probably derived from the 110 or 93 kDa contaminants from naturally proteolysed caldesmon. Based on these results the antibody epitope map shown in Figure 5 was constructed.

Western-blot analysis of bovine cardiac caldesmon with the monoclonal antibodies

Twelve monoclonal antibodies were selected from the array described above to investigate possible similarities and differences between bovine cardiac and chicken gizzard caldesmons. Antibodies with a low titre or having a low affinity for gizzard caldesmon were not selected. Western blotting of purified chicken gizzard smooth muscle caldesmon and partially-purified bovine ventricular muscle caldesmon is shown in Figure 6. As expected, all antibodies recognized the chicken gizzard protein. However, only monoclonal antibodies CDg-2, 3, 4 (result not shown), 5, 6, and 13 recognized the bovine cardiac protein; CDg-1, 7, 8, 9, 10, 11, and 12 failed to cross-react or cross-reacted only weakly with bovine cardiac caldesmon. In the results of an experiment similar to that shown in Figure 1(B), the cross-reactivity of the monoclonal antibody (CDg-3) with the strongest immunoreactivity to cardiac muscle caldesmon (see Figure 6B) was abolished following prior incubation of the antibody with chicken gizzard caldesmon (results not shown). When the Western-blot results shown in Figure 6 are interpreted with reference to the epitope map (Figure 5), they suggest that the bovine cardiac protein differs significantly in primary structure from the chicken smooth muscle protein, particularly in the C-terminal actin-, Ca^{2+} /calmodulin- and tropomyosin-binding domain. There may also be some differences in the N-terminal myosin-binding domain.

Localization of caldesmon in isolated cardiac myocytes

To study the specific localization of caldesmon in cardiac muscle, both polyclonal and monoclonal antibodies to caldesmon and secondary antibodies conjugated to TRITC were used to immunofluorescently label the protein in permeabilized cells. The

Table 1 Antibody characterization

The characterization of the heavy/light chain isotypes of the antibodies to caldesmon (CDg-1-CDg-13) was determined using a Screen/Isotyping kit (Boehringer-Mannheim) according to the manufacturer's protocol. †, two bands reacted.

Antibody	Heavy/light chain isotype	Chymotryptic peptides (kDa) ...	Immunoblot reactivity								NTCB peptides (kDa) ...	110	80	25	23
			110	85	60	35	29	28	22/20	11					
CDg-1	IgG ₁ /κ		+	+	—	—	—	+	—	—		+	—	+	—
CDg-2	IgG ₁ /κ		+	+	—	—	—	+	—	—		+	—	+	—
CDg-3	IgG _{2b} /κ		+	+	+	—	—	—	—	—		+	+	—	—
CDg-4	IgG ₁ /κ		+	+	—	—	—	—	—	—		+	+	—	—
CDg-5	IgG _{2b} /κ		+	+	—	+	+	—	—	—		+	+	—	—
CDg-6	not done		—	+	+	—	—	—	—	—		not done			
CDg-7	IgG _{2b} /κ		+	+	—	+	+	—	—	—		+	+	—	—
CDg-8	IgG ₁ /κ		+	—	—	+	+	—	—	—		+	+	—	—
CDg-9	IgG ₁ /κ		+	—	—	+	+	—	—	—		+	+	—	—
CDg-10	IgG ₁ /κ		—	—	—	+	+	—	+	+		—	—	—	+
CDg-11	IgG ₁ /κ		—	—	—	+	—	—	+	—		—	—	—	+
CDg-12	IgG ₁ /κ		—	—	—	+	—	—	+	—		—	—	—	+
CDg-13	IgG ₁ /κ		—	—	—	+	—	—	+	—		—	—	—	+

Figure 5 Epitope map for caldesmon monoclonal antibodies

This map was constructed on the basis of the results shown in Figure 4 (summarized in Table 1). The uppermost bar represents the primary structure of caldesmon with the major chymotryptic cleavage sites (▼) and NTCB digestion sites (△) indicated. The lines represent the proteolytic fragments obtained and the numbers refer to the epitopes mapped to that fragment (e.g. 1 = CDg-1, 2 = CDg-2).

polyclonal antibody to caldesmon revealed a striated pattern for the distribution of caldesmon in fixed rat (Figure 7A) and unfixed permeabilized rat (Figure 7B), rabbit (Figure 7C) and guinea pig (Figure 7D) cardiac myocytes. Labelling was also apparent around, but not within, the nucleus (see Figure 7D). A similar pattern of staining (Figures 8F and 8G) was found with two of the monoclonal antibodies to caldesmon (CDg-4, CDg-5) that cross-reacted with cardiac muscle caldesmon on Western blots (see Figure 6). The striated pattern of labelling was not seen (Figure 7H) with a third monoclonal antibody to caldesmon (CDg-8) that did not cross-react with cardiac muscle caldesmon on Western blots (see Figure 6). Secondary antibodies alone did not label the cardiac myocytes (results not shown). The results obtained in immunofluorescence experiments with a number of the monoclonal antibodies showed that striations were visible in cells labelled with CDg-2, CDg-4, -5 and -6 and in CDg-9, where the reactivity was weak but striations were still apparent (results not shown). These results were generally consistent with those obtained from bovine ventricular tissue using Western-blot analysis (Figure 6), although CDg-3, which strongly cross-reacted with bovine tissue, did not immuno-

Figure 6 Western blot of chicken gizzard and bovine ventricular caldesmon using monoclonal antibodies CDg-1-CDg-13

(A) Purified chicken gizzard caldesmon (0.6 µg/lane) and (B) partially-purified bovine ventricular caldesmon (144 µg/lane) were electrophoresed, transferred to nitrocellulose and incubated with the primary antibodies indicated below each lane, followed by a secondary antibody coupled to alkaline phosphatase to allow detection of the immunoreactive bands. *, polyclonal anti-(chicken gizzard caldesmon); #, absence of primary antibody.

fluorescently label rat cardiac myocytes. This may indicate that the CDg-3 epitope is inaccessible to the antibody in the native cardiac protein. It is unlikely to be due to species differences since CDg-3 cross-reacted strongly with rat uterine smooth muscle caldesmon on Western blots (results not shown).

The striated pattern of staining seen with the caldesmon antibody and the spacing of the striations ($\approx 2 \mu\text{m}$) were comparable with the sarcomere spacing in the myocytes revealed by antibodies to myosin (Figure 7E) or α -actinin. These results suggested that caldesmon is associated with the sarcomere in cardiac muscle. To explore this distribution in greater detail, dual-labelling experiments were carried out to allow comparison

Figure 7 Localization of caldesmon and myosin in cardiac myocytes from various mammalian sources

(A) Paraformaldehyde-fixed myocyte from rat heart, (B–D) unfixed myocytes, (B and E–H) Triton X-100-permeabilized myocytes from rat, (C) rabbit and (D) guinea pig heart. Cells were fixed or permeabilized and labelled with primary and secondary antibodies as described in the Materials and methods section. A polyclonal antibody to caldesmon was used in (A–D) and MM-21 in (E). The monoclonal antibodies to caldesmon CDg-4, CDg-5 and CDg-8 were used in (F–H) respectively. Goat (anti-rabbit TRITC)-conjugated secondary antibodies were used for (A–D); goat (anti-mouse TRITC)-conjugated secondary antibodies were used for (E–H). Scale bar = 10 μ m.

of the distributions of caldesmon and myosin or caldesmon and α -actinin in the same cells. Figures 8(A) and 8(B) are fluorescence micrographs from a single guinea pig cardiac myocyte labelled with the polyclonal caldesmon antibody and MM-21 respectively.

Figures 8(C), 8(D) and 8(E) allow a more detailed comparison of the two labels in a second cell. Figure 8(E) was created by overlaying the negatives of Figures 8(C) and 8(D). The two proteins did not co-localize but, instead, the caldesmon striations

Figure 8 Dual labelling of caldesmon and myosin and caldesmon and α -actinin in fixed and Triton X-100 permeabilized cardiac myocytes from various mammalian sources

Dual labelling of (A) caldesmon and (B) myosin in a single guinea pig cardiac myocyte. Dual labelling of (C) caldesmon and (D) myosin in a single rat cardiac myocyte. (E) was made by aligning and overlaying the negatives of (C) and (D) before printing. Co-localization of (F) caldesmon and (G) α -actinin in a single cardiac myocyte from guinea pig. To aid visualization, the negative of (G) was reversed to obtain a mirror image. Cells used in (A–G) were permeabilized (but not fixed) and labelled with primary and secondary antibodies as described in the materials and methods section. (H) Dual labelling of caldesmon (top) and myosin (bottom) using 11E10 in a paraformaldehyde-fixed rat single cardiac myocyte. Note the labelling of the A-band (A) but not the Z-line (Z) or the M-line (M) with 11E10 (bottom panel). (I) Dual labelling of a paraformaldehyde-fixed rat single cardiac myocyte with polyclonal anti-caldesmon and MM-21. For (A–H), an FITC-conjugated goat anti-rabbit secondary antibody was used for labelling the caldesmon primary antibodies and a TRITC goat anti-mouse secondary antibody was used for labelling the myosin and α -actinin primary antibodies. Dual-labelled cells were viewed using a fluorescence microscope with the appropriate band-pass filters and dichroic mirrors as described in the Materials and methods section. For (I), TRITC-conjugated secondary antibodies were used to label both caldesmon and myosin. Scale bars = 10 μ m.

lay between the striations labelled by the myosin antibody. These results indicate that caldesmon is localized at the centre of the I-band in cardiac muscle rather than in the A-band delineated by the myosin label. In Figures 8(F) and 8(G), the distribution of caldesmon in a guinea pig cardiac myocyte is compared with that

of α -actinin, a protein found in the Z-disks of striated muscle and consequently localized at the centre of the I-band. Comparison of Figures 8(F) and 8(G) lends further support to the conclusion that caldesmon is localized primarily in the centre of the I-band in cardiac muscle.

Based on the actin and myosin binding properties of caldesmon, we expected to see the caldesmon and myosin antibodies co-localized to the same structures in cardiac muscle cells. We would have expected the α -actinin label to lie between the striations labelled with the caldesmon antibody. As described above, this was clearly not the case. This unexpected finding motivated us to conduct additional experiments with the caldesmon and myosin antibodies to exclude reasons for artifactual labelling of the Z-lines by the caldesmon antibody. (i) As can be seen in Figures 7(F), 8(B), 8(D) and 8(E), the fluorescence labelling with MM-21 was confined to a fairly narrow band, indicating that the protein epitope recognized by MM-21 was located or accessible to the antibody only in the central portion of the cardiac thick filament. Similarly, caldesmon could be present in the A-band but inaccessible to the antibodies. (ii) When cardiac myocytes were permeabilized in Ca^{2+} -free solution, the average sarcomere length ($1.7 \pm 0.1 \mu\text{m}$, mean $\pm$ S.D.) of 93 sarcomeres measured in six cells fluorescently labelled with the α -actinin antibody was significantly less ($P = 0.005$) than those of 38 sarcomeres in three cells labelled with the MM-21 antibody and 72 sarcomeres in three cells labelled with the 11E10 myosin antibody, which were fixed before permeabilization ($2.1 \pm 0.3 \mu\text{m}$, mean $\pm$ S.D.). In cells with a short sarcomere length, localization of caldesmon at the ends of the thick filaments might make it appear to be localized at or near the Z-lines. (iii) Mis-registration of the fluorescence images made with different filter sets could make two proteins appear to be located in different places and (iv) The caldesmon antibody might be artifactually concentrated at the Z-lines. To rule out the first two possibilities, fixed cells (average sarcomere length $\approx 2 \mu\text{m}$, see above) were dual labelled with anti-caldesmon and a different myosin antibody (11E10). As can be seen in Figure 8(H), 11E10 labelled a broader band in the sarcomere than that labelled with MM-21 (compare with Figures 8B, 8D and 8E). The antibody did not label the Z-lines or the M-line in the fixed myocytes, however. This indicates that the caldesmon labelling was indeed present in the region of the Z-line (upper panel in Figure 8H) and not at the ends of the thick filaments. When exogenous caldesmon was added to cells along with anti-caldesmon labelling was clearly seen in the A-band, indicating that exogenously applied caldesmon could bind to the cardiac contractile proteins and be labelled by the antibody (G. C. Scott-Woo and G. J. Kargacin, unpublished work). Although it seems unlikely, it is possible that a slight mis-alignment of the FITC and TRITC images in Figures 8(C–G) could have resulted in the erroneous localization of caldesmon at the centre of the I-band. To rule out this possibility, dual-label experiments were done using TRITC secondary antibodies to label both primary antibodies. Although the specific localization of either of the primary antibodies cannot be determined under these conditions, the presence of one primary antibody at the centre of the I-band and the second primary antibody at the M-line would result in a striated pattern (in a single micrograph with a single set of filters), with striations spaced at $\approx 1 \mu\text{m}$ intervals. Figure 8(I) shows that this was the case when anti-caldesmon and MM-21 were used as primary antibodies. It seems unlikely that there was an artifactual concentration of the anti-caldesmon label at the Z-lines, since some of the caldesmon antibodies labelled the Z-lines but others did not (Figure 7). To further rule out this possibility, the concentrations of the polyclonal caldesmon antibody and secondary antibodies and the time of exposure of the cells were varied in some experiments. Primary antibody dilutions ranging from 1:100 to 1:1000 were used with exposure times of 5–24 h at 4°C . Secondary antibodies diluted 1:100 or 1:150 were also used with exposure times from 30–60 min. If either of the antibodies was artifactually concentrated at the Z-lines, perhaps

in the T-tubules, we would have expected to see an increase in fluorescence labelling when either the concentration or exposure time was increased. This, however, was not the case and no obvious differences in fluorescence intensity (extent of labelling) in any experiments were observed.

DISCUSSION

The results of this work clearly demonstrate the presence of caldesmon in cardiac myocytes from three mammalian species (rabbit, guinea pig and rat) and in bovine ventricular muscle. The apparent molecular mass of the cardiac *h*-caldesmon (≈ 150 kDa) was similar to that of gizzard *h*-caldesmon (≈ 141 kDa; see also Discussion in [2]); that of the cardiac *l*-caldesmon (≈ 70 kDa) was similar to that of gizzard *l*-caldesmon (70–80 kDa; see Discussion in [4]). The use of monoclonal antibodies to chicken gizzard smooth muscle caldesmon showed that chicken gizzard and bovine cardiac proteins share several epitopes but differ significantly in amino acid sequence, particularly within the C-terminal domain. Caldesmon was shown by Western blotting to be present in both atrial and ventricular myocytes isolated from rabbit cardiac muscle. The inability of Ban et al. [24] and Lu et al. [25] to detect caldesmon in cardiac tissue and cardiac myocytes in culture, respectively, may have been due to a relatively low abundance in the heart, as suggested by the weaker fluorescence of the isolated myocytes when the caldesmon labelling is compared with that of α -actinin or myosin (Figures 7 and 8). Comparison of Western blots of caldesmon in chicken gizzard and bovine ventricular tissue also suggests that the caldesmon content of the heart is significantly less than that of smooth muscle tissue (G. C. Scott-Woo, M. P. Walsh and G. J. Kargacin, unpublished work). It is also possible that the caldesmon antibodies used by Ban et al. [24] and Lu et al. [25] did not recognize the isoform of the protein found in cardiac cells. Finally it is possible that caldesmon may not be expressed in cardiac cells in culture; it is known that the morphological, electrophysiological, mechanical and functional properties and the expression of other cellular proteins can change with time in cardiac cells in culture [46–48].

In isolated adult cardiac myocytes, polyclonal and monoclonal antibodies to caldesmon localized to the region around the nucleus and to specific structures with a periodic spacing equal to the spacing of the sarcomeres. There also appeared to be some cytoplasmic labelling, which was not apparent in cells labelled with MM-21 or α -actinin antibodies. In some micrographs labelling with the caldesmon antibodies appeared somewhat punctate (see Figure 7G) and the punctate structures generally were associated with the striations that were also labelled with the antibodies. One might argue that the labelling seen with the polyclonal antibody to caldesmon was the result of non-specific labelling, incomplete washout of the primary or secondary antibodies in one part of the sarcomere (perhaps because of restricted diffusion in a region where filamentous structures are abundant) or labelling of a non-caldesmon epitope by the polyclonal antibody. These possibilities are unlikely, however, since a similar pattern of staining was seen with five of the monoclonal antibodies to caldesmon but not with seven others. Striations were not seen with antibodies to filamin and myosin light chain kinase (results not shown) or with secondary antibodies alone. Since both fixed, intact and unfixed, permeabilized myocytes were used in our experiments and the distribution of caldesmon was found to be similar under both conditions, it is unlikely that this pattern was the result of either fixation or permeabilization. It is also of note that Triton X-100-permeabilized myocytes (from both rabbit and rat heart), labelled with

either the polyclonal or monoclonal primary antibodies to caldesmon and with anti-rabbit or anti-mouse secondary antibodies, respectively, were able to contract rapidly in the presence of Ca^{2+} and ATP (G. J. Kargacin, unpublished work). This lends further support to the conclusion that a significant rearrangement of protein structures did not occur during the preparation or labelling of the permeabilized myocytes.

The presence of caldesmon at the centre of the I-band in the myocytes, revealed by the dual-label experiments with antibodies to caldesmon and myosin, and its apparent co-localization with α -actinin, suggests that caldesmon may be localized to the Z-disks. At present, however, the possibility that caldesmon is associated with the actin filaments in this region, consistent with its known actin and tropomyosin binding properties, cannot be ruled out. It is also possible that caldesmon is associated with structures not within the Z-disk itself but more peripheral to it. This would account for the punctate appearance of some of the caldesmon labelling (Figure 7G), the more abundant cytoplasmic staining that was apparent in the caldesmon labelled cells, and the presence of caldesmon around the nucleus (Figure 7D).

Our results argue against the hypothesis that caldesmon is directly involved in the regulation of the interaction of actin and myosin in relaxed cardiac myocytes, since it localized to a site that did not overlap with the thick filaments. It is perhaps significant, in this context, that the affinity of skeletal muscle heavy meromyosin for smooth muscle caldesmon is significantly lower than that of smooth muscle heavy meromyosin [17]. It is possible, however, that an interaction of caldesmon with both actin and myosin filaments could occur as the thick filaments approach the centre of the I-band during contraction. It has been suggested that caldesmon is involved in microfilament assembly in non-muscle cells [7,8] and the results presented here are consistent with a similar role for caldesmon in cardiac muscle. In light of the observation that caldesmon is localized to the centre of the I-band in cardiac myocytes and co-localized with α -actinin, it is of particular interest to note that Galazkiewicz et al. [8] suggested that caldesmon and α -actinin might act synergistically to regulate actin polymerization. The fairly high abundance of caldesmon in smooth muscle may be consistent with a regulatory and/or a structural role whereas its lower abundance in cardiac muscle may be consistent with only one of these roles.

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