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CALCIUM-INDUCED SPLITTING OF CONNECTIN/TITIN FILAMENTS: LOCALIZATION AND CHARACTERIZATION OF CALCIUM-BINDING SITES

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Background:

Connectin, also called titin, is a giant elastic protein with a chain weight of over 3000 kDa. It exists as a very thin filament linking a thick (myosin) filament with the Z-disc in a sarcomere of vertebrate striated muscle (Maruyama *et al.*, 1985; Fürst *et al.*, 1988), thereby being responsible for keeping the thick filaments centered within the sarcomere during force generation *in vivo* (Horowits *et al.*, 1987). During post-mortem ageing of meat at 4°C, on the other hand, the connectin filament is split into β-connectin (titin 2) and a 1200-kDa subfragment at a highly elastic N_2-line region (Takahashi *et al.*, 1992; Tanabe *et al.*, 1994), resulting in a loss of muscle elasticity. This is considered to be closely related to tenderization of meat. The same splitting of connectin filaments takes place *in vitro*, when myofibrils are treated with a solution containing 0.1 mM calcium ions and 70 μM leupeptin (Takahashi *et al.*, 1992), which is the most powerful protease-inhibitor. There appeared to be no activity of protease in our myofibril preparation, because the addition of casein, which is a good substrate for various proteases, did not affect the splitting of connectin filaments (Tatsumi *et al.*, 1996). We have found, by means of ^{45}Ca autoradiography, that connectin is a calcium-binding protein and that its binding sites are restricted to the β-connectin portion (Takahashi *et al.*, 1992). Based on these findings, we have proposed that the splitting of connectin filaments is caused non-enzymatically by the binding of large amounts of calcium ions to their N_2-line region. The same splitting of connectin filaments can be also induced by magnesium ions, whose requirement is, however, two orders of magnitude higher than calcium ions (Tatsumi *et al.*, 1996), suggesting that magnesium ions bind to the calcium-binding sites on β-connectin at a lower affinity. This homology supports our proposal that connectin filaments are split specifically by sarcoplasmic calcium-ions, whose concentration rises to 0.2 mM during post-mortem ageing of meat .

Objectives:

We determined the exact localization of calcium-binding sites on β-connectin and the amount of bound calcium-ions in order to clarify the splitting mechanism of connectin filaments.

Methods:

Fluorescence detection of calcium-binding domain

β-Connectin was prepared from chicken pectoral muscle according to the method of Kimura *et al.* (1984), and it was purified by hydroxyapatite column chromatography. The protein was digested by trypsin at 25°C, then applied to SDS-PAGE on linear 2～12% polyacrylamide gradient gels. Separated proteins were transferred electrophoretically onto polyvinylidene difluoride membranes, and they were subjected to a fluorescence calcium-binding assay using a quinoline calcium indicator Quin2 (Tatsumi *et al.*, 1997). Briefly, the transferred membranes were incubated in a solution containing 60 mM KCl, 1 mM $CaCl_2$, 5 mM $MgCl_2$ and 10 mM imidazole-HCl buffer (pH 6.8) for 1 h and then rinsed with 20% ethanol. After washing with deionized water, they were incubated with 1 mM Quin2 for 1 h, followed by thorough washing with deionized water. Fluorescent patterns were visualized by illumination with UV light and photographed through a green filter with Neopan 1600 Super Presto film.

Immunoelectron microscopy

Mechanically skinned single-fiber of chicken pectoral muscle was treated with 1% Triton X-100, followed by prefixation with 2% formalin. The fiber was treated with 20 mM glycine and 1% chicken egg albumin, and incubated with antiserum diluted 10-fold with PBS and with goat anti-rabbit IgG for 12 h at 4°C. After washing with PBS, the fiber was fixed in a 2% glutaraldehyde and 2% para-formaldehyde solution, post-fixed with 1% OsO_4, dehydrated with graded ethanol, and embedded in Epon 812. Sections of about 60 nm in thickness were stained with uranyl acetate and lead citrate and were examined under an electron microscope using an accelerating voltage of 75 kV.

Molar ratio of bound calcium ions to 400-kDa fragment

β-Connectin was digested by trypsin (1:1000 by weight) at 25°C for 5 min, and it was passed through a Bio-Gel A-50m column to purify the 400-kDa fragment. Binding of calcium ions to the 400-kDa fragment was quantitatively determined using a fluorescent calcium probe Rhod2 according to the method of Olwin and Storm (1985). Fluorescence emission spectra were acquired with 12.48 μM Rhod2 and 0.43 μM 400-kDa fragment in a solution containing 0.15 M KCl, 2 mM DTT, 1 mM $MgCl_2$ and 10 mM MOPS-NaOH buffer (pH 7.2) at 25°C. The excitation wave length was set at the Rhod2 maximum of 533 nm, and emission spectra were integrated from 550 to 700 nm of wavelength to calculate concentrations of free calcium ions and those bound to Rhod2. Both concentrations were then subtracted from the total concentrations to obtain those of calcium ions bound to the 400-kDa fragment. The dissociation constant of Rhod2 for calcium ions was assumed to be 1.0 μM for these calculations (Minta *et al.*, 1989).

Results and Discussions:

When β-connectin was treated with trypsin, it was first degraded into 1700-kDa and 400-kDa fragments, and the latter was then degraded into a 300-kDa fragment. A 200-kDa fragment appeared along with further degradation of the 1700-kDa fragment into a 1500-kDa fragment. These fragments were subjected to the fluorescence method using Quin2 to identify the calcium-binding domain of β-connectin. The 1700-kDa fragment was faintly fluorescent and the 1500-kDa fragment was completely negative. Calcium ions bound to the 400- and 300-kDa fragments, in addition to the 200-kDa fragment. These results indicate that calcium-binding sites on β-connectin are localized on the 400- and 200-kDa fragments. The 200-kDa fragment was not used for further experiments, because its antibodies were not produced.

Immunoreactivity of the antiserum against the 400-kDa fragment was determined by immunoblotting. Antibodies reacted specifically with α- and β-connectins in myofibrils, and with the 400- and 300-kDa fragments in tryptic digests of β-connectin, but not with the 1700-, 1500- and 200-kDa fragments at all. Immunoelectron microscopy showed that antibodies were heavily deposited at both edges of thick filaments, forming sharp and dense stripes, which were about 0.41 μm apart from the Z-disc at a sarcomere length of 2.4 μm; the 400-kDa fragment constitutes the N-terminal region of β-connectin, followed by the 1700-kDa portion, which attaches the thick filament. The results demonstrated, therefore, that the elastic region of β-connectin has an affinity for calcium ions. The calcium-binding sites are expected to be localized near the splitting position into β-connectin and the 1200-kDa subfragment, if one considers the presence of clusters of strong negative net charges, which potentially provide calcium-binding sites in the N_2-line region of β-connectin (Labeit & Kolmerer, 1995; Kolmerer *et al.*, 1996).

The 400-kDa fragment was purified from tryptic digests of β-connectin by gel permeation chromatography and subjected to quantitative measurements of calcium-binding using Rhod2. The saturation curve for increasing calcium ion concentrations yielded a dissociation constant of 0.1 μM for binding of calcium ions to the 400-kDa fragment; the molar ratio of bound calcium ions to the fragment was 12 : 1.

Conclusions:

The calcium-binding sites on β-connectin are restricted to the 400-kDa fragment, whose N-terminal is in contact with the C-terminal of the 1200-kDa subfragment, and 12 moles of calcium ions bind to 1 mole of the fragment. We conclude that connectin filaments are split by the binding of large amounts of calcium ions to the fragment.

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