Journal of Comparative Physiology B (2018) 188:919–927
https://doi.org/10.1007/s00360-018-1177-y

ORIGINAL PAPER

Soluble calcium-binding proteins (SCBPs) of the earthworm *Lumbricus terrestris*: possible role as relaxation factors in muscle

Prasath Thiruketheeswaran[1] · Ralf Huch[1] · Jochen D'Haese[1]

Received: 9 March 2018 / Revised: 10 July 2018 / Accepted: 18 July 2018 / Published online: 28 July 2018

Abstract

The soluble Ca^{2+}-binding protein (SCBP) from the earthworm *Lumbricus terrestris* was analyzed with regard to its role as a soluble muscle relaxation factor. The actomyosin ATPase activity was inhibited by the addition of decalcified SCBP as it binds Ca^{2+} stronger than the regulatory proteins associated with the actomyosin. Competitive $^{45}Ca^{2+}$-binding assays with decalcified actomyosin and SCBP showed that $^{45}Ca^{2+}$ is first bound to actomyosin and is subsequently taken over by SCBP with increasing incubation time. Ca^{2+} competition experiments carried out with $^{45}Ca^{2+}$ loaded SCBP and fragmented sarcoplasmic reticulum vesicles revealed that $^{45}Ca^{2+}$ bound to SCBP can be deprived by the ATP-dependent Ca^{2+} uptake of the sarcoplasmic reticulum. Furthermore, experiments in a diffusion chamber showed that the addition of SCBP significantly enhances the $^{45}Ca^{2+}$ flux in a concentration dependent manner. The amount of the Ca^{2+} flux increase tends to reach a maximum value of about 70%. With all protein components isolated from the obliquely striated muscle, our in vitro experiments consistently show that SCBP may accelerate muscle relaxation similar as assumed for vertebrate parvalbumin.

Keywords *Lumbricus terrestris* · Soluble calcium-binding protein (SCBP) · Sarcoplasmic reticulum · Competition for Ca^{2+} · Facilitated Ca^{2+} diffusion · Soluble relaxing factor

Introduction

Muscle contraction is triggered by an increase of the cytosolic-free Ca^{2+} concentration above the resting level (~ 10^{-7} M). For relaxation, new excitability Ca^{2+} must be removed from the actomyosin-associated regulatory proteins and transported out of the cytoplasm by membrane-bound Ca^{2+} ATPases (Rüegg 2012; Stammers et al. 2015).

In vertebrates, the high affinity Ca^{2+}-binding protein parvalbumin has been assumed to act as a soluble relaxing factor by supporting the Ca^{2+} depletion of troponin and the Ca^{2+} uptake by the sarcoplasmic reticulum (SR) (Gerday and Gillis 1976; Pechere et al. 1977; Haiech et al. 1979; Gillis 1985; Arif 2009).

In contrast to vertebrate muscle, parvalbumin could not be detected in invertebrates. Instead, soluble or sarcoplasmic Ca^{2+}-binding proteins (SCBP) were discovered and described in various invertebrate muscles. SCBPs are likewise to parvalbumin proposed to accelerate muscle relaxation by transporting Ca^{2+} from the myofibrils to the SR (Gerday 1988).

The amount of SCBP correlates with the contraction speed of the muscle type (Cox et al. 1976; Wnuk et al.1982; Cox 1990; Gao et al. 2006; White et al. 2011).

Protein sequencing data (Takagi et al. 1984, 1986) and X-ray crystallographic analysis (Cook et al. 1991) demonstrated that SCBPs belong to the EF-hand superfamily of calcium-binding proteins all sharing the common helix-loop-helix EF-hand domain for coordinative binding of Ca^{2+} ions (Kretsinger and Nockolds 1973; Mazumder et al. 2014).

SCBPs and parvalbumin are characterized by Ca^{2+}-Mg^{2+} mixed sites with a high affinity for Ca^{2+} and Mg^{2+}. Under physiological conditions where the Mg^{2+} concentration exceeds that of Ca^{2+}, these mixed sites are mainly occupied by Mg^{2+}. The Ca^{2+}–Mg^{2+} exchange rates of both proteins are crucial for the acceleration of Ca^{2+} removal during a single contraction–relaxation cycle. According to various

Communicated by I. D. Hume.

✉ Jochen D'Haese
dhaese@hhu.de

[1] Institute for Cell Biology, Department Biology, Heinrich-Heine-University Düsseldorf, Universitätsstr. 1, 40225 Düsseldorf, Germany

authors the exchange is not fast enough and therefore both proteins are considered as slow-onset buffers (Wnuk et al. 1982; Engelborghs et al. 1990; Schwaller 2010).

In previous studies, we have shown the presence of SCBPs in crude extracts of various muscles (leg muscles and the flight control muscles of the thorax) in the flies *Drosophila melanogaster* and *Calliphora erythrocephala* (Kiehl and D'Haese 1992). The results show that the specialized asynchronous muscles for flight, which contract more than once per nerve impulse, do not exhibit SCBP. However, in the synchronous steering muscles SCBP is present in high amounts. From the body wall muscle of the earthworm *Lumbricus terrestris*, three SCBP isoforms have been isolated and physicochemically characterized. In *L. terrestris*, more than a 1000-fold amount of SCBP has been calculated by sandwich ELISA in the body wall compared to the slow gizzard muscle (Huch and D'Haese 1992). Preliminary isometric measurements after single pulse and low-frequency stimulations of muscle stripes of the earthworm longitudinal and gizzard muscle indicate that in the longitudinal muscle the speed of relaxation is much faster than in the gizzard (Sturm et al. 1993). We could confirm a correlation between the amount of SCBP and speed of muscle contraction in the body wall and the gizzard muscle in *L. terrestris* by fluorescent in situ hybridization analysis (Thiruketheeswaran et al. 2016).

In the present study, we used SCBP, actomyosin, and fragmented SR all isolated from the earthworms' obliquely striated muscle to analyze these components for their Ca^{2+} competition. In addition, we investigated the effect of SCBP on the Ca^{2+} diffusion to get more insight into the physiological significance of high amounts of SCBP in this invertebrate muscle.

Materials and methods

Inhibition of actomyosin ATPase and competition between actomyosin and SCBP for Ca^{2+}

The actomyosin prepared according to D'Haese and Carlhoff (1987) and SCBP isolated as described elsewhere (Huch et al. 1988) were obtained from the body wall muscle of the earthworm *Lumbricus terrestris*. The ATPase activity of the actomyosin was determined as described by Carlhoff et al. (1987) in assay buffer containing 30 mM Tris-maleate pH 7.0, 20 mM KCl, 1 mM $MgCl_2$, and the reaction was started by the addition of ATP (1 mM final concentration). Varying amounts of $CaCl_2$ and EGTA were used to obtain free Ca^{2+} concentrations between 10^{-9} and 10^{-5} M (pCa^{2+}: 9 to 5). Calculations of free Ca^{2+} were done according to Jewell and Rüegg (1966) using an apparent K_D of 2×10^{-7} M for the Ca^{2+}-EGTA complex. Liberated phosphate was determined photometrically according to Fiske and Subbarow (1925).

To examine the influence of SCBP on the ATPase activity, SCBP was decalcified by the following procedure: SCBP-containing solutions were brought to 5 mM EGTA, dialyzed first against assay buffer, then against assay buffer containing approx. 10 g of the ion exchange resin Chelex-100 (BioRad, Richmond, USA).

Prior to the actomyosin competition experiment, SCBP and actomyosin were decalcified as described before except that competition buffer (10 mM Hepes, 10 mM histidine pH 7.0, 0.1 M KCl, 5 mM NaN_3) was used. After addition of 1 mM $MgCl_2$, samples of both protein solutions were mixed and preincubated (room temperature, 10 min) in equimolar concentration of 5 µM as was estimated using an apparent molecular mass of 20 kDa for SCBP (Huch et al. 1988) and 1 MDa for the actomyosin complex, the latter being calculated on the basis of the molecular ratios and the molecular masses of its components (myosin, actin, paramyosin, tropomyosin, and troponin) determined in SDS–PAGE (Carlhoff 1988). The reaction was initiated by pipetting 80 µl of starting solution [0.1 mM $^{45}CaCl_2$ (3700 Bq ml^{-1}), 10 mM MgATP] into the test tubes (total assay volume of each tube: 0.8 ml) followed by vigorous shaking, and stopped immediately (0 min) and after 1, 5 and 20 min by centrifugation (11000*g*, 5 min). The resulting actomyosin pellet was resuspended overnight in 0.4 ml of incubation medium according to Laemmli (1970) and the suspension was used for liquid scintillation counting (LSC, see below). For ultrafiltration of the supernatant to measure the Ca^{2+} bound to SCBP a Centricon 10 (Merck Millipore, Darmstadt, Germany) with a cut off of 10 kDa was used. Control experiments contained SCBP or actomyosin alone.

Competition between SCBP and SR for Ca^{2+}

Essentially, this experiment is a modified Ca^{2+} uptake assay using fragmented sarcoplasmic reticulum (FSR) vesicles prepared from earthworm body wall muscle according to Heilmann et al. (1977). SCBP was equilibrated in SR competition medium (10 mM Hepes, 10 mM histidine pH 7.0, 0.1 M KCl, 5 mM $MgCl_2$, 5 mM NaN_3, 10 µM $CaCl_2$, and 5 mM $K_2C_2O_4$) and dialyzed against the same buffer containing $^{45}Ca^{2+}$ (final activity: 7400 Bq ml^{-1}) overnight. The uptake of Ca^{2+} by FSR vesicles was measured by a filtration method as described by Martonosi and Feretos (1964) with adaptations according to Semich and Volmer (1985). FSR vesicles suspended in SR competition medium (protein concentration: 20 µg ml^{-1}) were preincubated under gentle stirring for 5 min at 25 °C. Thereafter, the radioactive competition medium with and without SCBP was added. After another 5 min, the Ca^{2+} uptake reaction was started by addition of ATP

(final concentration: 5 mM). Aliquots of 0.3 ml were taken after various periods of incubation and transferred into a disposable 1 ml syringe fitted with a 0.45 µm pore size filter (type SJHV; Merck Millipore). After filtration, the syringe filter with the retained FSR vesicles was washed with 1 ml of 0.1 M NaCl and taken for LSC. To determine the Ca^{2+} bound to SCBP the filtrate was further processed by ultrafiltration using Centricon 10 (Merck Millipore).

Measurement of facilitated Ca^{2+} diffusion

Ca^{2+} diffusion experiments were carried out essentially according to Feher (1983) in a three-chamber system. The effect of SCBP, present in the middle chamber, on the diffusion of Ca^{2+} was analyzed as the rate or flux at which $^{45}Ca^{2+}$ diffuses from the left to the right chamber.

The two outer chambers are 0.8 cm high and 0.8 cm in diameter, both containing a magnetic stir bar (6 mm × 3 mm) driven by micromotors placed below the chambers. The middle chamber is represented by a ring of varying thickness (0.2, 0.6, or 1.0 cm), which can be fixed between the outer chambers, thus providing variable diffusion distances. All diffusion experiments were performed at a diffusion distance of 0.2 cm unless otherwise indicated. Dialysis membranes (Spectra/Por®, molecular mass cutoff: 6–8 kDa, Spectrum Laboratories, Los Angeles, USA) fixed on both sides of the middle chamber separated the compartments from each other. Protein samples were introduced into the middle chamber as liquid 0.5% agar gels (temperature approx. 50 °C) in diffusion buffer (10 mM Hepes pH 7.0, 140 mM KCl, 0.5 mM $MgCl_2$, 10 µM $CaCl_2$), where they were allowed to gel for at least 30 min. During the experiment, the right chamber was perfused with diffusion buffer and the left chamber with the same buffer complemented with $^{45}Ca^{2+}$ (final radioactivity: 3700 Bq ml^{-1}). Carefully adjusted high precision pumps [type 112 (Beckman Coulter, Fullerton, USA) or P-500 (Pharmacia LKB, Uppsala, Sweden)] were used to ensure identical flow rates of 12 ml h^{-1} through both outer compartments. Fractions of 0.4 h (=4.8 ml) were collected from the perfundate of the right chamber and aliquots of 0.6 ml from each second fraction were taken for determination of radioactivity by LSC. The surrounding temperature was monitored continuously with a calibrated temperature sensor. The diffusion experiments were performed at a temperature in the range of 23 ± 3 °C. Calculations of the Ca^{2+} flux (I_{LR}) data were performed according to Feher (1984). Relative Ca^{2+} flux denotes the relation of I_{LR} in presence of SCBP divided by I_{LR} in presence of 0.5% agar: I_{LR} (SCBP)/I_{LR} (agar). Control diffusion experiments were performed with the non-calcium-binding protein bovine serum albumin (BSA, Cohn fraction V; Sigma).

Liquid scintillation counting (LSC)

The $^{45}Ca^{2+}$ radioactivity of syringe filters and liquid samples were measured in 4 ml of Ready Protein Plus scintillation mixture using a LS 5000 CE counter (Beckman Coulter).

Protein estimation

Protein concentrations were determined according to Bradford (1976) using a dye reagent concentrate (BioRad) and BSA as a standard.

Statistical analysis

Data represent the mean (± standard deviation, SD) of three independent experiments, each performed in triplicate. The statistical significance of differences between two means was determined by Student's paired *t* test. Data were considered significantly different if the two-tailed *p* value was < 0.05.

Results

Ca^{2+} bound to actomyosin is depleted by SCBP

The actomyosin used for these experiments showed a sigmoidal Ca^{2+} dependence of the ATPase activity with a Ca^{2+} sensitivity of 80–90% as calculated with the following equation according to Carlhoff and D'Haese (1987): 1 – [ATPase activity at 2 mM EGTA/ATPase activity at 0.1 mM Ca^{2+}] × 100 (%) (Fig. 1).

Actomyosin with full enzymatic activity (560 ± 20 nmol P_i min^{-1} mg^{-1}) was incubated with Ca^{2+} deprived SCBP in the presence of 10 µM Ca^{2+} (pCa^{2+}: 5). With increasing amounts of SCBP the ATPase activity of the actomyosin was progressively decreased to about 170 ± 10 nmol P_i min^{-1} mg^{-1} in presence of 100 µM SCBP (Fig. 1).

To analyze how SCBP and actomyosin compete for Ca^{2+}, a competition assay was conducted (see methods). Analysis of the $^{45}Ca^{2+}$ radioactivity in the actomyosin pellets obtained from the actomyosin–SCBP competition experiment revealed a reduction of the Ca^{2+} initially bound to actomyosin with increasing incubation time. The most drastic decrease was observed after 1 min of incubation: the mean counting rate dropped from slightly more than 26×10^3 cpm down to approx. 18×10^3 cpm. After 20 min the counting rate in the pellet was further reduced to about 15×10^3 cpm (Fig. 2a). By mass determination of the actomyosin pellet, it was ensured that the reduction of the counting rate was not due to a loss of sedimentation efficiency in the course of the experiment. Protein estimation of the supernatant revealed that actomyosin was sedimented to 95% under the assay conditions used.

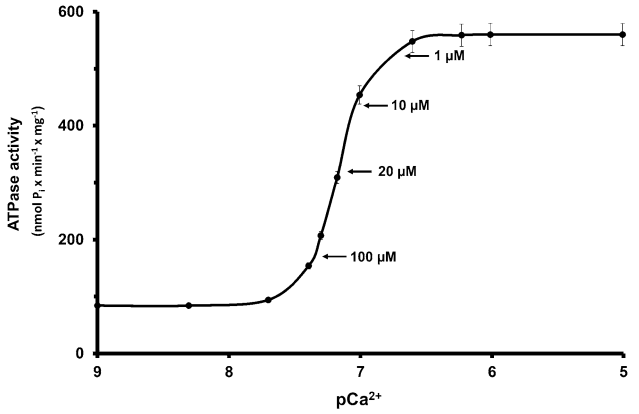

Fig. 1 Inhibition of actomyosin ATPase by SCBP. The ATPase activity of the actomyosin was determined as described in methods. A sigmoidal curve was obtained demonstrating a drastic decrease of enzymatic activity below a free Ca^{2+} concentration of 2×10^{-7} M. With the addition of increasing amounts of Ca^{2+}-free SCBP (indicated by arrows), the ATPase activity of the actomyosin was decreased. In the presence of 20 µM SCBP the ATPase activity was reduced to 319 ± 15 nmol P_i min^{-1} mg^{-1}, indicating a free Ca^{2+} concentration of about 9×10^{-8} M. With 100 µM SCBP the actomyosin ATPase activity was further reduced to 170 ± 10 nmol P_i min^{-1} mg^{-1} equivalent to a decrease down to about 6×10^{-8} M of free Ca^{2+}. In all preparations the actomyosin showed a Ca^{2+} sensitivity of about 80–90% as calculated with the following equation: 1 – [ATPase activity at 2 mM EGTA/ATPase activity at 0.1 mM Ca^{2+}] × 100 (%). Bars indicate mean ± SD of three independent experiments. The sample size for each determination of ATPase activity in the presence of SCBP was $N = 3$

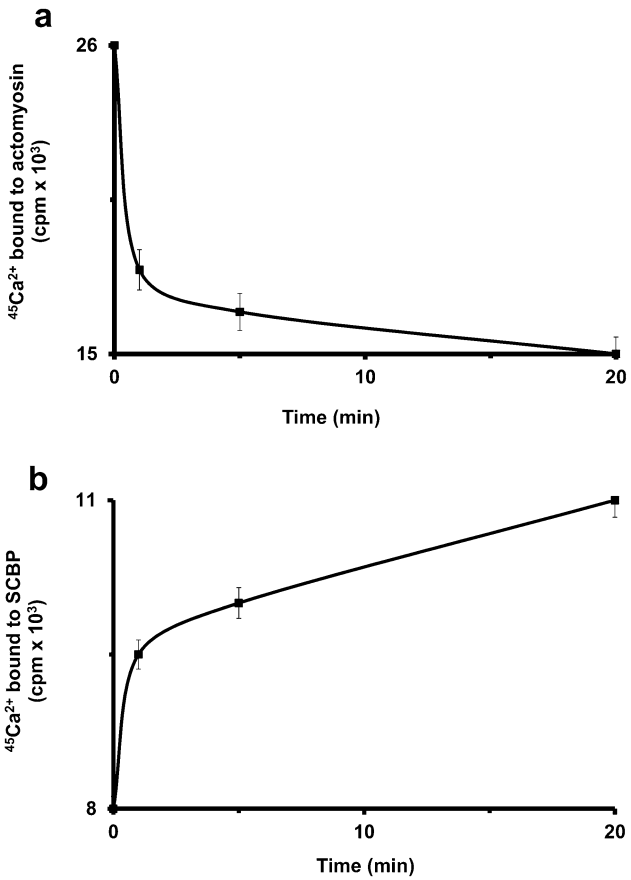

Fig. 2 Actomyosin–SCBP competition assay. Analysis of the $^{45}Ca^{2+}$ radioactivity in the actomyosin pellet (**a**) and SCBP supernatant (**b**) obtained from the actomyosin–SCBP competition experiment revealed a reduction of the $^{45}Ca^{2+}$ initially bound to actomyosin while at the same time the $^{45}Ca^{2+}$ uptake by SCBP increased with incubation time. The most drastic decrease was detected after one minute of incubation: the counting rate dropped from 26×10^3 cpm (0 min) down to approx. 18×10^3 cpm. After 20 min the counting rate in the pellet was further reduced to about 15×10^3 cpm. In contrast, within the SCBP-containing supernatant an increase in SCBP bound $^{45}Ca^{2+}$ was measured. $^{45}Ca^{2+}$ radioactivity was lowest, when actomyosin and SCBP were separated immediately after starting the competition reaction (8×10^3 cpm, 0 min) and increases with increasing incubation time (11×10^3 cpm, 20 min). Both competing proteins (actomyosin and SCBP) were present in equimolar concentration of approx. 5 µM. Bars indicate mean ± SD of three independent experiments

In contrast to actomyosin, SCBP is not sedimented and the alteration of Ca^{2+} bound to SCBP was measured by a subsequent ultrafiltration of the supernatant. Within the SCBP-containing retentate after ultrafiltration, $^{45}Ca^{2+}$ was accumulated. $^{45}Ca^{2+}$ concentration was lowest, when actomyosin and SCBP were separated immediately after starting the competition reaction (0 min). With longer incubation times an increase in radioactivity was measured (Fig. 2b). This result suggests, that Ca^{2+} first binds to actomyosin and subsequently is taken over by SCBP. In control experiments using actomyosin or SCBP alone both proteins showed the highest Ca^{2+} binding already at the start point (0 min) and also at the following time intervals.

SCBP facilitates Ca^{2+} diffusion

The Ca^{2+} diffusion experiments were first performed with 0.5% agar and bovine serum albumin (50 and 250 µM) which does not belong to the family of high affinity Ca^{2+}-binding proteins. A typical curve of an experiment with 0.5% agar shows a steep incremental phase turning into equilibrium or steady-state $^{45}Ca^{2+}$ flux after 8 to 10 h (Fig. 3a). This curve represents the control situation with a mean steady-state $^{45}Ca^{2+}$ flux (I_{LR}) = 1 (0.041 ± 0.003 pmol Ca^{2+} cm^{-2} s^{-1}) which was taken as a reference for all proteins tested. All measured $^{45}Ca^{2+}$ flux values are relative to the steady-state flux in agar. Bovine serum albumin revealed no difference to 0.5% agar alone in the middle chamber as far as the incremental phase of $^{45}Ca^{2+}$ radioactivity in the outflow from the right chamber and the steady-state Ca^{2+} flux are concerned.

Earthworm SCBP was analyzed in various concentrations (5, 20, 25, 50, 100 and 200 µM) in the Ca^{2+} diffusion experiment. The results demonstrate an increase in steady-state Ca^{2+}-flux as a function of SCBP concentration. Already 5 µM SCBP yielded an increase of the Ca^{2+} flux

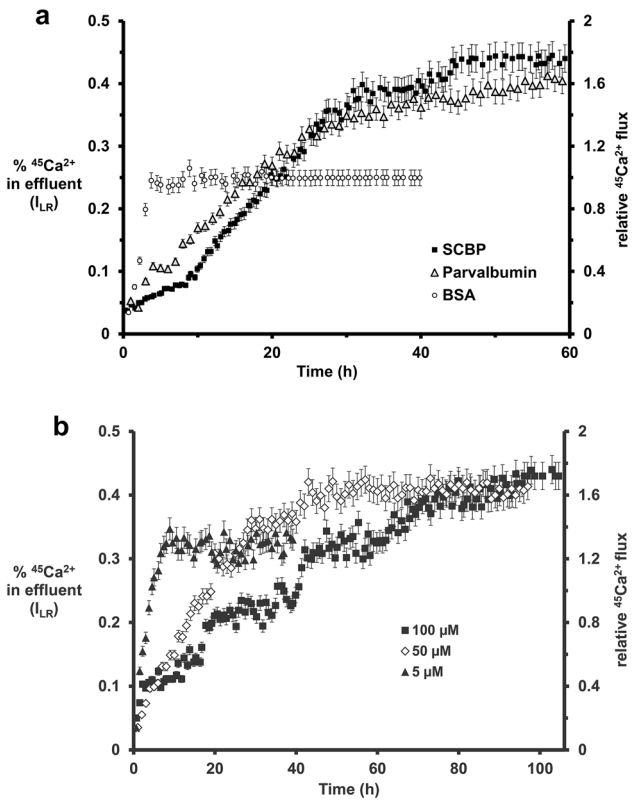

Fig. 3 Facilitated Ca^{2+} diffusion by SCBP and parvalbumin. **a** $^{45}Ca^{2+}$ flux (I_{LR}) values on the left ordinate are given in % which corresponds to $([^{45}Ca^{2+}]_R/[^{45}Ca^{2+}]_L) \times 100$. Relative $^{45}Ca^{2+}$ flux denotes the relation of I_{LR} in presence of SCBP, parvalbumin or bovine serum albumin (BSA) divided by I_{LR} in presence of 0.5% agar alone: I_{LR}(protein)/I_{LR}(agar). BSA (circles) revealed no difference to 0.5% agar alone, i.e., a steep incremental phase turning into a steady-state $^{45}Ca^{2+}$ flux (0.041 pmol cm^{-2} s^{-1}) was observed after 10 h. The resulting flux data for SCBP (squares) and parvalbumin (triangles) were 1.9 (0.077 pmol cm^{-2} s^{-1}) and 1.7 (0.072 pmol cm^{-2} s^{-1}), respectively. There was no significant difference between the fluxes with SCBP and parvalbumin (two sample *t* test $p > 0.05$). All protein solutions (25 µM) have been dialyzed against $^{45}Ca^{2+}$-containing diffusion buffer prior to the experiment (see "Materials and methods"). **b** With increasing concentration of SCBP the incremental phase of the $^{45}Ca^{2+}$ flux is prolonged, from about 10 h at 5 µM to about 100 h at 100 µM SCBP. As compared to the $^{45}Ca^{2+}$ flux in agar (I_{LR} [agar]) 5, 50 and 100 µM SCBP yielded in an increase of about 30% (relative flux 1.3; 0.053 pmol cm^{-2} s^{-1}), 60% (relative flux 1.6; 0.067 pmol cm^{-2} s^{-1}), and 70% (relative flux 1.7; 0.070 pmol cm^{-2} s^{-1}), respectively. Bars indicate mean ± SD of three independent experiments

of about 30% (mean relative flux 1.3, 0.053 pmol cm^{-2} s^{-1}) as compared to the control (0.5% agar). The amount of flux increase or the amount of diffusion facilitation brought about by SCBP tends to reach a maximum value of about 70% (relative flux 1.7, 0.070 pmol cm^{-2} s^{-1}) at 100 µM SCBP (Fig. 3a, b). This can be concluded from the concentration differences and the corresponding flux increase. In addition, the steady-state Ca^{2+} flux obtained from an experiment with 200 µM SCBP was not significantly higher (two sample *t* test $p > 0.05$, data not shown). With increasing concentration of SCBP the incremental phase of Ca^{2+} flux is prolonged, from approx. 10 h at 5 µM to about 100 h at 100 µM SCBP (Fig. 3b).

When tested in equimolar concentration (25 µM) the resulting equilibrium Ca^{2+} flux for parvalbumin from white muscle of the chub *Leuciscus cephalus* was not significantly different to SCBP (mean relative flux 1.7 corresponding to 0.072 pmol cm^{-2} s^{-1}, Fig. 3a).

It has been reported previously (Huch et al. 1988) that the SCBP isoforms differ in their Ca^{2+}-binding capacity. Using the method of equilibrium dialysis, the isoform $SCBP_2$ was found to bind 2 mol Ca^{2+} mol^{-1}, whereas $SCBP_3$ bound 3 mol Ca^{2+} mol^{-1}. The resulting flux data from experiments with purified isoforms (25 µM each) did not significantly differ (two sample *t* test $p > 0.05$). The Ca^{2+} fluxes were 0.072 ± 0.005 pmol cm^{-2} s^{-1} for $SCBP_2$ and 0.077 ± 0.004 pmol cm^{-2} 9 s^{-1} for $SCBP_3$ corresponding to relative fluxes of 1.74 and 1.87, respectively ($N = 3$, data not shown).

Ca^{2+} diffusion experiments with constant concentration of SCBP (100 µM) were performed to analyze the influence of the diffusion distance. At all three different diffusion distances tested (0.2, 0.6, and 1.0 cm) the steady-state Ca^{2+} flux increase was about 70% (Fig. 4). Applying the flux equations of Feher (1984) to our data, we calculated that SCBP increases the apparent Ca^{2+} diffusion coefficient by about 50%.

Ca^{2+} bound to SCBP is deprived by SR vesicles

The Ca^{2+} competition experiment carried out with SCBP and fragmented SR (FSR) vesicles as competing agents for Ca^{2+} was designed to analyze whether earthworm SR can take up Ca^{2+} previously bound to SCBP. As described in methods, the uptake of Ca^{2+} by FSR vesicles was measured by filtration according to Semich and Volmer (1985) in 1 ml syringe fitted with a 0.45 µm pore size filter. The $^{45}Ca^{2+}$ radioactivity remaining on the filters was analyzed after incubation of FSR vesicles with or without SCBP, both dependent on incubation time. In presence of 1 mg ml^{-1} SCBP (50 µM), that has been equilibrated against $^{45}Ca^{2+}$-containing buffer prior to the competition test, the mean counting rates after 20 min were about 19×10^3 cpm compared to approx. 4×10^3 cpm, when FSR (20 µg ml^{-1}) was incubated without SCBP (Fig. 5a). Protein determinations yielded no significant difference between the protein content of the SCBP solution and the SCBP-containing filtrates obtained after the competition assay implicating, that the additional amount of Ca^{2+} taken up by the FSR is not due to a direct interaction between SCBP and FSR. The control situation of FSR alone illustrates the background signal in the range of

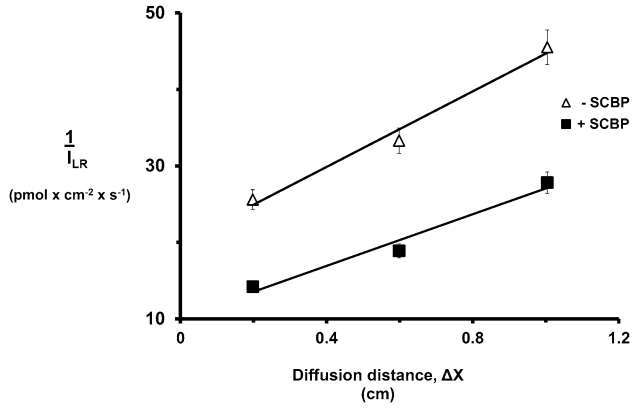

Fig. 4 Dependence of $^{45}Ca^{2+}$ flux on the diffusion distance. The inverse of the $^{45}Ca^{2+}$ flux ($1/I_{LR}$) is plotted against the thickness of the middle compartment (ΔX) for flux data obtained in the absence (blank triangles) and presence (black squares) of SCBP (100 µM) in diffusion buffer (see Methods). In the absence of SCBP the equilibrium or steady-state $^{45}Ca^{2+}$ flux was reached for 0.2 cm after 6–8 h (0.039 pmol cm^{-2} s^{-1}), for 0.6 cm after 16–18 h (0.030 pmol cm^{-2} s^{-1}), and for 1 cm after 30 h (0.022 pmol cm^{-2} s^{-1}). In the presence of SCBP, the steady-state $^{45}Ca^{2+}$ flux was reached for 0.2 cm after 72 h (0.070 pmol cm^{-2} s^{-1}), for 0.6 cm after 192 h (0.053 pmol cm^{-2} s^{-1}) and for 1 cm after 300 h (0.036 pmol cm^{-2} s^{-1}). The slope of $1/I_{LR}$ in this plot corresponds to D_aC from the equation $I_{LR} = D_aC/\Delta X$, where D_a is the apparent diffusion coefficient of $^{45}Ca^{2+}$ and C is the total $^{45}Ca^{2+}$ concentration (Feher 1984). Assuming a C of 1 µM the D_a is 3.8×10^{-5} and 5.8×10^{-5} cm^{-2} s^{-1} in 0.5% agar and 100 µM SCBP, respectively. The plot shows that SCBP enhances the apparent diffusion coefficient for $^{45}Ca^{2+}$. At all three different diffusion distances tested the obtained steady-state $^{45}Ca^{2+}$ flux increase in presence of SCBP was about 70%. Bars indicate mean ± SD of three independent experiments

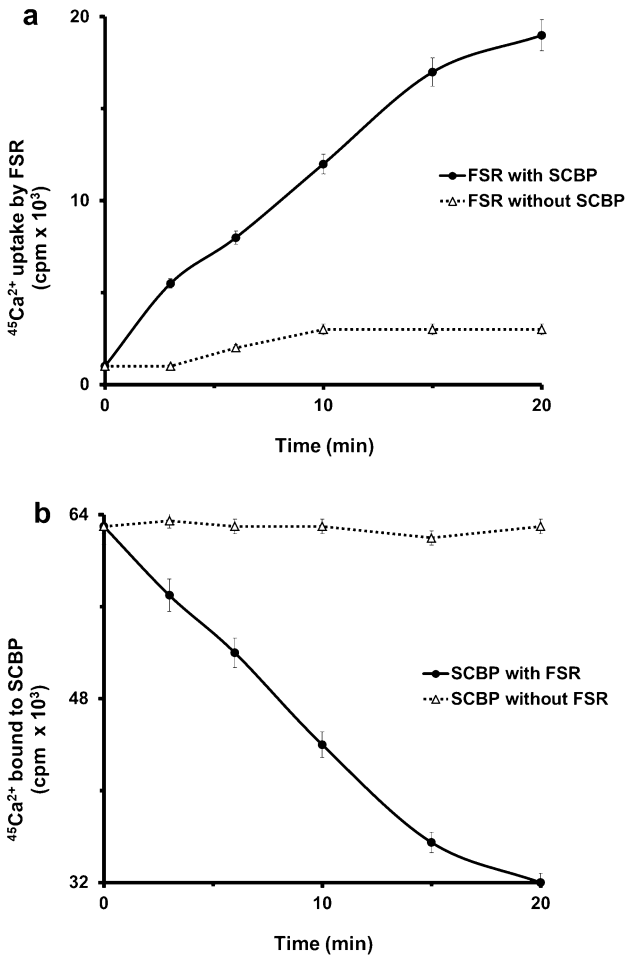

Fig. 5 SR-SCBP $^{45}Ca^{2+}$ competition experiment. **a** Time course of $^{45}Ca^{2+}$ uptake by fragmented SR (FSR) in presence of SCBP (black dots) or in incubation medium without SCBP (triangles). In presence of SCBP that has been equilibrated against $^{45}Ca^{2+}$-containing buffer prior to the competition test (see methods), the counting rates after 20 min were about 19×10^3 cpm compared to about 4×10^3 cpm, when FSR was incubated without SCBP. Protein concentration: 1 mg ml^{-1} (=50 µM SCBP) and 20 µg ml^{-1} (FSR). Assay volume: 1 ml. **b** Time course of $^{45}Ca^{2+}$ deprivation from SCBP by FSR. SCBP was incubated with FSR (black dots) or alone (triangles). The SCBP-containing filtrate obtained after filtration to separate FSR from SCBP was subsequently ultrafiltrated leaving SCBP enriched within the retentate. The counting rate in the control experiment (SCBP without FSR) was constant with incubation time, about 63×10^3 cpm. In contrast, when SCBP was incubated with FSR, the amount of $^{45}Ca^{2+}$ radioactivity in the retentate was reduced to about 32×10^3 cpm after 20 min. Bars indicate mean ± SD of three independent experiments

4×10^3 cpm caused by unspecific binding to the filters. The SCBP-containing filtrate obtained after filtration to separate FSR from SCBP was subsequently ultrafiltrated leaving SCBP enriched within the retentate. The counting rate in the control experiment (SCBP without FSR) was constant with incubation time, with a mean of 63×10^3 cpm (Fig. 5a). In contrast, when SCBP was incubated with FSR, the amount of $^{45}Ca^{2+}$ radioactivity in the retentate was reduced to a mean of 32×10^3 cpm after 20 min (Fig. 5b). Therefore, it can be concluded that under the experimental conditions the Ca^{2+}-binding state of SCBP is reduced by about 50% as a consequence of the ATP-driven Ca^{2+}-pumping action of the SR vesicles. Thus, the source for the additional amount of $^{45}Ca^{2+}$ taken up by the FSR vesicles is SCBP.

Discussion

Two physiological functions have been proposed and are under continuous discussion for soluble Ca^{2+}-binding proteins (SCBP) and parvalbumin (PV) during muscle relaxation namely as Ca^{2+} buffer and Ca^{2+} shuttle transporting Ca^{2+} between myofibrils and sarcoplasmic reticulum (SR). Both roles result from kinetic considerations of their ability to bind and exchange Ca^{2+} and Mg^{2+}, respectively (Wnuk et al. 1982). Evidence supporting a role as buffer for SCBPs comes from structural studies which showed no significant Ca^{2+}-dependent conformational changes (Engelborghs et al. 1990; Cook et al. 1993; Sillen et al. 2003) in contrast to

"Ca^{2+} sensors" (e.g., calmodulin; Chin and Means 2000; Johnson 2006), where binding of Ca^{2+} induces a conformational change which enables them to interact with specific targets. In equilibrium dialysis and Scatchard plot analysis of the earthworm SCBP isoforms, $SCBP_2$ bound two Ca^{2+} with a K_D of 1.5×10^{-7} M and one Ca^{2+} with 1×10^{-5} M. For $SCBP_3$ the binding of three Ca^{2+} with a K_D of 1.3×10^{-7} M has been shown (Huch et al. 1988). Considering the different selectivity and affinity constants for Ca^{2+} (Zot and Potter 1984; Celio et al. 1996; Schwaller 2010), three and two mixed Ca^{2+}–Mg^{2+} sites can be derived for $SCBP_2$ and $SCBP_3$, respectively. Under resting conditions where the free Mg^{2+} is several magnitudes higher than Ca^{2+} ([Ca^{2+}]: about 0.01 µM, [Mg^{2+}]: 0.5–1 mM; Berridge et al. 2000; Romani and Scarpa 1992) it is likely that the mixed Ca^{2+}–Mg^{2+} binding sites are occupied by Mg^{2+}. As shown for PV from frog muscle fibers and the SCBP of the marine annelid *Nereis* the dissociation rate of Mg^{2+} at the mixed Ca^{2+}-Mg^{2+} binding sites are too slow so that Ca^{2+} is taken up by SCBP with a delay (Engelborghs et al. 1990; Hou et al. 1992). Therefore the rate of Ca^{2+} uptake by SCBP is determined by the rate of dissociation of Mg^{2+} from SCBP. Measuring the time dependent binding of Ca^{2+} in a mixture of SCBP and actomyosin both pretreated to be in a Ca^{2+}-free and Mg^{2+}-loaded form the added Ca^{2+} is first bound to a higher extent by the actomyosin and is thereafter progressively bound by SCBP. Apparently, the slow off-dissociation rate of Mg^{2+} from SCBP allows Ca^{2+} first to bind to Ca^{2+} specific sites of the regulatory proteins of the dually regulated earthworm actomyosin (troponin C and myosin light chains; D'Haese and Ditgens 1980) to trigger muscle contraction. Thereafter, SCBP is able to inhibit the enzymatic activity of body wall actomyosin in a chelator-like manner. As shown in our assay in Fig. 1, the addition of Ca^{2+}-deprived and Mg^{2+}-loaded SCBP led to a dose-dependent decrease of the free Ca^{2+} exhibiting a stronger binding to SCBP than to the Ca^{2+}-binding proteins associated with the actomyosin. This function is related to their Ca^{2+}-buffering feature (Schwaller 2010) and would be of importance in situations of "Ca^{2+} stress" in the cytosol, such as prolonged cell activation with high Ca^{2+} levels. Our experimental setup did not allow for short time measurements. The spatial arrangement of the actomyosin and SR in the earthworm muscle probably has great influence on the Ca^{2+} movements. In our assays, the concentrations of actomyosin and SCBP were chosen to correspond closely to the proportions of both components in muscle but for experimental reason the concentrations were about 10 times lower. This may be the reason why the kinetic of actomyosin decalcification by SCBP appears very slow. But it enabled us to detect a shift of Ca^{2+} from actomyosin to SCBP which is nearly complete within the first minute (Fig. 2). Use of higher concentrations is difficult as the actomyosin in rigor state was used with a three-dimensional network of actin and reassembled myosin. The Ca^{2+} depletion of actomyosin has been so far only shown for PV with isolated skeletal myofibrils (Gerday and Gillis 1976). Furthermore, it was demonstrated that after prolonged contraction the removal of Ca^{2+} in PV-deficient mice was much slower than in wild type muscle (Raymackers et al. 2000).

During the relaxation phase, a Ca^{2+} movement from the contractile proteins to the SR takes place. Our results obtained from the Ca^{2+} diffusion experiments demonstrate that the unidirectional Ca^{2+} flux is increased by Ca^{2+}-loaded SCBP. The maximum amount of facilitation of Ca^{2+} diffusion (approx. 70%) implicates a saturation effect, which can be explained by a mutual hindrance of the SCBP molecules with increasing concentrations. In vivo SCBP may start to facilitate Ca^{2+} diffusion to the SR, provided that SCBP is Ca^{2+} saturated. As the exchange rates at the mixed Mg^{2+}–Ca^{2+} sites are not fast enough during a single twitch, repeated stimulations are needed to provide a Ca^{2+} saturated SCBP to facilitate Ca^{2+} diffusion. For longitudinal muscle a tetanic contraction and a catch-like activity of the earthworm muscle has been shown (Tashiro and Yamamoto 1971). In these cases, SCBP could accelerate the relaxation rate after a prolonged intracellular Ca^{2+} increase by facilitation of Ca^{2+} transport from myofilaments to the SR. Considering facilitated Ca^{2+} diffusion two properties of the transporting protein are of major interest, which are its Ca^{2+}-binding capacity and its affinity for Ca^{2+}. Indications for the significance of Ca^{2+}-binding affinity were gained by Feher et al. (1989) from comparative diffusion experiments performed with calmodulin, a ubiquitous Ca^{2+} sensor protein (Haeseleer and Palczewski 2002) and the intestinal calbindin-D9K which facilitates the absorption of Ca^{2+} (Balmain 1991). The effect of calmodulin was considerably less than that of calbindin-D9K. The authors attributed this difference to the lower association constant for calmodulin. Assuming an apparent K_D of 2.5×10^{-6} M, it is conceivable that calmodulin was not fully saturated with Ca^{2+} (Zot and Potter 1984; Feher et al. 1989). Thus, as the incremental phase of $^{45}Ca^{2+}$ flux is dependent on SCBP concentration and prolonged with increasing SCBP concentration (Fig. 3b) this phase may represent the process of equilibration of SCBP with $^{45}Ca^{2+}$. This can also be concluded from the finding, that dialysis of the protein against the diffusion buffer prior to the experiment resulted in a shortening of the incremental phase by about 50%.

When regarding SCBP as a Ca^{2+}-transporting molecule it is of interest to consider the interaction at the interface between cytosol and the SR. This issue was addressed by the Ca^{2+} competition experiment using SCBP and fragmented SR (FSR) vesicles. It has been shown by Stössel and Zebe (1968), that FSR vesicles from earthworm body wall muscle are able to inhibit the actomyosin ATPase by lowering the Ca^{2+} concentration. The vesicular fraction we

used for Ca^{2+} competition showed Ca^{2+} uptake properties, which were dependent on ATP and free Ca^{2+} concentration very similar as FSR from vertebrate skeletal muscle (Rüegg 2012). The Ca^{2+} competition experiment showed that SCBP enhances Ca^{2+} uptake by FSR, which can be explained by dissociation of Ca^{2+} ions from SCBP that compensate for the reduction of Ca^{2+} in the medium due to the activity of the SR Ca^{2+} ATPase. In a previous study it was suggested that PV may activate Ca^{2+} uptake by directly binding to SR (Ushio and Watabe 1994). However, our filtrate experiments clearly showed that a direct interaction between SCBP and fragmented SR (FSR) can be excluded. The SCBP-containing filtrate obtained after filtration to separate FSR from SCBP was subsequently ultrafiltrated leaving SCBP enriched within the retentate. Protein determinations yielded no significant difference between the protein content of the applied SCBP solution and the SCBP-containing ultrafiltrates obtained after the competition assay implicating, that the additional amount of Ca^{2+} taken up by the FSR is not due to a direct interaction between SCBP and FSR.

Though in lower amounts PV has been shown to be present also in various other non-muscle tissues like brain and kidney (Berchtold et al. 1984; Heizmann 1988; Bastianelli 2003; Belge et al. 2007; Olinger et al. 2012). Likewise the presence of SCBP has also been described in neuronal tissue (Hermann and Cox 1995; Kelly et al. 1997; Thirukeetheeswaran et al. 2016). In these organs the soluble Ca^{2+} binding proteins may function differently and play a role as a cytosolic Ca^{2+} buffer, as protectors against high Ca^{2+} levels, Ca^{2+} shuttle facilitating Ca^{2+} diffusion or even as Ca^{2+} sensors (Schwaller 2010).

In three different approaches using competition assays and Ca^{2+} diffusion experiments, our results consistently demonstrated a possible involvement of SCBPs in the relaxation mechanism of the fast obliquely striated muscle fibers of *Lumbricus terrestris*.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

References

Arif SH (2009) A Ca^{2+}-binding protein with numerous roles and uses: parvalbumin in molecular biology and physiology. Bioessays 31:410–421

Balmain N (1991) Calbindin-D9k. A vitamin-D-dependent, calcium-binding protein in mineralized tissues. Clin Orthop Relat Res 265:265–276

Bastianelli E (2003) Distribution of calcium-binding proteins in the cerebellum. Cerebellum 2:242–262

Belge H, Gailly P, Schwaller B, Loffing J, Debaix H, Riveira-Munoz E, Beauwens R, Devogelaer JP, Hoenderop JG, Bindels RJ, Devuyst O (2007) Renal expression of parvalbumin is critical for NaCl handling and response to diuretics. Proc Natl Acad Sci USA 104:14849–14854

Berchtold MW, Celio MR, Heizmann CW (1984) Parvalbumin in non-muscle tissues of the rat. Quantitation and immunohistochemical localization. J Biol Chem 259:5189–5196

Berridge MJ, Lipp P, Bootman MD (2000) The versatility and universality of calcium signalling. Nat Rev Mol Cell Biol 1:11–21

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Carlhoff D (1988) Dissertation. Charakterisierung der Proteine aus der schräggestreiften Muskulatur des Regenwurms *Lumbricus terrestris* mit besonderer Berücksichtigung der Myosin-gekoppelten Ca^{2+}-Regulation. Heinrich-Heine-University, Düsseldorf

Carlhoff D, D'Haese J (1987) Slow type muscle cells in the earthworm gizzard with a distinct, Ca^{2+}-regulated myosin isoform. J Comp Physiol B 157:589–597

Celio MR, Pauls T, Schwaller B (1996) Guidebook to the calcium-binding proteins. Oxford University Press, New York

Chin D, Means AR (2000) Calmodulin: a prototypical calcium sensor. Trends Cell Biol 10:322–328

Cook WJ, Ealick SE, Babu YS, Cox JA, Vijay-Kumar S (1991) Three-dimensional structure of a sarcoplasmic calcium-binding protein from *Nereis diversicolor*. J Biol Chem 266:652–656

Cook WJ, Jeffrey LC, Cox JA, Vijay-Kumar S (1993) Structure of a sarcoplasmic calcium-binding protein from amphioxus refined at 2.4 A resolution. J Mol Biol 229:461–471

Cox JA (1990) Calcium vector protein and sarcoplasmic calcium binding proteins from invertebrate muscle. In: Dedman JR, Smith VL (eds) Stimulus-response coupling: the role of intracellular calcium. Telford Press, Caldwell, pp 85–110

Cox JA, Wnuk W, Stein EA (1976) Isolation and properties of a sarcoplasmic calcium-binding protein from crayfish. Biochemistry 15:2613–2618

D'Haese J, Carlhoff D (1987) Localization and histochemical characterization of myosin isoforms in earthworm body wall muscle. J Comp Physiol B 157:171–179

D'Haese J, Ditgens A (1980) Double regulation in the obliquely striated muscle of *Lumbricus terrestris*. Musc Res Cell Motility 1:208

Engelborghs Y, Mertens K, Willaert K, Luan-Rilliet Y, Cox JA (1990) Kinetics of conformational changes in *Nereis* sarcoplasmic calcium-binding protein upon binding of divalent ions. J Biol Chem 265:18809–18815

Feher JJ (1983) Facilitated calcium diffusion by intestinal calcium-binding protein. Am J Physiol 244:C303–C307

Feher JJ (1984) Measurement of facilitated calcium diffusion by a soluble calcium-binding protein. Biochim Biophys Acta 773:91–98

Feher JJ, Fullmer CS, Fritzsch GK (1989) Comparison of the enhanced steady-state diffusion of calcium by calbindin-D9K and calmodulin: possible importance in intestinal calcium absorption. Cell Calcium 10:189–203

Fiske CH, Subbarow Y (1925) The colorimetric determination of phophorus. J Biol Chem 66:375–400

Gao Y, Gillen CM, Wheatly MG (2006) Molecular characterization of the sarcoplasmic calcium-binding protein (SCP) from crayfish *Procambarus clarkii*. Comp Biochem Physiol B Biochem Mol Biol 144:478–487

Gerday C (1988) Soluble calcium binding proteins in vertebrate and invertebrate muscles. Calcium and calcium-binding Proteins. Springer, Berlin, pp 23–39

Gerday C, Gillis JM (1976) Proceedings: the possible role of parvalbumins in the control of contraction. J Physiol 258:96P–97P

Gillis JM (1985) Relaxation of vertebrate skeletal muscle. A synthesis of the biochemical and physiological approaches. Biochim Biophys Acta 811:97–145

Haeseleer F, Palczewski K (2002) Calmodulin and Ca^{2+}-binding proteins (CaBPs): variations on a theme. Adv Exp Med Biol 514:303–317

Haiech J, Derancourt J, Pechere JF, Demaille JG (1979) Magnesium and calcium binding to parvalbumins: evidence for differences between parvalbumins and an explanation of their relaxing function. Biochemistry 18:2752–2758

Heilmann C, Brdiczka D, Nickel E, Pette D (1977) ATPase activities, Ca^{2+} transport and phosphoprotein formation in sarcoplasmic reticulum subfractions of fast and slow rabbit muscles. Eur J Biochem 81:211–222

Heizmann CW (1988) Parvalbumin in non-muscle cells. Calcium and calcium binding proteins. Springer, Berlin, pp 93–101

Hermann A, Cox JA (1995) Sarcoplasmic calcium-binding protein. Comp Biochem Physiol B Biochem Mol Biol 111:337–345

Hou TT, Johnson JD, Rall JA (1992) Effect of temperature on relaxation rate and Ca^{2+}, Mg^{2+} dissociation rates from parvalbumin of frog muscle fibres. J Physiol 449:399–410

Huch R, D'Haese J (1992) Quantification of the soluble calcium-binding protein (SCBP) in various muscle tissues of the terrestrial oligochaete *Lumbricus terrestris*. Soil Biol Biochem 24:1231–1235

Huch R, D'Haese J, Gerday C (1988) A soluble calcium-binding protein from the terrestrial annelid *Lumbricus terrestris*. J Comp Physiol B 158:325–334

Jewell BR, Rüegg JC (1966) Oscillatory contraction of insect fibrillar muscle after glycerol extraction. Proc R Soc B 164:428–459

Johnson CK (2006) Calmodulin, conformational states, and calcium signaling. A single-molecule perspective. Biochemistry 45:14233–14246

Kelly LE, Phillips AM, Delbridge M, Stewart R (1997) Identification of a gene family from *Drosophila melanogaster* encoding proteins with homology to invertebrate sarcoplasmic calcium-binding proteins (SCPS). Insect Biochem Mol Biol 27:783–792

Kiehl E, D'Haese J (1992) A soluble calcium-binding protein (SCBP) present in *Drosophila melanogaster* and *Calliphora erythrocephala* muscle cells. Comp Biochem Physiol B 102:475–482

Kretsinger RH, Nockolds CE (1973) Carp muscle calcium-binding protein. II. Structure determination and general description. J Biol Chem 248:3313–3326

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Martonosi A, Feretos R (1964) Sarcoplasmic reticulum. I. The uptake of Ca^{2+} by sarcoplasmic reticulum fragments. J Biol Chem 239:648–658

Mazumder M, Padhan N, Bhattacharya A, Gourinath S (2014) Prediction and analysis of canonical EF-hand loop and qualitative estimation of Ca^{2+} binding affinity. PLoS One 9:e96202

Olinger E, Schwaller B, Loffing J, Gailly P, Devuyst O (2012) Parvalbumin: calcium and magnesium buffering in the distal nephron. Nephrol Dial Transplant 27:3988–3994

Pechere JF, Derancourt J, Haiech J (1977) The participation of parvalbumins in the activation-relaxation cycle of vertebrate fast skeletal-muscle. FEBS Lett 75:111–114

Raymackers JM, Gailly P, Schoor MC, Pette D, Schwaller B, Hunziker W, Celio MR, Gillis JM (2000) Tetanus relaxation of fast skeletal muscles of the mouse made parvalbumin deficient by gene inactivation. J Physiol 2:355–364

Romani A, Scarpa A (1992) Regulation of cell magnesium. Arch Biochem Biophys 298:1–12

Rüegg JC (2012) Calcium in muscle contraction: cellular and molecular physiology. Springer, Berlin

Schwaller B (2010) Cytosolic Ca^{2+} buffers. Cold Spring Harb Perspect Biol 2:13

Semich R, Volmer H (1985) Calcium-uptake by sarcoplasmic reticulum prepared from the asynchronous flight muscles of *Phormia terraenovae*. Comp Biochem Physiol B 80:805–812

Sillen A, Verheyden S, Delfosse L, Braem T, Robben J, Volckaert G, Engelborghs Y (2003) Mechanism of fluorescence and conformational changes of the sarcoplasmic calcium binding protein of the sand worm *Nereis diversicolor* upon Ca^{2+} or Mg^{2+} binding. Biophys J 85:1882–1893

Stammers AN, Susser SE, Hamm NC, Hlynsky MW, Kimber DE, Kehler DS, Duhamel TA (2015) The regulation of sarco(endo) plasmic reticulum calcium-ATPases (SERCA). Can J Physiol Pharmacol 93:843–854

Stössel W, Zebe E (1968) Zur intrazellulären Regulation der Kontraktionsaktivität. Pflüger`s Arch 302:38–56

Sturm H, D'Haese J, Heide G (1993) Contraction modes of the gizzard muscle and body wall muscles in the earthworm *Lumbricus*. J Muscle Res Cell Motil 14:262

Takagi T, Kobayashi A, Konishi K (1984) Amino-acid sequence of sarcoplasmic calcium-binding protein from scallop (*Patinopecten yessoensis*) adductor striated muscle. Biochim Biophys Acta 787:252–257

Takagi T, Kazuhiko K, Cox JA (1986) Amino acid sequence of two sarcoplasmic calcium-binding proteins from the protochordate *Amphioxus*. Biochemistry 25:3585–3592

Tashiro N, Yamamoto T (1971) The phasic and tonic contraction in the longitudinal muscle of the earthworm. J Exp Biol 55:111–122

Thiruketheeswaran P, Kiehl E, D'Haese J (2016) Soluble calcium-binding proteins (SCBPs) of the earthworm *Lumbricus terrestris*: molecular characterization and localization by FISH in muscle and neuronal tissue. Histochem Cell Biol 146:635–644

Ushio H, Watabe S (1994) Carp parvalbumin binds to and directly interacts with the sarcoplasmic reticulum for Ca^{2+} translocation. Biochem Biophys Res Commun 199:56–62

White AJ, Northcutt MJ, Rohrback SE, Carpenter RO, Niehaus-Sauter MM, Gao Y, Wheatly MG, Gillen CM (2011) Characterization of sarcoplasmic calcium binding protein (SCP) variants from freshwater crayfish *Procambarus clarkii*. Comp Biochem Physiol B Biochem Mol Biol 160:8–14

Wnuk W, Cox J, Stein EA (1982) Parvalbumin and other sarcoplasmic Ca^{2+}-binding proteins. In: Cheung WY (ed) Calcium and cell function, vol II. Academic Press, New York, pp 243–278

Zot HG, Potter JD (1984) The role of calcium in the regulation of the skeletal muscle contraction-relaxation cycle. In: Sigel H (ed) Metal ions in biological systems, vol 17. Dekker, New York, pp 381–410