



Characterization of sarcoplasmic calcium binding protein (SCP) variants from freshwater crayfish *Procambarus clarkii*

Alexandra J. White^a, Michael J. Northcutt^a, Suzanne E. Rohrback^a, Robert O. Carpenter^a, Margaret M. Niehaus-Sauter^a, Yongping Gao^b, Michele G. Wheatly^b, Christopher M. Gillen^{a,*}

^a Department of Biology, Kenyon College, Gambier, OH 43022, USA

^b Department of Biological Sciences, Wright State University, Dayton, OH 45435, USA

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ABSTRACT

Sarcoplasmic calcium binding protein (SCP) is an invertebrate EF-hand calcium buffering protein that has been proposed to fulfill a similar function in muscle relaxation as vertebrate parvalbumin. We have identified three SCP variants in the freshwater crayfish *Procambarus clarkii*. The variants (pcSCP1a, pcSCP1b, and pcSCP1c) differ across a 37 amino acid region that lies mainly between the second and third EF-hand calcium binding domains. We evaluated tissue distribution and response of the variants to cold exposure, a stress known to affect expression of parvalbumin. Expression patterns of the variants were not different and therefore do not provide a functional rationale for the polymorphism of pcSCP1. Compared to hepatopancreas, expression of pcSCP1 variants was 100,000-fold greater in axial abdominal muscle and 10-fold greater in cardiac muscle. Expression was 10–100 greater in fast-twitch deep flexor and extensor muscles compared to slow-twitch superficial flexor and extensors. In axial muscle, no significant changes of pcSCP1, calmodulin (CaM), or sarcoplasmic/endoplasmic reticulum Ca-ATPase (SERCA) expression were measured after one week of 4 °C exposure. In contrast, large decreases of pcSCP1 were measured in cardiac muscle, with no changes in CaM or SERCA. Knockdown of pcSCP1 by dsRNA led to reduced muscle activity and decreased expression of SERCA. In summary, the pattern of pcSCP1 tissue expression is similar to parvalbumin, supporting a role in muscle contraction. However, the response of pcSCP1 to cold exposure differs from parvalbumin, suggesting possible functional divergence between the two proteins.

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1. Introduction

Sarcoplasmic calcium binding proteins (SCPs) are high-affinity EF-hand Ca^{2+} buffers expressed most highly in skeletal muscles (Hermann and Cox, 1995). SCPs are found exclusively in invertebrates, and have been identified in crustaceans, insects, annelids, mollusks, cephalochordates and other groups (Cox et al., 1976, 1981; Cox and Stein, 1981; Takagi and Cox, 1990; Pauls et al., 1993; Kelly et al., 1997). Crayfish SCP is a dimer of 22 kDa subunits, with each subunit containing three functional Ca^{2+} binding sites (Cox et al., 1976). Two subunits, α and β , have been purified from crayfish muscle and characterized (Wnuk and Jauregui-Adell, 1983).

The binding properties of crayfish SCP dimers are complex (Wnuk et al., 1979). Two sites are Ca^{2+} -specific and four are Ca^{2+} - Mg^{2+} sites. There is evidence for both positive and negative cooperativity in the Ca^{2+} - Mg^{2+} sites. Binding constants for Ca^{2+} are $\sim 10^{-7}$ M in the presence of 1 mM of Mg^{2+} and $\sim 10^{-8}$ M in its absence. Mg^{2+} binds with lower affinity than Ca^{2+} (binding constant $\sim 10^{-5}$ M).

Sequence identities among SCP proteins from different species are low, and multiple isoforms are present in many species, suggesting considerable evolutionary modification of the protein. Lobster has α and β subunits with similar amino acid composition to the crayfish peptides (Wnuk and Jauregui-Adell, 1983). Two SCP peptides that differ from each other across their entire sequences are expressed in shrimp (Takagi and Konishi, 1984a, 1984b). Seven isoforms of a SCP protein, differing across a 17 amino acid segment that encompasses the first EF-hand domain, are found in amphioxus (*Branchiostoma lanceolatum*) (Takagi and Cox, 1990).

SCP has been proposed to function in a role analogous to that of vertebrate parvalbumin. Both proteins are Ca^{2+} buffers expressed at very high levels in fast-twitch skeletal muscle. Parvalbumin increases muscle relaxation rate without affecting activation kinetics (Cox et al., 1976; Muntener et al., 1995; Schwaller et al., 1999). In resting muscle, parvalbumin binds mainly Mg^{2+} . During contraction, it slowly releases Mg^{2+} and binds Ca^{2+} , thus enhancing the relaxation rate without competing with troponin C for Ca^{2+} during activation (Berchtold et al., 2000; Wang and Metzger, 2008). The metal binding properties of SCP suggest that it could play a similar role in invertebrate muscle. However, despite the similarities between parvalbumin and SCP, structural differences between the proteins call into question whether they

* Corresponding author. Tel.: +1 740 427 5399; fax: +1 740 427 5741.

E-mail address: gillenc@kenyon.edu (C.M. Gillen).

serve identical functions. Parvalbumin is smaller (~12 kDa) than SCP and does not form dimers. Further, metal binding by parvalbumin is simpler than by SCP. Each parvalbumin molecule has two EF-hand Ca^{2+} binding sites, and little cooperativity is observed (Schwaller, 2010).

We previously characterized a SCP transcript from the freshwater crayfish *Procambarus clarkii* (originally called pcSCP1, here renamed pcSCP1a) (Gao et al., 2006). We now report the cloning of two additional SCP variants (pcSCP1b and pcSCP1c). The three variants differ from each other across a 123 b.p. region. The protein coded for by pcSCP1b matches the previously reported β subunit, while the pcSCP1a and pcSCP1c code for proteins that match the α subunit equally well.

One explanation for polymorphism of pcSCP1 transcripts is that they are differentially expressed to meet differing functional needs across tissues or in response to environmental stresses. Another possibility is that a combination of functional properties is required within particular tissues to meet complex Ca^{2+} binding needs. We tested the differential tissue expression hypothesis by measuring mRNA expression across tissues and during environmental stress. We also compared expression patterns of pcSCP1 to those of vertebrate parvalbumin measured in other studies. If parvalbumin and pcSCP1 fulfill similar functional roles, then similar expression patterns are expected. Because parvalbumin has previously been shown to be induced by cold exposure in skeletal muscle (Nelson et al., 2003), we evaluated responses of pcSCP1 to the cold. Finally, in a preliminary direct assessment of pcSCP1 function, we examined the effects of pcSCP1 knockdown by RNAi.

2. Materials and methods

2.1. Animals

Male and female freshwater crayfish *P. clarkii* ranging from 10 to 20 grams were obtained from Niles Biological, Inc. (Sacramento, CA, USA). Crayfish were maintained in 40 L aquaria with filtered aerated tap water at 23 °C and a 16 h:8 h light–dark cycle. They were fed twice weekly with shrimp pellets. For cold exposure experiments, animals were randomly assigned to groups held at 23 °C or 4 °C for one week. We have previously shown that one week exposure to 4 °C induces changes in calcium-handling genes in *P. clarkii* (Gao et al., 2009a). Four trials of cold exposure were performed, with 3 control and 3 cold-acclimated crayfish per trial. Cardiac muscle, axial abdominal muscle, antennal gland, hepatopancreas, and gill were collected from decerebrated animals. Tissues were frozen immediately in liquid nitrogen and stored at –80 °C.

2.2. Hemolymph cation measurements

The effect of cold exposure on hemolymph Ca^{2+} and Mg^{2+} was determined in separate groups of crayfish exposed one week to 4 °C and 23 °C. Hemolymph was sampled using a 27 gauge needle inserted into the arthroidal membrane of a walking leg. Samples were diluted 1:100 with ultrapure water and analyzed using a 500-DX chromatography system (Dionex, Sunnyvale, CA, USA) with CS12a cation-exchange column, AS50 autosampler, and CSRS-Ultra 4 mm recyclable suppressor. Samples (25 μL) were eluted with 18 mmol L^{-1} methanesulfonic acid at a flow rate of 1 mL min^{-1} . Ca^{2+} and Mg^{2+} were quantified by reference to standards (Spex Certiprep, Metuchen, NJ, USA) using Peaknet software (Dionex).

2.3. Standard PCR

Total RNA was isolated using STAT-60 reagent (Iso Tex Diagnostics), as specified by the manufacturer and quantified by nanospectroscopy (ND-1000, Nanodrop Technologies). Genomic DNA was removed using the TURBO DNA-free kit (Ambion, Austin, TX, USA). One μg of DNA-free total RNA was reverse transcribed to cDNA with random hexamers using

the Taqman Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). Gene-specific primers were designed with the Primer Express TM software (Applied Biosystems), synthesized (Operon), and optimized and tested for amplification efficiency by assessment of serially diluted cDNA (Table 1). Standard PCR reactions were run (Platinum Supermix or Platinum Supermix High Fidelity, Invitrogen) with 200 nM primer concentration and one μL of cDNA in 20 μL reactions. Negative controls with templates from reverse transcriptase reactions with enzyme omitted were included.

To identify pcSCP1 variants, we amplified cDNA from axial muscle using primers that amplify the full coding sequence (SCP-full, Table 1). PCR products were analyzed by agarose gel electrophoresis. Selected PCR products were ligated into the PCR 2.1 vector (Invitrogen), the vector was transformed into *E. coli*, and plasmid was purified (Qiagen). Selected clones were sequenced from both ends by automated sequencing (Retrogen).

2.4. qPCR

Quantitative real-time PCR reactions (SYBR Green, Applied Biosystems) were performed in 96-well plates on an ABI 7500 system (Applied Biosystems). To specifically amplify pcSCP1 variants, we designed forward primers specific to each variant and used these with the same reverse primer (Table 1; SCP1a, SCP1b, and SCP1c). cDNA was synthesized as above. Twenty μL reactions were run containing 2 μL of cDNA and primers at 900 nM, except for 18sRNA primers which were at 200 nM. cDNA was diluted 10 fold for reactions with 18 s rRNA and pcSCP1 primers, due to the high expression level of these transcripts. Cycling conditions were: 50 °C for 2 min then 95 °C for 10 min for one cycle, followed by 40 cycles of 95 °C for 15 s then 60 °C for 1 min. Negative controls (cDNA synthesis reactions with enzyme omitted) were run on every plate. To check for non-specific amplification, melting curves were run for each plate. Also, selected real-time PCR products were analyzed by agarose gel electrophoresis and sequenced (Retrogen).

2.5. Statistics and analysis

Threshold cycle (C_t) was determined as the cycle at which fluorescence above a baseline signal was first detected. Samples were analyzed in triplicate and the fold changes were calculated based on the relative quantification $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001). For each cDNA, ΔC_t was calculated by subtracting average C_t of the endogenous control (18 s rRNA) reactions from the average C_t of the target. $\Delta\Delta C_t$ was calculated as the difference between the ΔC_t s in two conditions, and fold change in expression was calculated as

Table 1
Quantitative PCR primers.

Primer set	Primers (5' to 3')
SCP-full	F: TGCGGCTTCGGCTTCTGAGAAACAAGAGGT R: GCGAAGGCCGCCAGGTTGC
SCP1a	F: GGAGTTCAAGCAGGCTGTGAGAAGAAC R: GATACCGGGCAAGGCTGATG
SCP1b	F: CCCGCGTGCTTCAAGACTGTTATTAGC R: GATACCGGGCAAGGCTGATG
SCP1c	F: GTATGTGTGGGCAAGGGCTTTGACACC R: GATACCGGGCAAGGCTGATG
SCP-3prime	F: TGAAGGAGGTGCAGTAAGAAGC R: TGTGGTGCCTGTCTATTGC
SCP-RNAi	F: TGCGGCTTCGGCTTCTGAGAAACAAGAGGT R: GATACCGGGCAAGGCTGATG
18 s	F: TGGTGCATGGCCGTTCTTA R: AATTGCTGAGATCCGTCGAC
SERCA	F: CTTGACCCACGTAAGGAAG R: CAGATAGCCTCAGCAGTTGCCCT
CaM	F: GGCAATGGAACGATCGACTT R: AACACCTGAAGGCTTCCT

$RQ = 2^{(-\Delta\Delta Ct)}$. Error bars for RQ values were calculated based on the range of RQs calculated from the $\Delta\Delta Ct$ values $\pm$ standard errors. Analysis of variance (ANOVA) was used to evaluate differences in ΔCt between tissues, treatments, and pcSCP1 variants. The data met the assumptions for ANOVA based on the Anderson-Darling normality test and Bartlett's test of equal variances, except where noted. Because we have found systematic differences in ΔCt values between trials, we included trial as a variable in the ANOVA models whenever it was appropriate. Tukey's post-hoc tests were used to make pairwise comparisons.

2.6. RNAi knockdown

We followed the general strategy used by others who have successfully knocked down gene expression in crustaceans with dsRNA injections (Liu et al., 2006; Lugo et al., 2006; Tiu et al., 2007; Hui et al., 2008). We generated a 560 b.p. dsRNA spanning from b.p. –64 in the 5' untranslated region to b.p. 496 of the pcSCP1a transcript using the SCP-RNAi primer set (Table 1). The dsRNA includes the entire variable region, the entire open reading frame 5' to the variable region, and 151 b.p. 3' to the variable region. DNA templates for dsRNA synthesis were made using the BLOCK-iT RNAi TOPO® Transcription Kit (Invitrogen) and dsRNA was synthesized using the BLOCK-iT RNAi TOPO® Transcription Kit or the Megascript T7 kit (Ambion).

Ten crayfish (~15 g each) were injected with ~25–40 ng of SCP-dsRNA in 100 μ l of crayfish saline (205 mM NaCl, 6.8 mM KCl, 11.4 mM $CaCl_2$, 1.9 mM $MgCl_2$, and 2.0 mM $NaHCO_3$) in three separate trials. Eleven negative controls were injected with saline alone or with a 1.85 kb dsRNA from *Xenopus* elongation factor 1 α gene (Megascript, Ambion). Injections were made through the arthroidal membrane at the base of a walking leg with a 27 gauge needle. Injected animals were held at 23 °C. Forty-eight hours after dsRNA injection, crayfish activity was rated by observers blind to the treatment and tissues were collected for assessment of gene expression. To assess the tail flip response, we lightly tapped animals on the dorsal side and observed the resultant response (Kellie et al., 2001). We assessed the claw raise response by holding animals firmly at the junction of the thorax and observing how long the chelae remained raised (Hayes, 1977; Wigginton et al., 2010). Animals were rated as: normally active (2), impaired (1), or alive but immobile or nearly immobile (0). No differences were observed between the negative controls and they were pooled for analysis. Differences between SCP-dsRNA injected animals and controls were evaluated by two-sample t-tests.

3. Results

3.1. Detection of pcSCP1 variants

We sequenced the 579 b.p. coding region of three separate clones of pcSCP1 from *P. clarkii* axial abdominal muscle cDNA (Fig. 1a). Nucleotide sequences of the three clones are identical except for a 123

b.p. variable region from b.p. 222 to b.p. 345. Across this region, pcSCP1a (GenBank accession no. **JF692202**) and pcSCP1c (**JF692204**) are 74% identical, while pcSCP1b (**JF692203**) has lower identity to both pcSCP1a (65%) and pcSCP1c (67%). Sequencing of additional clones from both axial abdominal and cardiac muscle did not reveal any additional variants. The pcSCP1a, pcSCP1b, and pcSCP1c sequences most likely result from alternative splicing, because the three sequences match exactly with each other outside of the 123 b.p. variable region. Repeated attempts to clone the genomic DNA that encodes the variable region were unsuccessful. Thus the precise genetic mechanism underpinning the variants is not yet determined.

Sequence analysis of the entire amino acid sequences of the crayfish SCPs revealed much higher identity to each other than to SCPs from other groups. pcSCP1a, pcSCP1b, and pcSCP1c are 91–93% identical to each other and to SCP from narrow clawed crayfish, *Astacus leptodactylus* (**P05946**). Variant pcSCP1a exactly matches the previously reported pcSCP1 sequence (**ABB58783**, Gao et al., 2006), except for 3 amino acid substitutions within a 6 amino acid stretch, with 98% overall identity. Identity to two variants of SCP from the shrimp *Penaeus* sp. (**P02635** and **P02636**) is between 76% and 83%, with SCP1b having somewhat lower identity (76–78%) than SCP1a and SCP1c (79–83%). All three variants show lower identity to salmon louse *Lepeophtheirus salmonis* (**ADD24234**, 63–65%) and *Drosophila melanogaster* SCP (**NP_001015389**, 53%). A set of *Daphnia pulex* ESTs code for a protein with moderate identity to pcSCP1 (**UGID:3461313**, 55%).

The variable region of the pcSCP1 protein spans 37 amino acids that are almost entirely between the second and third EF-hand Ca^{2+} binding domains (Fig. 1b). pcSCP1a is more identical to pcSCP1c across this region (68%) than it is to pcSCP1b (58%), while pcSCP1b and pcSCP1c share 65% identity. These differences include several non-conservative amino acid sequences. For instance, pcSCP1b has 6 unique substitutions, including residue 95 (tyrosine replaces phenylalanine), residue 101 (cysteine replaces alanine) and residue 108 (arginine replaces asparagine). pcSCP1c also has 6 unique substitutions, including residue 84 (lysine replaces glutamine) and residue 104 (valine replaces threonine).

We compared the predicted amino acids sequences of the pcSCP1a, pcSCP1b, and pcSCP1c variants to the amino acid composition of purified crayfish SCPI (α_2 dimer) and SCPIII (β_2 dimer) determined biochemically by Wnuk and Jauregui-Adell (1983). The β subunit best matches pcSCP1b. Both have one extra tyrosine, serine, cysteine, and arginine compared to the α subunit and the proteins predicted by pcSCP1a and pcSCP1c. The α subunit matches pcSCP1a and pcSCP1c equally well and thus could represent proteins coded for by pcSCP1a, pcSCP1c, or a mixture of pcSCP1a and pcSCP1c.

3.2. Tissue distribution

To determine tissue distribution of the variants, we performed qPCR using variant-specific primers (Table 1; SCP1a, SCP1b, and SCP1c). First,

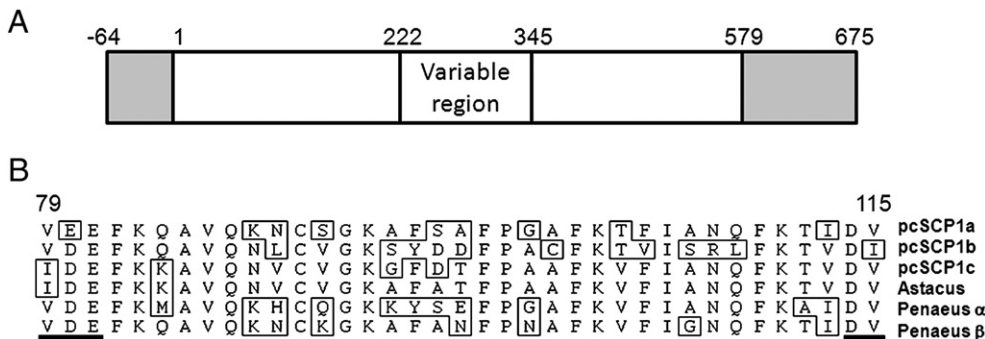


Fig. 1. A. Map of pcSCP1 variant nucleotide sequences. Non-coding regions are shaded. pcSCP1a, pcSCP1b, and pcSCP1c match exactly except for a 123 b.p. variable region. B. Map of the variable region of the amino acid sequences from the pcSCP1 variants, *Astacus leptodactylus* (**P05946**) and *Penaeus* sp. (**P02635** and **P02636**). Underlined regions represent portions of the second and third EF-hand calcium-binding domains. Boxed residues represent divergence from the consensus sequence.

we tested the specificity of the variant-specific qPCR primer sets with traditional PCR reactions using plasmid DNA templates containing the full sequence of each variant. Each variant-specific primer set produced amplicons from its corresponding plasmid and not from plasmids containing the other variants (not shown). Additionally, we evaluated selected qPCR products by agarose gel electrophoresis and sequencing and found all to match the predicted size and sequence. Finally, melting curves were examined for each qPCR plate, and no discrepancies in melting temperatures were identified.

Quantitative PCR assays revealed that the three variants were expressed in skeletal (axial abdominal) and cardiac muscle at approximately 100,000 fold and 10 fold above the level in hepatopancreas, respectively (Fig. 2). The variants were also detectable in antennal gland at approximately the same level as in hepatopancreas (not shown). No differences were identified among the variants (ANOVA, $F_{\text{tissue}} = 143$, $F_{\text{variant}} = 0.69$, $F_{\text{interaction}} = 0.17$, $p_{\text{tissue}} < 0.001$, $p_{\text{variant}} = 0.505$, $p_{\text{interaction}} = 0.953$).

All three variants were most highly expressed in the deep flexor and extensor portions of axial tail muscle compared to the superficial flexor and extensor regions (Fig. 3). No differences were observed among the variants (ANOVA, $F_{\text{tissue}} = 22.0$, $F_{\text{variant}} = 0.91$, $F_{\text{interaction}} = 0.35$, $p_{\text{tissue}} < 0.001$, $p_{\text{variant}} = 0.409$, $p_{\text{interaction}} = 0.909$). Expression in the deep flexor was approximately 100 fold above expression in the superficial flexor (Tukey Test, $p < 0.001$), while expression in the deep extensor was approximately 10 fold above the superficial extensor (Tukey Test, $p = 0.016$). Tukey tests revealed no significant differences between deep extensor and deep flexor or between superficial extensor and superficial flexor. Bartlett's test of equal variances revealed different variances among ΔCt values for the tissues ($p = 0.036$).

3.3. Cold exposure

Hemolymph Mg^{2+} was increased 89% from 1.2 ± 0.1 mM to 2.3 ± 0.3 mM after 7 days of exposure to 4°C . Hemolymph Ca^{2+} was unchanged ($23^\circ\text{C} = 4.6 \pm 0.2$ mM, $4^\circ\text{C} = 5.0 \pm 0.2$ mM). In axial muscle, expression of all three pcSCP1 variants was slightly elevated after cold exposure (Table 2). Significant differences in ΔCt values were found between trials, but there were no significant interactions between trial and other factors. When trial is included in the ANOVA, the effect of cold exposure on pcSCP1 nearly reaches statistical significance but there are no differences among the variants (ANOVA, $F_{\text{temp}} = 3.14$, $F_{\text{variant}} = 0.78$, $F_{\text{trial}} = 105.22$, $p_{\text{temp}} = 0.081$, $p_{\text{variant}} = 0.465$, $p_{\text{trial}} < 0.001$). In contrast, cold exposure did not change expression in any tissue of two other proteins that interact with Ca^{2+} , the Ca^{2+} sensor calmodulin (CaM) and

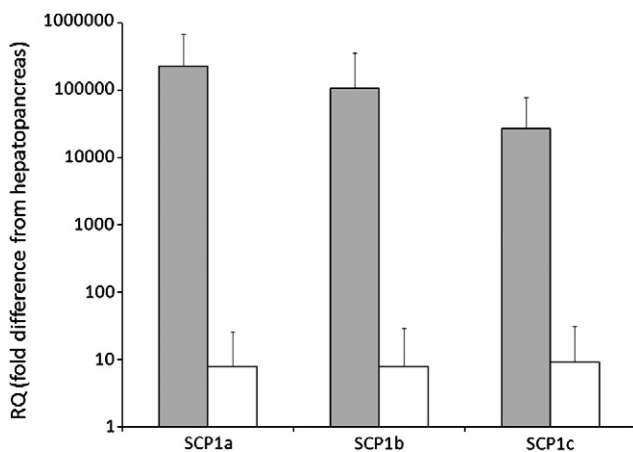


Fig. 2. Tissue expression of the pcSCP1 variants using variant-specific qPCR primers. Gray bars are axial abdominal muscle; open bars are cardiac muscle. Values are fold differences in expression (RQ) in comparison to hepatopancreas ($n = 6$ per tissue; mean $\pm$ SEM). See text for statistical analysis.

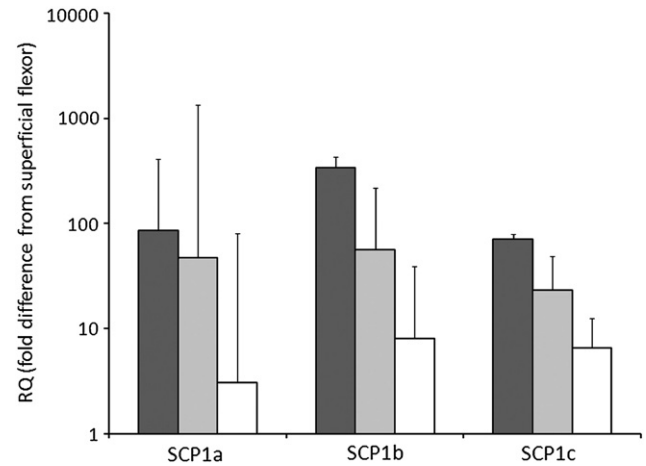


Fig. 3. Expression of the pcSCP1 variants using variant-specific qPCR primers in axial abdominal muscle subtypes. Dark gray bars are deep flexor, middle gray bars are deep extensor, and open bars are superficial extensor. Values are fold differences in expression (RQ) in comparison to superficial flexor ($n = 6$ per tissue; mean $\pm$ SEM). See text for statistical analysis.

SERCA. Ct values for 18 s rRNA were not affected by cold exposure ($\text{Ct}_{\text{room}} = 12.4 \pm 0.19$, $\text{Ct}_{\text{cold}} = 12.8 \pm 0.20$, Two sample T -test, $p = 0.118$).

All three variants of SCP were undetectable in cardiac muscle following cold exposure. In qPCR experiments, amplification of pcSCP1 from cardiac muscle of cold-acclimated crayfish resulted in ΔCt values that were not different from negative controls (Table 2). Agarose gel electrophoresis of selected qPCR products confirmed that pcSCP1 was absent from cardiac muscle of cold-acclimated animals and present in room temperature controls (Fig. 4).

3.4. RNAi knockdown of SCP

Expression of SCP was significantly decreased by about 50% in the SCP-dsRNA injected animals (Fig. 5). To avoid interference with the injected dsRNA, we monitored pcSCP1 expression using pcSCP1c-specific primers (Table 1, SCP1c) and primers that amplify all three variants 3' to the pcSCP1a dsRNA (Table 1, SCP-3prime). Similar decreases in SCP were observed after qPCR with the SCP1c-specific primers (ANOVA, $F_{\text{treatment}} = 4.77$, $F_{\text{trial}} = 11.40$, $p_{\text{treatment}} = 0.043$, $p_{\text{trial}} = 0.001$) and with the SCP-3prime primers (ANOVA, $F_{\text{treatment}} = 5.38$, $F_{\text{trial}} = 18.51$,

Table 2
Effect of one week cold exposure on gene expression.

Gene	Tissue ^a	23 °C		4 °C		RQ ^c
		N	ΔCt^b	N	ΔCt	
CaM	Hepato	7	12.09 ± 0.63	7	13.14 ± 0.58	0.48
	Cardiac	6	11.69 ± 0.35	6	11.09 ± 0.17	1.52
	Gill	7	10.57 ± 0.16	7	10.25 ± 0.15	1.25
	Axial	5	10.36 ± 0.94	5	11.00 ± 0.62	0.64
	Antennal	7	12.81 ± 0.53	7	11.94 ± 0.23	1.83
SERCA	Cardiac	6	11.77 ± 0.74	6	11.34 ± 0.34	1.35
	Axial	6	7.54 ± 1.37	6	7.48 ± 0.55	1.04
	Antennal	7	18.09 ± 0.50	7	17.55 ± 0.31	1.45
SCP1a	Cardiac	6	15.98 ± 0.94	5	n/a ^d	
	Axial	12	13.03 ± 1.99	12	12.51 ± 2.02	1.43
SCP1b	Cardiac	6	16.61 ± 0.55	4	n/a	
	Axial	12	13.92 ± 2.26	12	12.24 ± 2.55	3.20
SCP1c	Cardiac	6	15.05 ± 1.13	4	n/a	
	Axial	12	12.86 ± 2.08	12	11.10 ± 2.18	3.39

^a Abbreviations are: "hepato" for hepatopancreas, "axial" for axial abdominal muscle, "cardiac" for cardiac muscle, and "antennal" for antennal gland.

^b Values are mean $\pm$ SEM.

^c RQ represents fold increase due to cold exposure.

^d n/a refers to conditions where Ct values for the target were not different from negative controls.

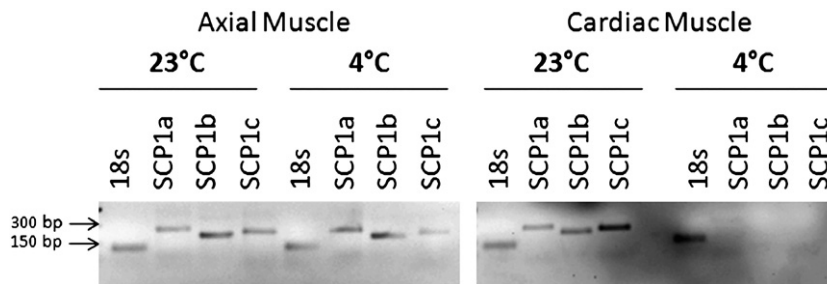


Fig. 4. Analysis of amplicons from qPCR reactions with variant specific pcSCP1 primers on a 1% agarose gel. cDNAs were produced from RNAs isolated from axial and cardiac muscle after one week exposure to 4 °C or 23 °C. Predicted sizes of the amplicons are: 18 s (101 b.p.), pcSCP1a (256 b.p.), pcSCP1b (199 b.p.), and pcSCP1c (231 b.p.).

$p_{\text{treatment}} = 0.033$, $p_{\text{trial}} < 0.001$). SERCA expression was also decreased by about 50% in SCP-dsRNA injected animals (ANOVA, $F_{\text{treatment}} = 7.13$, $F_{\text{trial}} = 4.34$, $p_{\text{treatment}} = 0.016$, $p_{\text{trial}} = 0.030$). In contrast, there was a trend toward increased CaM expression (ANOVA, $F_{\text{treatment}} = 3.60$, $F_{\text{trial}} = 6.77$, $p_{\text{treatment}} = 0.075$, $p_{\text{trial}} = 0.007$).

Crayfish injected with SCP-dsRNA were substantially less active than control animals. On a scale where 2 represents normal activity and 0 represents nearly immobile, control animals were rated 1.9 ± 0.1 (SEM) while SCP-dsRNA animals were rated 1.0 ± 0.21 (Two-Sample *T*-test, $T = 3.86$, $p = 0.002$). In response to manual stimulation, most SCP-dsRNA injected animals were able to produce single tail-flips, but were often unable to generate a series of tail-flips. Also, most SCP-dsRNA injected animals were able to take a defensive position with their claws above their bodies, but apparently fatigued quickly and were unable to sustain the position for longer than two seconds.

4. Discussion

4.1. SCP variant sequences

Multiple forms of SCP are expressed in many invertebrates, but the functional basis for this polymorphism is not well understood (Hermann and Cox, 1995). We report here the molecular cloning of three SCP variants from the freshwater crayfish *P. clarkii*. Because their nucleotide sequences match each other exactly outside the variable region, these variants are most likely the product of alternative splicing. The pcSCP1 variants provide an opportunity to explore the basis of SCP polymorphism in a model system that is well characterized at both molecular and physiological levels.

Alternative splicing can produce transcripts coding for proteins with dramatic or subtle functional differences (Stamm et al., 2005). In the case of the pcSCP1 variants, one possibility is that the variants vary in their ion binding affinities, a difference that has been seen among splice variants

of other proteins (Stetefeld and Ruegg, 2005; Gimenez and Forbush, 2007). Comparisons to other EF-hand Ca^{2+} binding proteins show that differences in binding affinity are possible even though most of the differences between the three pcSCP1 variants lie outside the EF-hand domains. In both parvalbumin and troponin C, amino acid substitutions outside the EF-hand domains affect binding affinity (Gillis et al., 2003; Tikunova and Davis, 2004; Erickson and Moerland, 2006). In conjunction with functional differences, alternatively spliced transcripts often have differential cell, tissue, or developmental expression patterns (Payne and Forbush, 1994; Stamm et al., 2005). However, we report here that there are no differences in tissue distribution or in the response to cold exposure among the pcSCP1 variants. We also compare expression patterns to those of the vertebrate Ca^{2+} binding protein parvalbumin, because SCP has been argued to have functional similarities to parvalbumin (Cox et al., 1976).

4.2. Tissue distribution

The overall pattern of pcSCP1 expression is similar to that of vertebrate parvalbumin, with some important exceptions. Transcripts of the three SCP variants were found to be most highly expressed in axial abdominal muscle, with some expression in cardiac muscle, and very low expression in hepatopancreas and antennal gland. These findings are consistent with those of Cox and colleagues (Cox et al., 1976). High expression of SCP in skeletal muscle parallels the expression pattern of parvalbumin. However, the presence of SCP in cardiac muscle represents a difference from parvalbumin, which is not usually expressed in this tissue (Wang and Metzger, 2008). This difference in cardiac muscle expression could be due to differences between parvalbumin and SCP function or differences between regulation of contraction in invertebrate versus vertebrate hearts.

We further refined the localization of pcSCP1 within abdominal muscle, which is divided into superficial “tonic” muscle with slow-twitch properties and deep “phasic” muscle with fast-twitch properties (Kennedy and Takeda, 1965a, 1965b). Our results demonstrate considerably higher expression of pcSCP1 in the deep, fast muscles of both the flexor and extensor systems, demonstrating the association of SCP with fast-twitch muscle. Parvalbumin is also most highly expressed in fast-twitch muscles (Blum et al., 1977). Overall, the tissue distribution pattern supports the hypothesis that SCP, like parvalbumin, serves to increase relaxation rate.

In fish, parvalbumin isoforms are differentially regulated across muscles with different functional properties (Wilwert et al., 2006; Coughlin et al., 2007; Brownridge et al., 2009). In contrast, we find no evidence for such differential tissue expression of the crayfish SCP variants. Patterns of expression among tissues and abdominal muscle sub-types were the same for all three variants. Further, mRNA for all three variants appears to be expressed at approximately the same level. Based on the ΔCt values for room-temperature acclimated crayfish, pcSCP1a and pcSCP1c mRNA are approximately 2-fold more abundant than pcSCP1b mRNA (Table 2). This finding contrasts somewhat with Wnuk and Jauregui-Adell (Wnuk and Jauregui-Adell,

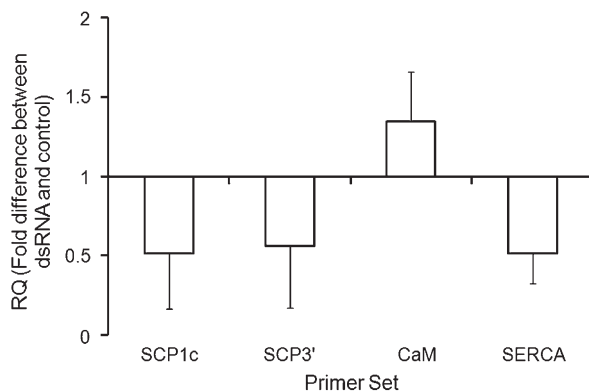


Fig. 5. Gene expression after knockdown of pcSCP1 by RNAi. Values are fold differences in expression (RQ) after injection of dsRNA in comparison to control ($n = 10$ SCP-dsRNA, $n = 11$ control; mean $\pm$ SEM). See text for statistical analysis.

1983), who found 14-fold higher expression of α -subunit dimers compared to β -subunit dimers. Regulation at the translational level could explain this discrepancy.

4.3. Cold exposure

Cold exposure is thought to affect Ca^{2+} balance of ectotherms (Godiksen and Jessen, 2002; Nelson et al., 2003). We see no evidence of changes in hemolymph Ca^{2+} in response to one week of cold exposure. This result does not rule out challenges to extracellular Ca^{2+} homeostasis, but it does suggest that crayfish are able to meet a cold challenge. Additionally, effects on intracellular Ca^{2+} balance are also possible. Our finding are consistent with those showing only moderate changes in hemolymph Ca^{2+} during the pre- and postmolt phases of the molting cycle, a time of considerable challenge to organismal Ca^{2+} balance (Wheatly and Hart, 1995).

In contrast to Ca^{2+} , hemolymph Mg^{2+} was nearly doubled in cold-exposed crayfish. The significance of this rise is unclear. In most marine crustaceans, hemolymph Mg^{2+} is maintained below the external level. Because high hemolymph Mg^{2+} is correlated with low activity levels, hemolymph Mg^{2+} regulation may be a factor in the ability of crustacean groups to maintain activity and survive in polar regions (Frederich et al., 2000). However, levels of Mg^{2+} in freshwater crayfish are much lower than in marine crustaceans, and it is unknown whether the measured changes in Mg^{2+} would affect activity of freshwater crayfish.

Previous work in crayfish has shown that cold exposure leads to changes in expression of Ca^{2+} -handling proteins similar to those observed during postmolt. For example, both cold exposure and postmolt cause increases in expression of mRNA encoding the plasma membrane Ca^{2+} transporters NCX (Na^+ - Ca^{2+} exchanger) and PMCA (Plasma membrane Ca^{2+} ATPase) (Wheatly et al., 2007; Gao et al., 2009a). In contrast, we report here that one week of exposure to 4 °C caused little change in expression of CaM, SERCA, and pcSCP1 mRNA in abdominal muscle (Table 2). This differs from the response of each of these genes during postmolt, where CaM and SERCA are upregulated and pcSCP1 is downregulated (Gao et al., 2006, 2009b; Wheatly et al., 2007). Thus, while the plasma membrane Ca^{2+} transporters NCX and PMCA respond similarly to cold and postmolt, other Ca^{2+} handling proteins show differing responses to these conditions.

The response of pcSCP1 variants to the cold contrasts with the response of vertebrate parvalbumin. In axial abdominal muscle, we observed trends toward only small increases in expression of the pcSCP1 variants during cold exposure. In contrast, Nelson and colleagues found ~6 fold increased expression of parvalbumin after cold exposure in goldfish (Nelson et al., 2003). In cardiac muscle, we observed dramatic decreases in pcSCP1 expression. While parvalbumin is generally not found in cardiac tissues, it is expressed in hearts of Antarctic fish (Laforet et al., 1991), suggesting increased demand for parvalbumin in cardiac muscles of animals living in cold environments. Thus, in vertebrates, cold exposure appears to increase parvalbumin levels in both skeletal and cardiac muscle, while we see little change of SCP in skeletal muscle and large decreases in cardiac muscle.

The physiological rationale for decreased cardiac pcSCP1 is unclear. Heart rate decreases in response to cold exposure in crayfish (Goudkamp et al., 2004). If cold exposure also tends to decrease the amplitude of cardiac Ca^{2+} transients, then decreased pcSCP1 expression could be a mechanism to maintain amplitude of Ca^{2+} transients and contraction strength. Results from experiments where parvalbumin is expressed in cardiac tissue support this interpretation. Mammalian cardiomyocytes generally express little or no parvalbumin, and relaxation rate is increased when parvalbumin is expressed by gene transfer (Wahr et al., 1999). However, at high levels of cardiomyocyte parvalbumin expression, contraction amplitude is decreased in conjunction with a decreased amplitude of the Ca^{2+} transient (Coutu and

Metzger, 2002). Thus, decreases in cardiac Ca^{2+} buffering proteins could offset cold-induced reductions in contraction strength.

4.4. RNAi knockdown of pcSCP1

The pcSCP1a dsRNA we injected would be expected to affect expression of all three pcSCP1 variants because it contained substantial sequence exactly matching these sequences on both sides of the variable region. In support of this, we observed a ~50% knockdown using qPCR primers specific to pcSCP1c and primers that amplify all three variants. Our finding of reduced muscular activity in dsRNA injected animals is consistent with a role of pcSCP1 in relaxation kinetics of skeletal muscle. Further physiological analysis is required to more specifically characterize this effect.

Intriguingly, SERCA mRNA was also decreased while there was a trend towards increased CaM levels in RNAi treated animals. One possibility is that reduction in pcSCP1 levels alters cellular Ca^{2+} dynamics, triggering changes in SERCA and CaM expression. Off-target effects are a potential problem in dsRNA experiments (Seinen et al., 2010). However, sequence analysis using BLASTN 2.2.24+ (Altschul et al., 1997) and ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) reveals no regions of overlap between the pcSCP1 dsRNA sequence and *P. clarkii* SERCA and CaM.

5. Summary

While SCP mirrors parvalbumin in some characteristics, there are several differences. Similar to parvalbumin, pcSCP1 expression is much higher in fast-twitch compared to slow-twitch muscles. However, we find substantial pcSCP1 expression in crayfish cardiac muscle, while parvalbumin is not expressed in hearts of most vertebrates. Also, axial muscle pcSCP1 does not increase during cold exposure to the same extent as fish parvalbumin. Moreover, pcSCP1 decreases in cardiac muscle upon cold exposure, while parvalbumin increases. These differences are not entirely unexpected, because there are substantial differences in structure between crustacean SCP and vertebrate parvalbumin, suggesting that there may also be functional differences (Wnuk et al., 1979; Schwaller, 2010).

Expression patterns of the newly identified pcSCP1 variants are essentially identical. Thus, the hypothesis of differential expression to meet differing functional needs across tissues or in response to cold exposure is not supported by this work. Measurements of metal-binding kinetics of the three variants are needed to reveal whether the variants have functional differences.

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