Distribution of the Ca^{2+}-binding S100A1 Protein at Different Sarcomere Lengths of Slow and Fast Rat Skeletal Muscles

B. Maco[1], B. Uhrík[1], C. W. Heizmann[2]

1 *Institute of Molecular Physiology and Genetics, Slovak Academy of Sciences, Bratislava, Slovakia*
2 *Division of Clinical Chemistry and Biochemistry, Department of Pediatrics, University of Zurich, Zurich, Switzerland*

Abstract. The localization of S100A1 in rat soleus (*SOL*) and extensor digitorum longus (*EDL*) muscles was studied immunocytochemically at different sarcomere lengths (stretched, relaxed and contracted) at the ultrastructural level. The muscle fibres were contracted by application of 15 mmol/l caffeine. Following aldehyde fixation, dehydration and embedding in Lowicryl HM20 (−35 °C) ultrathin sections were incubated with rabbit polyclonal antiserum against S100A1. Goat antirabbit secondary antibodies conjugated with 10 nm gold particles were used to visualize antigen sites. Relative areas of Z-lines, A- and I-bands were estimated from longitudinal sections by the point counting method. The highest densities of the particles were found at the Z-lines. A higher incidence of S100A1 antigen sites in I-bands than in A-bands and a higher density of S100A1 in lateral parts of A-bands (with actin and myosin filaments overlapping) compared with the central area of A-bands are consistent with an interaction of S100A1 with F-actin in skeletal muscles. Antigen sites were also present at M-lines and at distinct locations of the sarcoplasmic reticulum.

Key words: Ca^{2+}-binding proteins — S100A1 — F-actin — Myofibrils — Muscle cells — Sarcoplasmic reticulum

Introduction

Calcium ions play an important role in the regulation of numerous cellular processes (Breier et al. 1998; Sulová et al. 1998; Lipská and Radzyukevich 1999; Kamouchi et al. 1999; for a review see Berridge 1997). The role of Ca^{2+} ions in intracellular communication is mediated by Ca^{2+}-binding proteins (for a review see Heizmann

Correspondence to: Dr. B. Uhrík, Institute of Molecular Physiology and Genetics, Slovak Academy of Sciences, Vlárska 5, 833 34 Bratislava, Slovakia. Tel.: +421-7-5477 2111, Fax: +421-7-5477 3666, E-mail: umfguhrk@savba.savba.sk

1992; Schäfer and Heizmann 1996). Expression and intracellular distribution of various Ca^{2+}-binding proteins may be tissue-specific (Bastianelli et al. 1999).

S100A1 protein, a member of the S100 protein subfamily of EF-hand calcium-binding proteins is highly expressed in heart and skeletal muscle tissues (Kato and Kimura 1985; Kiewitz et al. 2000; for a review see Donato 1999). The role of S100A1 in muscle cells is not yet clear. Comparative studies in skeletal muscles of rodents demonstrated high concentrations of S100A1 in slow twitch fibres and low but detectable levels of this protein in fast twitch fibres (Haimoto and Kato 1987; Zimmer 1991). Our previous study (Maco et al. 1997) showed that, besides in elements of the sarcoplasmic reticulum, in slow (m. soleus – *SOL*) and fast (m. ext. digitorum longus – *EDL*) skeletal muscles S100A1 is mainly localized in myofibrils at all levels of the sarcomere with the highest density at Z-lines. Recently, a Ca^{2+}-dependent interaction of S100A1 with F-actin of smooth muscle cells has been demonstrated (Mandinova et al. 1998) implying a possible role of S100A1 during the contraction-relaxation cycle. If a similar interaction in skeletal muscle cells is assumed, differences in distribution of S100A1 between *SOL* (high levels of S100A1) and *EDL* (low levels of S100A1), between I- and A-bands, and between different parts of A-bands at different sarcomere lengths may be expected. This assumption was tested in the present immunocytochemical study in rat *EDL* and *SOL*. Consistent with the assumed interaction, the results showed a higher incidence of S100A1 antigen sites in I-bands than in A-bands and a higher density of S100A1 in lateral parts of A-bands (with actin and myosin filaments overlapping) as compared with the central area of A-bands.

Materials and Methods

Rat *EDL* and *SOL* muscles were dissected in Tyrode's solution. Three pairs of *SOL* and *EDL* were used, one pair of the muscles was exposed to 15 mmol/l caffeine in Tyrode's solution for 2 min to elicit contracture.

The muscles were fixed for 2 hours with a mixture of 1% paraformaldehyde and 0.025% glutaraldehyde in 0.1 mol/l phosphate buffer (PBS), washed in 50 mmol/l glycine in PBS for 3×15 min, dehydrated in ethanol series at a low temperature (30% at 0 °C; 50% at −20 °C; 70%, 90%, 100% at −35 °C) and embedded in Lowicryl HM20 (polymerized under UV light at −35 °C).

Ultrathin sections were placed on Formvar-coated copper grids, floated on drops of 0.5% Tween 20 in Dulbecco's Modified Eagle's Medium supplemented with 5% fetal calf serum (DMEM), washed in PBS, preincubated in 5% goat normal serum (GNS) in DMEM for 10 min, and incubated for 60 min with rabbit polyclonal antiserum against S100A1 in DMEM (diluted 1:400) supplemented with 5% GNS. Purified polyclonal antiserum was obtained by immunization of rabbits with recombinant human S100A1 produced in *E. coli* cultures (Ilg et al. 1996). These polyclonal antibodies are specific and do not cross-react either with other S100 or Ca^{2+}-binding proteins or with other cellular proteins.

Goat antirabbit secondary antibodies conjugated with 10 nm gold particles

in DMEM (diluted 1:25) were applied for 60 min to visualize antigen sites. The sections were then treated with 2% glutaraldehyde in PBS for 2 min to stabilize binding of antibodies, and contrasted with 2% uranylacetate in 50% metanol for 2×35 min and with lead acetate for 10 min. The specificity of immunolabelling was ascertained by omitting primary antibodies during the labelling procedure.

To determine the distribution of antigen sites gold particles were counted and related to areal densities of structural components of myofibrils. Half of the A-bands (from the A/I boundary to the M-line but without the M-line) were divided into four parts to compare shifts in particle distribution between fibres of different sarcomere lengths. Since the density of S100A1 antigen sites in EDL was very low, averaging of the numbers of gold particles from individual electronograms was not possible and the particles were counted directly on the screen of the electron microscope. The areal densities of Z-lines and I- and A-bands of myofibrils on longitudinal sections, corresponding to their volume densities, differed according to the sarcomere lenghts, and were estimated by the point counting method (Weibel and Bolender 1973). This estimation made it possible to detect differences in the distribution of antigen sites along the sarcomeres of the myofibrils.

Three different blocks from each muscle were used. From each block, 3 different grids with sections were prepared. The gold particles were counted in different sections from the same specimen and their numbers were: 3244 (*EDL*, contracted), 1093 (*EDL*, relaxed), 2300 (*EDL*, slightly stretched), 2251 (*SOL*, contracted), 2692 (*SOL*, relaxed), 1100 (*SOL*, slightly stretched).

Results

Fig. 1 shows the ultrastructural appearance and immunogold marking in a stretched *EDL* (Fig. 1a,b) and in a relaxed *SOL* (Fig. 1c,d).

Muscles with fibres of three different sarcomere lengths were evaluated both for *SOL* and *EDL*. Z-lines in *SOL* and *EDL* occupied 5.53±0.28% and 4.07±0.42% (±SEM) of the myofibrillar area, respectively. The relative numbers of immunogold particles over Z-lines were 17.60±2.16% for *SOL* and 14.63±1.18 for *EDL*. The percentages of S100A1 antigen sites over Z-lines were more than 3 times higher than the area occupied by Z-lines.

Areas occupied by Z-lines, I- and A-bands and the relative numbers of immunogold particles differed according to the sarcomere lenghts. The results are summarised in Table 1.

It can be seen that at each sarcomere length the percentages of gold particles over I-bands are higher than the relative area of the I-band. This is true both for *SOL* and *EDL* but it is more pronounced for *SOL*, especially if the muscles with similar I-band areas are compared (2^{nd} column of *SOL* and 1^{st} column of *EDL*).

Relative distributions of gold particles along A-bands are shown in Table 2.

The results in Table 2 show that the highest densities of S100A1 antigen sites in both muscles and at all sarcomere lengths may be found in A-band parts No. 1 and No. 2, i.e. in the area of interdigitation of actin and myosin filaments. With

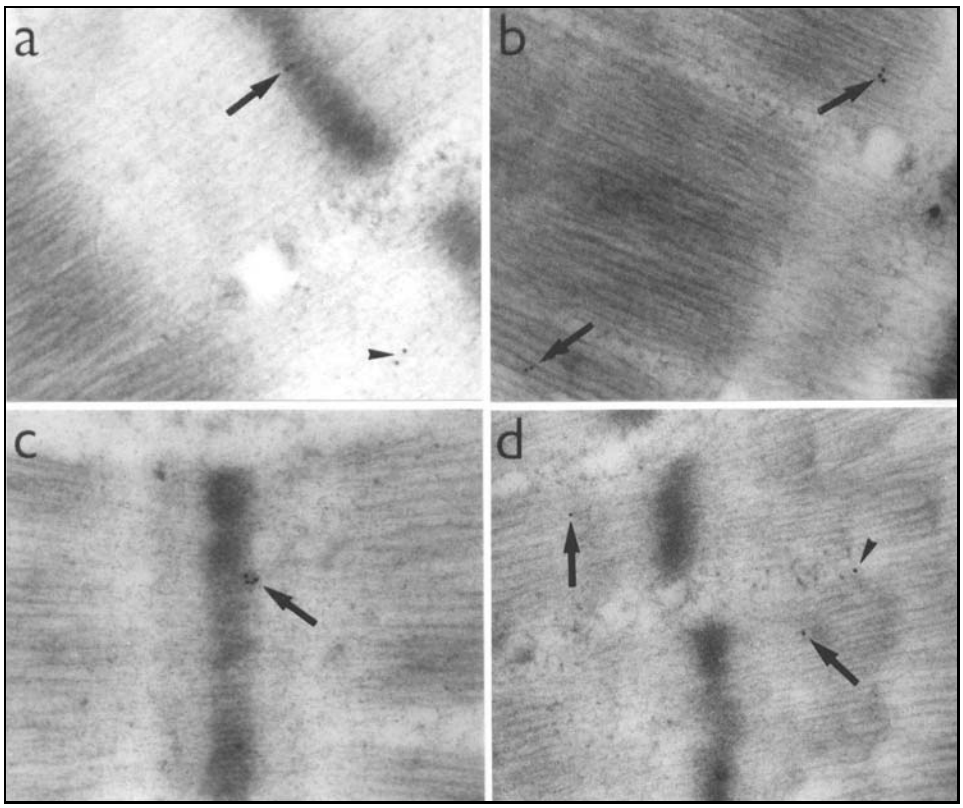

Figure 1. Ultrastructural appearance and immunogold marking in a stretched *EDL* (a, b) and in a relaxed *SOL* (c, d). a – immunogold at the Z-line (arrow) and in the central part of the half of the I-band (arrowhead); b – immunogold at different levels of the A-band; c – immunogold at the Z-line; d – immunogold at the A/I boundary (arrows) and at the sarcoplasmic reticulum (arrowhead). Magnification: ×68,000 (a, c) and ×55,000 (b, d)

the sarcomere shortening, a decrease in relative immunogold particle densities in the A-band part No. 4 is pronounced. This part corresponds to the central area of A-bands without actin filaments. The differences in *EDL* between *a, b, c* and *d; f* and *h; i* and *l; d* and *h, l* are significant ($p < 0.05$; ANOVA test). In *SOL* the differences between *a, b* and *d; e* and *g, h; i* and *j, k; k* and *l; a* and *i* are significant, between *c* and *k* ($p < 0.07$)*; g* and *k; d* and *l* ($p < 0.08$) are marginally significant.

The relative distribution of S100A1 antigen sites in I-bands could be determined in the relaxed or stretched muscles. In both *SOL* and *EDL* the central part of half of the I-bands showed the highest densities of immunogold particles.

Another frequent location of S100A1 antigen sites were terminal cisternae of the sarcoplasmic reticulum (SR) and the elements of the longitudinal SR between A-bands of neighbouring myofibrils. Since the quality of fixation adequate for im-

Table 1. Relative areas (in %) and relative numbers (in %) of immunogold particles over Z-lines, I- and A-bands of muscles with different sarcomere lengths (mean±SEM)

	SOL			*EDL*		
	contracted	relaxed	stretched	contracted	relaxed	stretched
I-band area	9.8±0.5	16.3±1.2	42.0±0.7	16.8±1.4	20.0±1.3	37.9±1.3
Gold particles over I-band	15.8±2.0	45.5±1.7	55.3±1.9	22.9±1.8	20.6±5.0	62.3±0.7
A-band area	84.1±0.4	78.4±1.0	52.8±1.1	78.3±1.3	76.3±1.4	58.5±1.3
Gold particles over A-band	63.2±1.9	36.3±1.6	31.1±2.8	62.5±0.6	66.8±3.7	21.0±1.3
Z-line area	6.1±0.2	5.3±0.5	5.2±0.4	4.9±0.1	3.7±0.3	3.6±0.2
Gold particles over Z-line	21.0±0.4	18.2±0.7	13.6±0.9	14.6±1.3	12.6±1.2	16.7±0.8

Table 2. Relative numbers (in %) of immunogold particles along A-bands of muscles with different sarcomere lengths (mean±SEM)

A-band parts	*SOL*			*EDL*		
	contracted	relaxed	stretched	contracted	relaxed	stretched
1	29.2 ± 1.3^{a}	32.4 ± 3.3^{e}	34.9 ± 0.9^{i}	37.8 ± 2.8^{a}	23.5 ± 4.0^{e}	39.2 ± 4.4^{i}
2	28.0 ± 2.4^{b}	26.2 ± 1.0^{f}	21.0 ± 3.3^{j}	30.7 ± 1.9^{b}	33.5 ± 2.8^{f}	24.6 ± 2.6^{j}
3	25.1 ± 2.8^{c}	22.7 ± 1.9^{g}	16.1 ± 2.3^{k}	23.8 ± 3.6^{c}	27.2 ± 2.3^{g}	18.4 ± 1.8^{k}
4	17.7 ± 3.7^{d}	18.7 ± 3.9^{h}	28.0 ± 1.6^{l}	7.7 ± 0.6^{d}	15.8 ± 0.9^{h}	17.8 ± 1.8^{l}

A-band parts are numbered from A/I boundary to M-line

munocytochemistry did not preserve SR membranes satisfactorily we could not estimate the volume densities of SR. However about 10.5% of gold particles in *EDL* and 10.8% in *SOL* were associated with vesicular structures in intermyofibrillar spaces. According to data published by others (Davey and O'Brien 1978; Davey and Wong 1980) SR volume in rat *EDL* occupies between 6 to 9.3% and in *SOL* between 5.3 to 5.6% suggesting a relatively high density of S100A1 antigen sites in SR.

Discussion

Using the same blocking protocol, the density of immunogold is much higher in *SOL* than in *EDL*, in accordance with the results of biochemical studies. There it has been shown that S100A1 levels in slow-twitch skeletal muscles are much higher

than in fast-twitch muscles (Haimoto and Kato 1987; Zimmer 1991).

As found in the present study, the percentages of gold particles over I-bands were higher than the relative area of I-bands in both *SOL* and *EDL* at all sarcomere lengths (Table 1). This finding is consistent with the association of S100A1 with F-actin as described by Mandinova et al. (1998).

Further support to the existence of a Ca^{2+}-dependent interaction of S100A1 with actin is given by the relative numbers of immunogold particles along A-bands of muscles with different sarcomere lengths (Table 2). Higher densities of S100A1 antigen sites at places of interdigitation of actin and myosin filaments, and especially the relative decrease of immunogold particles in the central part of the A-bands in muscles with shorter sarcomeres (the bottom line in Table 2) agree well with the expected shifts of S100A1 along the sarcomere.

The presence of S100A1 antigen sites along the sarcomere suggests an association of S100A1 with contractile filaments and/or with some other components associated with myofibrils. It is not clear as yet if this association has also a functional significance in the activation of contraction.

In addition to a possible affinity to actin, the higher densities of antigen sites in the middle of half of the I-bands may reflect an interaction of S100A1 with some glycogenolytic and glycolytic enzymes described by others (Zimmer et al. 1995; Landar et al. 1996), as it is known that these types of enzymes bind preferentially to F-actin (Arnold and Pette 1968; Pette 1975).

The highest densities of immunogold particles seen at Z-lines may result from a combination of S100A1 binding to F-actin and to CapZ, the actin capping protein (Ivanenkov et al. 1996).

In both *SOL* and *EDL*, antigen sites were also detected at M-lines in the present study. This location may be of interest as the site of titin kinase (for a review see Labeit et al. 1997) and the possibility that this enzyme is regulated by S100A1 in a similar way as twitchin kinase is regulated in invertebrates (Heierhorst et al. 1996).

The presence in elements of the sarcoplasmic reticulum of S100A1 antigen sites observed in our study corresponds to similar findings reported by others (Haimoto and Kato 1987), and may be related to S100A1-stimulation of Ca^{2+}-induced Ca^{2+} release from isolated sarcoplasmic reticulum vesicles (Fanò et al. 1989), to increased caffeine-induced Ca^{2+} release by S100A1 in permeabilized skeletal muscle fibres (Weber et al. 1997), and to interaction of S100A1 with the ryanodine receptor (Treves et al. 1997).

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