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Ultrastructural Distribution of the S100A1 Ca²⁺-Binding Protein in the Human Heart

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Summary

Impaired calcium homeostasis and altered expression of Ca²⁺-binding proteins are associated with cardiomyopathies, myocardial hypertrophy, infarction or ischemia. S100A1 protein with its modulatory effect on different target proteins has been proposed as one of potential candidates which could participate in these pathological processes. The exact localization of S100A1 in human heart cells on the ultrastructural level accompanied with biochemical determination of its target proteins may help clarify the role of S100A1 in heart muscle. In the present study the distribution of the S100A1 protein using postembedding (Lowicryl K4M) immunocytochemical method in human heart muscle has been determined quantitatively, relating number of antigen sites to the unit area of a respective structural component. S100A1 antigen sites have been detected in elements of sarcoplasmic reticulum (SR), in myofibrils at all levels of sarcomere and in mitochondria, the density of immunolabeling at Z-lines being about 3 times and at SR more than 5 times higher than immunolabeling of remaining structural components. The presence of the S100A1 in SR and myofibrils may be related to the known target proteins for S100A1 at these sites.

Key words

Human heart muscle • Ca²⁺-binding proteins • S100A1 • Immunocytochemistry

Introduction

Calcium ions as second messengers play an important role in the regulation of large number of cellular processes such as muscle contraction, secretion, cell division, cell cycle progression, energy production, proliferation, gene transcription and differentiation (Berridge 1997). The key molecules to transduce Ca²⁺

signaling are Ca²⁺-binding proteins mainly belonging to the large EF-hand protein superfamily (Heizmann and Hunziker 1991, Kawasaki *et al.* 1998).

Since the first isolation of S100 proteins from bovine brain (Moore 1965) they have become the largest subfamily of EF-hand Ca²⁺-binding proteins (Zimmer *et al.* 1995, Schäfer and Heizmann 1996, Heizmann and Cox 1998, Donato 1999). S100 proteins are acidic proteins of low molecular weight (10-12 kDa) and are

characterized by two distinct EF-hand domains with different affinities for Ca^{2+} . To date, 19 different proteins which display various degree of amino acid sequence homology, have been assigned to the S100 protein family (Donato 1999). A new S100 nomenclature is based on the clustered organization of at least 13 S100 genes on human chromosome 1q21 (Engelkamp *et al.* 1993, Heizmann and Schäfer 2001) and mouse chromosome 3, respectively (Ridinger *et al.* 1998).

S100 proteins show tissue- and cell-specific expression, a characteristic they share with most other EF-hand Ca^{2+} -binding proteins but not with the multifunctional and ubiquitously expressed calmodulin (Crivici and Ikura 1995). S100 proteins mainly exert their modulatory effect through Ca^{2+} -regulated interactions with intracellular specific target proteins.

Although both S100A1 and S100B were originally assumed to be neuronal specific proteins, it is now clear that S100A1 is specifically expressed in slow-twitch skeletal and heart muscles (for review see Heizmann and Cox 1998, Donato 1999).

Ultrastructural localization studies of the S100A1 in skeletal (Haimoto and Kato 1987, Maco *et al.* 1997, 1999, 2000a) and heart muscle cells (Haimoto and Kato 1988) have been done mostly in rodents and have localized S100A1 in myofibrils associated with actin and myosin filaments and with Z-lines, in the sarcoplasmic reticulum, in mitochondria, along the sarcolemma, and in fascia adherens of the intercalated discs.

Expression and localization of S100 proteins in human tissues may differ from that in rodent tissues (Engelkamp *et al.* 1992). However, no corresponding ultrastructural studies have been performed in human heart. Changes in expression of S100A1 in human cardiomyopathy (Remppis *et al.* 1996) and a right ventricular upregulation of S100A1 in chronic pulmonary hypertension (Ehlermann *et al.* 2000) indicate that more attention should be paid to the role of S100A1 and probably also of other calcium binding proteins in cardiac diseases. In addition, the increased level of S100A1 in the serum after myocardial infarction with the possibility to differentiate between infarction and angina pectoris (Usui *et al.* 1990) suggests a potential diagnostic value of this protein.

The importance of a distinct subcellular location of S100A1 for the cell function under normal and pathologic conditions would be more evident if quantitative data on the distribution of antigens in relation to structure would be available. In the present ultrastructural study the distribution of S100A1 protein using immunocytochemical method in human heart was

determined quantitatively, relating number of antigen sites to the unit area of a respective structural component.

Methods

Tissue preparation

Pieces of human heart muscle from the right atrium were obtained during surgery of a 34-years old man with a mitral valve disease in accordance with Helsinki Declaration of 1975.

Fixation and preparation of sections

The heart biopsy samples were immediately fixed for 1 h at 4 °C in 0.5 % glutaraldehyde (Fluka, Buchs, Switzerland) and 3 % paraformaldehyde (Merck, New Jersey, USA) in 10 mmol/l phosphate buffer solution (PBS) containing (mmol/l): Na_2HPO_4 (10), NaH_2PO_4 (10), NaCl (85.5), NaN_3 (20), pH 7.4. Fixed specimens were then dehydrated in ethanol, embedded in Lowicryl K4M (Polysciences, Eppelheim, Germany) and polymerized under UV light at -40 °C. Grey/silver sections were mounted on parlodion-coated copper grids (200 mesh). Sections were washed with 2 % BSA (Boehringer, Mannheim, Germany) in 10 mmol/l PBS before incubation with antibodies (see below) and stained with 6% aqueous uranyl acetate (Fluka, Buchs, Switzerland) and lead citrate.

EM immunocytochemistry

Ultrathin sections were labeled with purified polyclonal antiserum obtained by immunization of goats with recombinant human S100A1 protein produced in *E. coli* cultures (Ilg *et al.* 1996). The serum was diluted 1:300 with 2 % BSA in PBS and applied for one hour. The sections were then washed in PBS, and exposed for 1 h to the rabbit anti-goat secondary antibody coupled with 15-nm gold (Aurion, Wageningen, Netherland) and diluted 1:20 with 2 % BSA in PBS. Stainings with primary antibodies preabsorbed with human recombinant S100A1 protein were used as control.

Prior to application all antibodies were sonicated in water (Sonorex, TK 52H, Berlin, Germany) for 2-3 s. The sections were examined in a Philips EM 400 (Holland) electron microscope at 80 kV, photographed (negatives Kodak SO-163, USA), and printed with total magnification of 17 500× for immunogold counting.

Particle counting

The densities of gold particles on different levels of sarcomere (Z-line, I-band, and A-band), sarcoplasmic reticulum, mitochondria and sarcoplasm of human heart muscle cells were obtained from longitudinal sections.

Three different blocks of tissue were used. From each block 3 different grids with sections were prepared. Sixteen micrographs were taken from each grid and printed at total magnification of 17 500 \times .

Areas (μm^2) of immunolabeled structural components (Ac) of myofibrils were estimated by the point counting method (Weibel and Bolender 1973) from each micrograph: $Ac = \sum p_x \times (d/mag)^2$ where p is

number of points of two-dimensional test lattice for particular structural components of myofibrils; d is distance of two neighboring points of the test grid (μm), and mag is total magnification of a micrograph. The density of immunolabeling was expressed as the number of gold particles per unit area of the respective structural component. Background labeling was estimated in a similar manner.

Table 1. Immunogold labeling of individual blocks at particular structural components.

	Gold particles/ μm^2		
	Block 1	Block 2	Block 3
Z-line	0.2592 \pm 0.0188	0.2883 \pm 0.0659	0.6931 \pm 0.0802
I-band	0.0318 \pm 0.0246	0.1249 \pm 0.0364	0.3336 \pm 0.0243
A-band	0.0501 \pm 0.0087	0.0920 \pm 0.0141	0.1454 \pm 0.0117
mitochondria	0.0618 \pm 0.0134	0.1249 \pm 0.0289	0.2946 \pm 0.0110
sarcoplasmic reticulum	0.4531 \pm 0.2003	0.8973 \pm 0.3145	1.1794 \pm 0.0418
remaining sarcoplasm	0.1079 \pm 0.0577	0.0584 \pm 0.0158	0.1642 \pm 0.0211

Label over empty plastic of 0.1839 particles/ μm^2 has been subtracted. Each block is represented by three sets of data pooled from 16 micrographs obtained from individual grids with sections. Data are expressed as number of gold particles per μm^2 ; mean values \pm S.E.M.

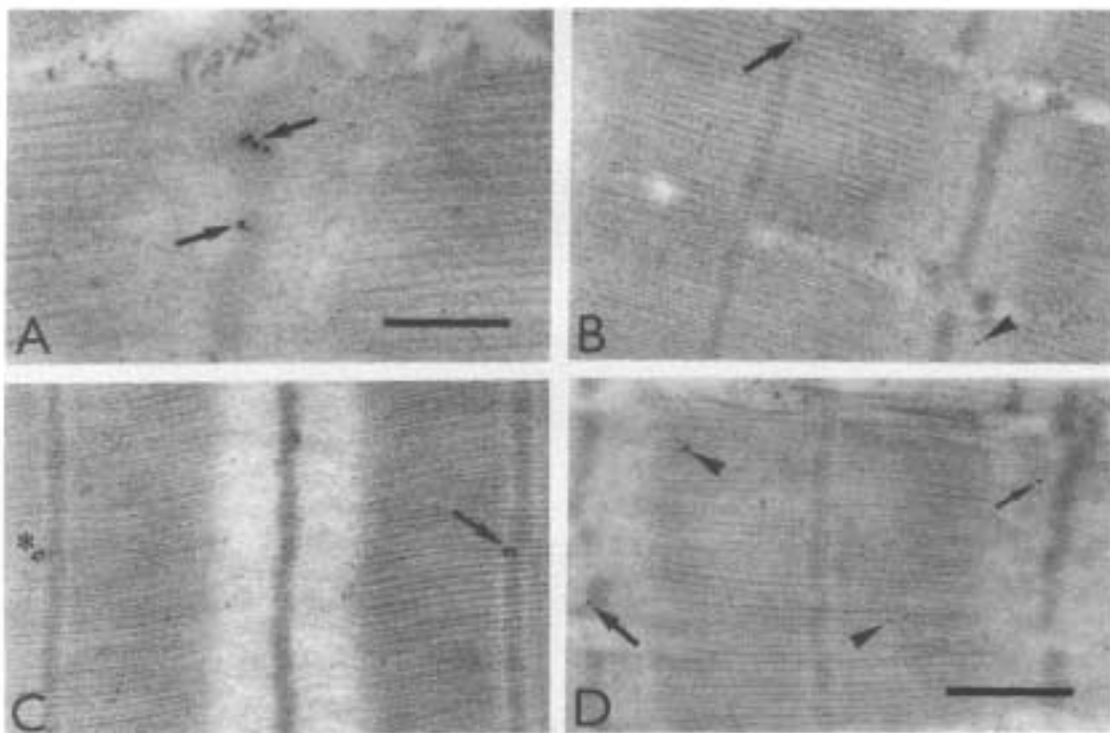


Fig. 1. Ultrastructural localization of S100A1 protein in human heart muscle at different levels of sarcomere. (A) Presence of gold particles on Z-line (arrows); (B) on I-band (arrowhead) and M-line (arrow); (C) on M-line (arrow) and on H-band (asterisk); (D) on A-band (arrowheads), I-band (thin arrow) and Z-line (thick arrow). Bars: A = 0.3 μm ; B-D = 0.5 μm .

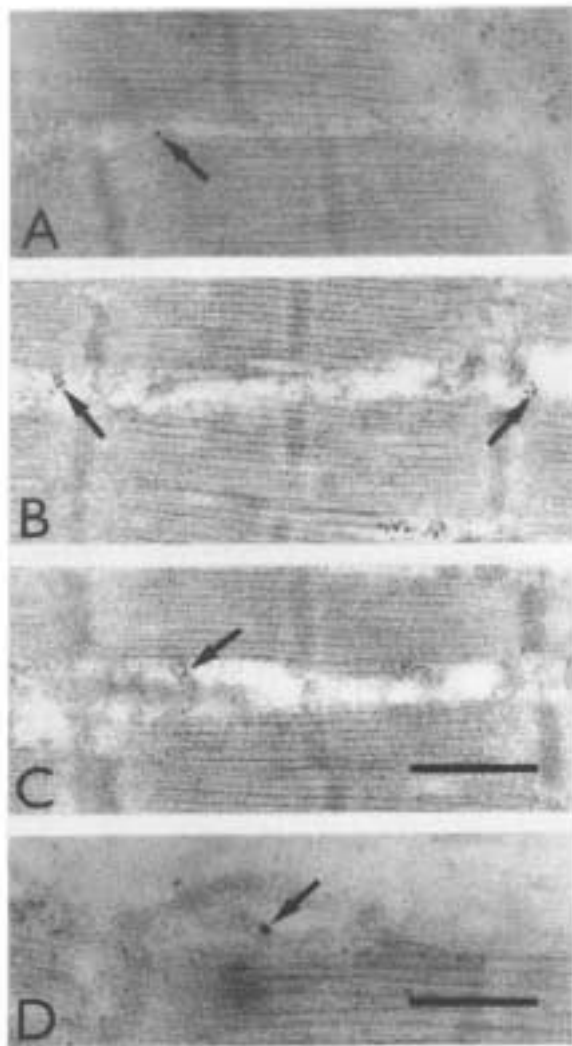


Fig. 2. The presence of S100A1 protein in elements of the sarcoplasmic reticulum (arrows). Bars: A-C = 0.5 μm ; D = 0.3 μm .

Results

Immunogold electron microscopy of S100A1 distribution in longitudinal sections of normal human heart showed that antigen sites for S100A1 protein were localized mainly in myofibrils at all levels of sarcomere (Fig. 1) and in elements of sarcoplasmic reticulum (SR) (Fig. 2).

The staining with immunogold on other structural components as intercalated discs, sarcolemma and nuclei was sparse. In an effort to suppress nonspecific immunolabeling as much as possible some specific labeling could have been suppressed as well. However, no immunolabeling occurred in controls (Fig. 3).

Immunogold particles were not uniformly distributed but occurred preferentially at particular locations. As summarized in Table 1, the most preferred

locations were Z-lines (Figs 1A and 1D) and elements of SR (Fig. 2).

A lower density of S100A1 protein was observed on thin and thick myofilaments (Figs 1B and 1D). Within the A-bands a slightly higher density of immunogold particles was detected on M-lines (Figs 1B and 1C).

To compensate for differences in immunolabeling between individual blocks (Table 1) resulting probably from different time intervals between sectioning and immunolabeling or from differences in blocking conditions, the immunogold densities at particular structural components were normalized to corresponding immunogold densities of mitochondria.

The normalized densities are shown in Figure 4. It is evident that density of immunolabeling on Z-line is about 3 times and in case of SR more than 5 times higher than immunolabeling of remaining structural components. These differences are significant ($p < 0.05$).

Discussion

A precise control of Ca^{2+} homeostasis in cardiac muscle cells is a prerequisite for normal function and its derangement results in various pathological states. Ultrastructural localization studies of S100A1 may contribute to elucidation of subcellular mechanisms of Ca^{2+} -signaling and give us insight into processes resulting in pathological heart.

To our knowledge, the ultrastructural localization and quantitative evaluation of S100A1 distribution in normal human heart has been performed for the first time in the present study. Although S100A1 may be found in different compartments of the heart cell, two areas of the highest occurrence, the Z-line and the elements of the sarcoplasmic reticulum, could be identified. Total amount of S100A1 protein expressed by human heart is 1.84 ± 0.5 $\mu\text{g}/\text{mg}$ protein which is about twofold higher than that of human skeletal muscle (Kato and Kimura 1985).

A relatively sparse immunogold staining in the present study may result from several reasons: a) strong blocking conditions during specimen preparation; b) the lower S100A1 expression in atrial than in ventricular myocardium (as described during mouse heart development, Kiewitz *et al.* 2000); c) the lower S100A1 expression in the right heart in comparison with the left ventricle (as described for porcine myocardium, Remppis *et al.* 1996). It is not clear if the underlying mitral valve disease could have an effect on the S100A1 expression in the right atrium. There were no clinical signs of right atrium dysfunction.

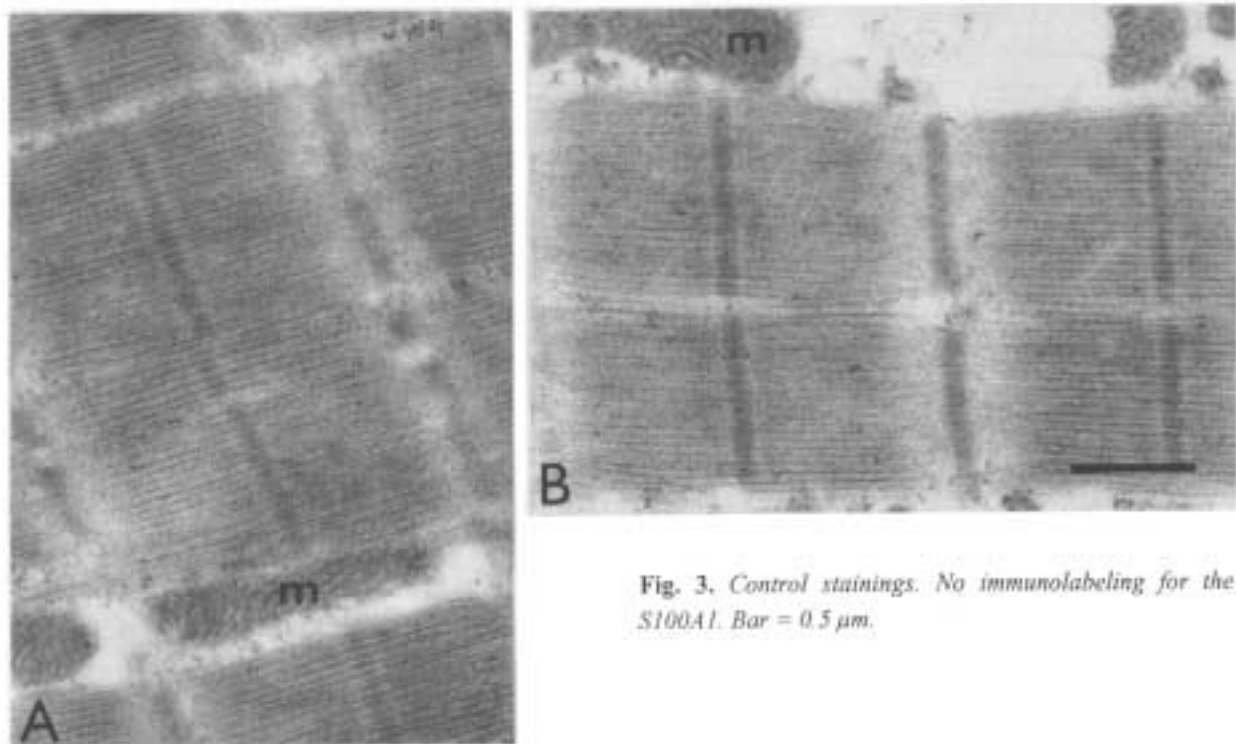


Fig. 3. Control stainings. No immunolabeling for the S100A1. Bar = 0.5 μ m.

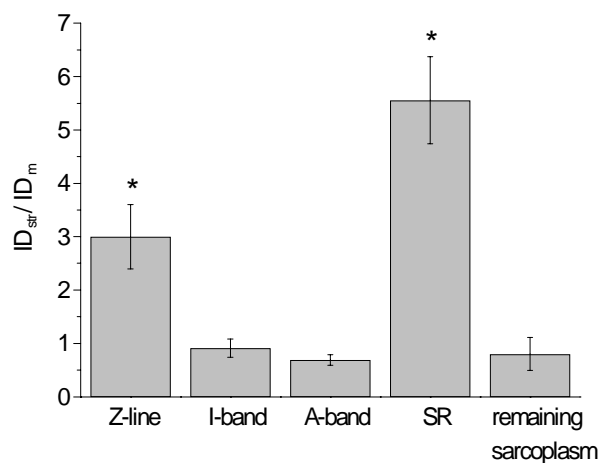


Fig. 4. Normalization of immuno-gold densities at particular structural components (ID_{str}) to corresponding immunogold densities of mitochondria (ID_m). Data are means \pm S.E.M.; * $p < 0.05$ with respect to the I-band, A-band and remaining sarcoplasm.

The presence of antigen sites for S100A1 along the sarcomere (Figures 1A-D) suggests an association of S100A1 with contractile filaments and/or with some other components associated with myofibrils. While an

interaction of S100A1 with skeletal muscle myosin heavy chain has been detected by gel overlay technique (Zimmer 1991), an interaction with cardiac myosin heavy chain has not been observed (Donato *et al.* 1989). These differences could be due to the different myosin isoforms in cardiac and skeletal muscle fibers. Recently Mandinova *et al.* (1998) have shown a direct interaction of S100A1 protein with rabbit skeletal F-actin in Ca²⁺-dependent manner in vitro and with actin stress fibers of human smooth muscle cells.

Location of antigen sites at M-lines (Figs 1B and 1C) may be interesting since M-lines are a site of titin kinase (Labeit *et al.* 1997) which is equivalent to twitchin kinase in invertebrates. Twitchin kinase is regulated by S100A1 and it is assumed that cardiac titin may be regulated in a similar way (Heierhorst *et al.* 1996, 1997). The high concentration of immunogold particles seen at the Z-line (Figs 1A and 1D) may result from a combination of interaction of S100A1 binding to F-actin and CapZ, the actin capping protein (Ivanenkov *et al.* 1996). S100A1 protein interacts with CapZ protein at the same binding site as phosphatidylinositol 4-monophosphate (PIP), a regulator of CapZ activity. It is assumed that S100A1 protein modulates the inhibitory

effect of PIP on activity of CapZ protein and can participate in the regulation of length, orientation and/or anchoring of actin filaments to the Z-line (Caldwell *et al.* 1989, Xu *et al.* 1999).

The location of S100A1 antigens at the sarcoplasmic reticulum observed in our study (Fig. 2) corresponds to similar findings from mouse (Haimoto and Kato 1988) and porcine heart (Donato *et al.* 1989) or rat skeletal muscle (Haimoto and Kato 1987, 1999, Maco *et al.* 1997, 2000a). It may be related to S100A1 stimulation of Ca²⁺-induced Ca²⁺ release from isolated SR vesicles (Fanò *et al.* 1989b, Marsili *et al.* 1992), to increased caffeine-induced Ca²⁺ release by S100A1 in permeabilized fast and slow twitch skeletal muscle fibers (Weber *et al.* 1997) and to interaction of S100A1 with the ryanodine receptor (Treves *et al.* 1997). The localization of S100A1 at the level of SR could also be related to its stimulatory effect on the basal activity of adenylate cyclase (Fanò *et al.* 1989a) associated with the sarcolemma, T-tubules and junctional SR where this enzymatic complex has been identified histochemically (Slezak and Geller 1984; for review see Schulze and Buchwalow 1998).

The growing list of S100A1 target proteins also includes some enzymes of energy metabolism such as glycogen phosphorylase a and phosphoglucomutase (for review see Zimmer *et al.* 1995, Landar *et al.* 1996) in slow-twitch skeletal muscle. Although many glycogenolytic and glycolytic enzymes are preferentially bound to F-actin (Arnold and Pette 1968) both above-

mentioned enzymes are associated with the membranes of SR (Entam *et al.* 1976, Lee *et al.* 1992). Recently, other glycolytic enzymes were also found to be localized on cytoplasmic surface of rabbit skeletal and heart SR vesicles and it was suggested that the ATP generated by SR-associated glycolytic enzymes may be coupled to SR active calcium transport (Xu and Becker 1998).

Another hypothesis how to explain the presence of S100A1 antigen sites at the level of SR is provided by gel-overlay experiments performed by Zimmer (1991) who found an interaction of S100A1 with different SR proteins. Two of these proteins with the molecular weights of 30 kDa and 100 kDa could be tentatively identified as phospholamban and Ca²⁺-ATPase (SERCA2), respectively. Thus S100A1 may not only be involved in the Ca²⁺ release from SR but could also play a role in the calcium uptake into SR. In such a case S100A1 would be an interesting candidate to explain the impaired Ca²⁺-homeostasis in dilated cardiomyopathy (Minamisawa *et al.* 1999).

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