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CARP PARVALBUMIN BINDS TO AND DIRECTLY INTERACTS WITH THE SARCOPLASMIC RETICULUM FOR Ca^{2+} TRANSLOCATION

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SUMMARY: Interaction between two relaxing factors, the sarcoplasmic reticulum and parvalbumin, in carp fast skeletal muscle was investigated. Immunoblotting using an anti-parvalbumin antibody revealed that parvalbumin bound to the light sarcoplasmic reticulum isolated from carp fast skeletal muscle in the presence of Ca^{2+}. Parvalbumin enhanced Ca^{2+} uptake activity of the light sarcoplasmic reticulum. Furthermore, using a photoreactive cross-linker, we detected a protein in the light sarcoplasmic reticulum which bound to parvalbumin in a Ca^{2+}-dependent manner. These results suggest that parvalbumin may directly interact with the sarcoplasmic reticulum in contraction-relaxation cycle of carp fast skeletal muscle.
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In vertebrate skeletal muscle, Ca^{2+} released from Ca^{2+} release channels of the sarcoplasmic reticulum (SR) binds to regulatory proteins of myofibrils, thus triggers muscle contraction. Ca^{2+} is taken up again into the SR by Ca^{2+}-ATPase during relaxation (1). Since Briggs (2) identified a low molecular weight Ca^{2+}-binding protein, parvalbumin, as a soluble relaxing factor in the skeletal muscle, many investigators have claimed that parvalbumin has an important role in regulation of muscle contraction-relaxation cycle in concert with the SR (3-6). However, details in the mechanisms underlying the interaction between the SR and parvalbumin still remain ambiguous. We previously found that the SR purified from carp fast

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skeletal muscle contained a Ca^{2+}-binding protein of Mr = about 10 kDa (7,8). The purposes of this study are to reveal the interaction between the SR and parvalbumin.

MATERIALS AND METHODS

The light SR were prepared from fast skeletal muscle of carp (*Cyprinus carpio*, 0.7 - 0.9 Kg in body weight) as reported previously (7), except that homogenizing buffer contained 10 mM 3-(N-morpholino)propanesulfonic acid (MOPS)-KOH (pH 7.2), 60 mM KCl and 10 μM phenylmethylsulfonyl fluoride (PMSF).

To examine the effects of washing with Ca^{2+}-free buffer on parvalbumin bound to the light SR, a portion of the light SR pellet was suspended in buffer containing 10mM MOPS-KOH (pH 7.2), 60 mM KCl, 2 mM $MgCl_2$, 10 μM PMSF and 1 mM EGTA, and centrifuged at 100,000 x g for 1 h. The same washing procedure was repeated to remove bound parvalbumin. The resulting pellet was suspended in and dialyzed against the same buffer without EGTA for 20 h and used for the subsequent analyses. 10 mM MOPS-KOH (pH 7.2) containing 60 mM KCl, 2 mM $CaCl_2$ and 10 μM PMSF was used for the control experiment in the presence of Ca^{2+}. Supernatant fraction was obtained by centrifugation at 100,000 x g for 1 h from carp muscle homogenate.

Parvalbumin was isolated by the "TCA" method of Tanokura *et al.* (9), purified by the method of Pechère *et al.* (10), and used for raising an antibody in rabbit. The resulting antibody was affinity-purified by using a parvalbumin-conjugated TSK gel AF-Tresyl 650 S (TOSOH) column. Protein concentration was determined by the method of Lowry *et al.* (11) using bovine serum albumin as a standard.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (12) for the Tris-glycine system or the method of Schägger and Jagow (13) for the Tris-tricine system. Samples were applied to the gels at 20 μg/lane. Immunoblotting was carried out as described previously (8).

Ca^{2+} uptake of the carp light SR was measured as described previously (14) with slight modifications. The reaction mixture contained 10 mM MOPS-KOH (pH 7.2), 0.5 mg of light SR protein/ml, 100 mM KCl, 0.1 mM $^{45}CaCl_2$ (0.74 kBq/ml), 5 mM $MgCl_2$, and 0.5 mg/ml parvalbumin. Parvalbumin was replaced by 0.1 mM EGTA in the control experiment. Parvalbumin was previously dialyzed against 10 mM MOPS-KOH (pH 7.2) containing 5 mM $MgCl_2$, 10 μM PMSF, and 5 mM EGTA for 24 h, and then against the same buffer without EGTA for 20 h to remove Ca^{2+} from parvalbumin.

Biotin-labeled parvalbumin was cross-linked with the SR as follows: 10 mg/ml parvalbumin was dialyzed against 5 mM MOPS-KOH (pH 7.8) containing 10 μM PMSF, 5 mM EGTA, and 2 mM $MgCl_2$ for 24 h, and then against the same buffer without EGTA for 20 h to remove Ca^{2+} from parvalbumin. To the inner solution was added 1/20 volume of 10 mg N-hydroxysuccinimidobiotin (Sigma)/ml dimethylsulfoxide. The mixture was dialyzed against 10 mM MOPS-KOH (pH 7.2) containing 2 mM $MgCl_2$ for 20 h and concentrated to 10 mg protein/ml by Ultrafree C3LGC (Millipore). To biotin-labeled parvalbumin thus prepared was added sulfosuccinimidyl-6(4'-azido-2'-nitrophenyl-amino)hexanoate

(Sulfo-SANPAH, Pierce) to 1 mM in the dark. The solution was incubated for 20 h at 4 °C and dialyzed against 10 mM MOPS-KOH (pH 7.2) containing 2 mM $MgCl_2$ for 20 h. The mixture containing 0.5 mg Sulfo-SANPAH/biotin-labeled parvalbumin/ml, 0.5 mg the light SR protein/ml, 10 mM MOPS-KOH (pH 7.2), 0.1 mM $MgCl_2$, and 0.1 mM $CaCl_2$ or 0.1 mM EGTA was incubated at 20 °C for 10 min. Subsequently, the mixture was exposed to an ultraviolet lamp (365 nm in wavelength) at 20 °C for 20 min and centrifuged to remove unbound biotin-labeled parvalbumin. The resulting pellet was washed again and suspended in a distilled water, subjected to SDS-PAGE, and stained by an avidin-biotin system. The mixture without the SR was treated as above in the presence of Ca^{2+} and used as a control.

RESULTS AND DISCUSSION

SDS-PAGE patterns (Tris-tricine system) and the following immunoblotting analysis in Fig. 1 revealed that the anti-parvalbumin antibody recognized parvalbumin isoforms among water-soluble proteins of carp muscle (Fig. 1, lane a). The same results were obtained in native-PAGE and the following immunoblotting analysis (data not shown). This antibody reacted with the about 10 kDa proteins in supernatant fraction, demonstrating that the proteins concerned would be parvalbumin. Then, bound parvalbumin was removed from the light SR by washing in the presence of EGTA, but not in the presence of Ca^{2+} (lanes b and c, respectively), suggesting that parvalbumin would be translocated and interact with the SR in a Ca^{2+}-dependent manner.

To elucidate the physiological role of parvalbumin in Ca^{2+} uptake of the SR, exogenous parvalbumin was added to the light SR washed in the presence of EGTA and Ca^{2+} uptake was measured. Ca^{2+} uptake with the light SR and parvalbumin at the same concentration of 0.5 mg protein/ml showed no differences from that with the light SR alone during first 30 s ($P>0.5$ in Student's *t*-test)(Fig. 2). The quantity of Ca^{2+} uptake was hardly changed after 1 min in the absence of parvalbumin, while it increased by the addition of parvalbumin. Ca^{2+} uptake after 3 min was 117.6 ± 2.3 nmol Ca^{2+}/mg protein in the absence of parvalbumin, while the uptake was

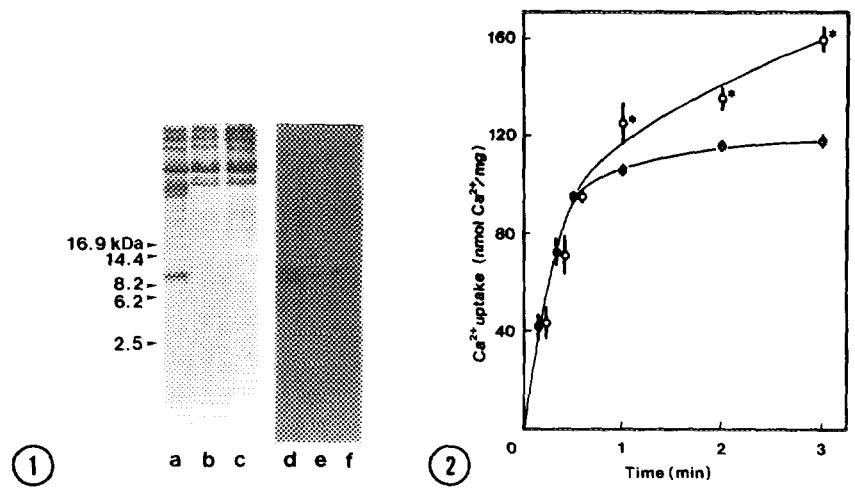
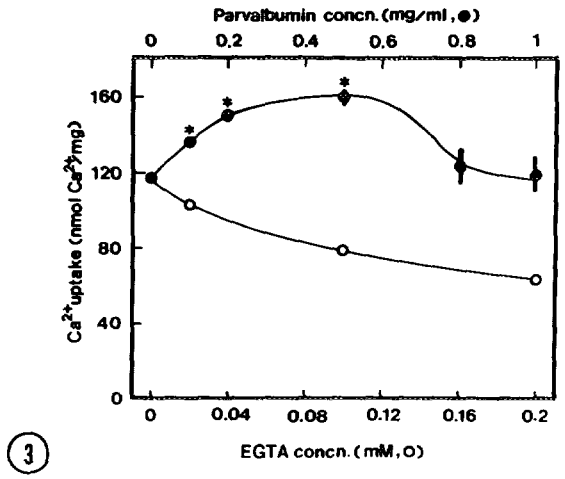


Fig. 1. Removal of bound parvalbumin from the carp light SR by washing treatment in the absence of Ca^{2+}. Supernatant fraction and the light SR from carp fast muscle were subjected to SDS-PAGE with Tris-tricine system (13), followed by an immunoblotting analysis. Patterns stained with Coomassie brilliant blue (lanes a,b and c); patterns after staining with the anti-parvalbumin antibody (d, e and f). Supernatant fraction (a and d); the light SR washed with Ca^{2+}-containing solution (b and e); the light SR washed with EGTA-containing solution (c and f). Numerals on the left represent Mr of peptides derived from horse-heart myoglobin (Sigma).

Fig. 2. Ca^{2+} uptake of the carp light SR in the absence (filled circles) and presence (open circles) of parvalbumin. The data are presented as a mean value with S.D. from 6 determinations. The values with asterisks are significantly different from those without parvalbumin ($P<0.01$ in Student's *t*-test).

159.7 ± 4.2 nmol Ca^{2+}/mg protein in its presence. Then, Ca^{2+} uptake of the light SR was measured for 3 min at various concentrations of parvalbumin up to 1 mg/ml (Fig. 3). Since Ca^{2+} binds to parvalbumin at a ratio of 2 mol/mol (15), the corresponding concentration of EGTA to chelate Ca^{2+} instead of parvalbumin was used for control experiments. EGTA apparently reduced the quantity of Ca^{2+} uptake. However, Ca^{2+} uptake was enhanced in the presence of parvalbumin at concentrations up to 0.5 mg/ml, significantly different from those in its absence ($P<0.01$ in Student's *t*-test). The protein weight ratio of parvalbumin to the light SR which gave the maximal Ca^{2+} uptake rate was about 1. This value is in the range of 0.95 - 1.30 for *in vivo* weight ratio, calculated from 8 - 11 mg/g of frog muscle for the SR (16) and 8.4 mg/g of carp muscle for parvalbumin (17).



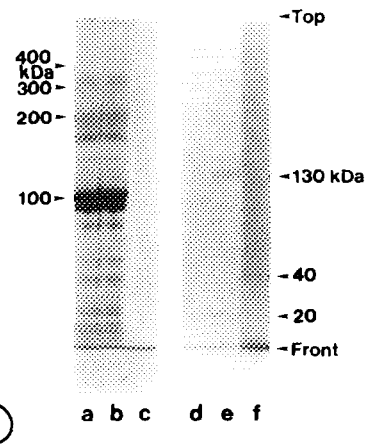


Fig. 3. Ca^{2+} uptake of the carp light SR in 3 min at various concentrations of parvalbumin (filled circles) or EGTA (open circles). The data are shown as a mean value with S.D. from 6 determinations. The values with asterisks are significantly different from those without parvalbumin. The concentrations of parvalbumin and EGTA in the upper and lower abscissa, respectively, match with each other in their molar ratios for binding Ca^{2+} of 1 and 2 mol Ca^{2+} / mol, respectively.

Fig. 4. Effect of Ca^{2+} on cross-linking between the carp light SR and biotin-labeled parvalbumin. The cross-linking reaction was performed in the absence (lanes a and d) or presence of Ca^{2+} (lanes b and e). Parvalbumin alone was also treated with the cross-linking reagent in the presence of Ca^{2+} (lanes c and f). Patterns after staining Coomassie brilliant blue (lanes a, b and c); patterns after staining by the avidin-biotin system (lanes d, e and f).

To confirm binding and interaction of parvalbumin with the carp SR, biotin-labeled parvalbumin at a concentration of 0.5 mg/ml was cross-linked to the light SR (0.5 mg/ml) using a photo-reactive reagent, Sulfo-SANPAH, and subjected to SDS-PAGE (Fig. 4). Coomassie brilliant blue staining revealed that protein profiles for the light SR were not markedly altered irrespective of the absence and presence of Ca^{2+} (Fig. 4, lanes a and b). The lane where only biotin-labeled parvalbumin was applied exhibited one band at the dye front (lane c). Parvalbumin-binding protein(s) in the carp SR transferred to a polyvinylidene difluoride membrane was detected by an avidin-biotin system (Fig. 4, lanes d, e, and f). Faint bands of about 20 and 40 kDa appearing in the absence of Ca^{2+}

probably corresponded to the dimer and tetramer of parvalbumin, respectively (lane d), because the same bands were detected when only biotin-labeled parvalbumin was applied (lane f). An application of 0.1 mM Ca^{2+} introduced two new bands, one with 130 kDa and the other on the top of the gel (lane e). Since Sulfo-SANPAH is water-soluble and membrane-impermeable, parvalbumin might be cross-linked to the domain of the 130 kDa protein exposed to the sarcoplasm. The band detected on the top suggest that a part of parvalbumin might be cross-linked to the SR membrane and not solubilized.

The present results suggest that parvalbumin would not only act as a carrier for Ca^{2+} from troponin C to the SR in carp muscle but also activate Ca^{2+} uptake by directly binding to the 130 kDa protein of the SR, thus shortening a recovery period prior to the subsequent contraction.

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