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BINDING OF PARATROPOMYOSIN TO CONNECTIN FRAGMENTS INFLUENCED BY CALCIUM IONS FROM CHICKEN SKELETAL MUSCLES

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Background

Paratropomyosin is exclusively located at the A-I junction region of sarcomeres in living and pre-rigor skeletal muscles and translocated by 0.1 mM calcium ions to thin filaments (Hattori and Takahashi, 1988), where paratropomyosin may inhibit actin and myosin interaction and induce weakening of rigor linkages formed between actin and myosin. Such weakening of rigor linkages allows lengthening of the rigor-shortened sarcomeres (Yamanoue and Takahashi, 1988) and accounts for the increased tenderness of meat during postmortem ageing. So far, it has not been known what protein or other substance interacts with paratropomyosin at the A-I junction, or why paratropomyosin is released from the region by increased calcium ion concentrations. We have shown that paratropomyosin bound to β-connectin (titin 2) and 400 kDa fragment which was a portion of N-terminal side of β-connectin, and suggested that paratropomyosin interacts with connectin filaments at the A-I junction region (Yamanoue, *et al.*, 1998a; Yamanoue, *et al.*, 1998b). On the other hand, the physiological concentration of calcium ions varies from 10^{-7} M to 10^{-5} M during the contraction-relaxation cycle in living muscles (Ebashi, 1972) and the concentration increases to 2×10^{-4} M in postmortem muscles (Nakamura, 1973). The influences of these changes in calcium ion concentration on the interaction of paratropomyosin with the connectin fragments are unclear.

Objectives

The objective in this study was to determine the influence of calcium ions on the interaction of paratropomyosin with the connectin fragments which were β-connectin and 400 kDa fragment.

Methods

Proteins. Chicken breast muscle was used. Paratropomyosin was purified with a hydroxyapatite column by the method described previously (Yamanoue *et al.*, 1998a). β-Connectin was prepared by the method of Kimura and Maruyama (1983) and purified according to Itoh *et al.* (1986). Separation of 400 kDa fragment from β-connectin preparation digested by α-chymotrypsin was done by the method of Kawamura *et al.* (1995), with omission of adding of 0.1% SDS.

Turbidity measurement. Paratropomyosin was mixed with the connectin fragments in a solution described in the legends of figures. Various pCa values were calculated under the assumption that the binding constant of EGTA in the Tris-maleate buffer (pH 6.8) would be 5.0×10^{5} M^{-1} (Ogawa, 1968), and achieved by varying the total $CaCl_2$ at a constant 2 mM EGTA. Above 0.1 mM calcium ions the solutions were prepared by adding of various amounts of $CaCl_2$. After incubation of the mixture for 30 min at 25℃, turbidity was determined by measuring the absorbance at 320 nm with a spectrophotometer (UV-2200A, Shimadzu Co., Kyoto). Mean value from two experiments was expressed.

Microscopic observation. The mixtures of paratropomyosin and the connectin fragments were observed under a phase contrast microscope (OPTIPHOT, Nikon Co., Tokyo) and photographed with a Neopan F film (Fuji Photo Film, Tokyo).

Calcium binding. After SDS-polyacrylamide gel electrophoresis (SDS-PAGE) by the procedure of Laemmli (1970) with a 2-15% gel, the separated proteins were electrotransferred onto a PVDF membrane. Binding of calcium ions to a protein was detected with the fluorescent quinoline Ca^{2+} indicator, quin2, by the method of Tatsumi *et al.* (1992).

Results and discussions

Figure 1 shows changes in turbidities of the mixtures in adding various amounts of paratropomyosin to the connectin fragments in the presence of 1 mM EGTA or 1 mM $CaCl_2$. Turbidity in the presence of 1 mM EGTA was increased with added amount of paratropomyosin up to 4 times to β-connectin by weight ratio tested here (approximately 240 : 1 molar ratio), and the increase was suppressed in the presence of 1 mM $CaCl_2$ (Yamanoue *et al.*, 1998a). When paratropomyosin was added to 400 kDa fragment up to 4 : 1 by weight ratio (approximately 47 : 1 molar ratio), turbidity was increased almost linearly to 0.036 in the presence of 1 mM EGTA. The increase was also suppressed to 0.025 in the presence of 1 mM $CaCl_2$, but the suppression was less than that of the mixture of paratropomyosin and β-connectin. Addition of various amounts of bovine serum albumin to β-connectin gave no effect on turbidity.

In examining of the mixture of paratropomyosin and β-connectin by a phase contrast microscope, aggregates formed in the mixture were observed (Fig. 2A). Aggregates of β-connectin (0.1 mg/ml) without paratropomyosin were rarely observed (data not shown). In the presence of 1 mM EGTA, the aggregates in small projections of irregular conformations were formed in adding of paratropomyosin at 1:1 by weight ratio to β-connectin and enlarged aggregates were observed frequently with increase of added paratropomyosin to 4 : 1 by weight ratio (Fig. 2A-a, b). However, in the presence of 1 mM $CaCl_2$ smaller aggregates were formed and the number of the aggregates was less than that in the presence of 1 mM EGTA (Fig. 2A-c, d). In the case of adding of 65 and 325 μg/ml paratropomyosin to 400 kDa fragment (65 μg/ml), small and spherical aggregates were formed and the number of the aggregates increased with increase of added paratropomyosin under both conditions containing 1 mM EGTA and 1 mM $CaCl_2$ (Fig. 2B). Also, the

number of aggregates formed in the presence of 1 mM $CaCl_2$ (Fig. 2B-g, h) was less than that in the presence of 1 mM EGTA (Fig. 2B-e, f). Thus, the limited formation of aggregates by 1 mM $CaCl_2$ seems to attribute to the suppression of increase in turbidity by 1 mM $CaCl_2$ as shown in Fig. 1.

Paratropomyosin was added to the connectin fragments with various calcium ions from 0 mM to 10 mM (Fig. 3). Turbidities were changed in slightly higher level when the Tris-maleate buffer was used. Turbidity of the mixture of paratropomyosin and β-connectin was little changed below 3.5×10^{-7} M calcium ions (pCa 6.45). With increase of free calcium ions, it increased almost linearly and reached a maximum value of 0.040 at pCa 4.42 (3.8×10^{-5} M), and then decreased sharply to 0.017 at pCa 4.00 (1.0×10^{-4} M). When paratropomyosin was added to 400 kDa fragment, turbidity changed in a similar pattern, but above 10^{-4} M calcium ions the extent of the decrease was less than that of the mixture of paratropomyosin and β-connectin. These results show that the interaction of paratropomyosin with the connectin fragments was drastically changed by the increase of calcium ion concentration.

Figure 4 shows binding of calcium ions to β-connectin preparation, which included of several degraded fragments of β-connectin in this experiment, 400 kDa fragment and paratropomyosin on a PVDF membrane. When the membrane was exposed to UV light, both β-connectin preparation and 400 kDa fragment were fluorescent. However, no fluorescence was detected in the region of paratropomyosin. This result suggests that changes in the interaction of paratropomyosin with the connectin fragments are attributed to binding of calcium ions to the connectin fragments, not to paratropomyosin.

Conclusions

We showed that the interaction of paratropomyosin with β-connectin and 400 kDa fragment was influenced by various calcium ion concentrations and weakened by above 10^{-4} M calcium ions which corresponds to postmortem condition of calcium ions of skeletal muscles. Thus, weakening of the interaction of paratropomyosin with connectin filaments by increased calcium ion concentration seems to release paratropomyosin from connectin filaments to translocate onto thin filaments, where paratropomyosin may weaken the rigor-linkages between actin and myosin, thereby inducing meat tenderization.

Literature

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Data in the form of figures

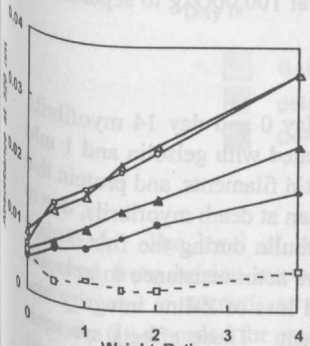

Fig. 1. Effect of the Amount of Paratropomyosin on Turbidities of the Mixtures of Paratropomyosin and Connectin Fragments.
Various amounts of paratropomyosin were mixed with β-connectin (○, ●, ▲, 50 μg/ml) and 400 kDa fragment (△, 50 μg/ml) in 0.1 M NaCl, 50 mM MOPS buffer (pH 7.0), and 1 mM EGTA (open) or 1 mM $CaCl_2$ (closed). Bovine serum albumin (□) was also mixed with β-connectin in the presence of 1 mM EGTA.

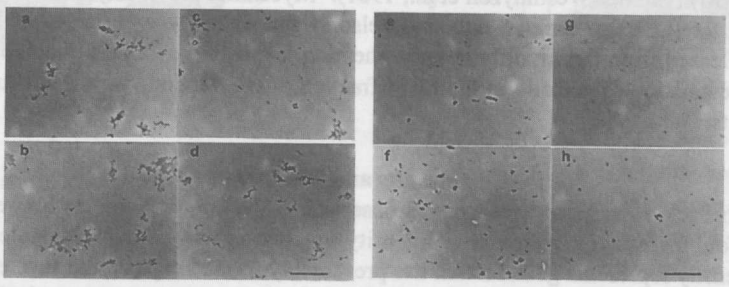

Fig. 2. Phase Contrast Micrographs of the Aggregates Formed between Paratropomyosin and Connectin Fragments.
Various amounts of paratropomyosin were mixed with β-connectin (A; 0.1 mg/ml) and 400 kDa fragment (B; 65 μg/ml) in 0.1 M NaCl, 50 mM MOPS buffer (pH 7.0), and 1 mM EGTA (a, b, e, and f) or 1 mM $CaCl_2$ (c, d, g and h). After incubation for 30 min at 25℃, the mixture was observed under a phase contrast microscope. a and c, 0.1 mg/ml paratropomyosin; b and d, 0.4 mg/ml paratropomyosin; e and g, 65 μg/ml paratropomyosin; f and h, 325 μg/ml paratropomyosin. Bar, 20 μm.

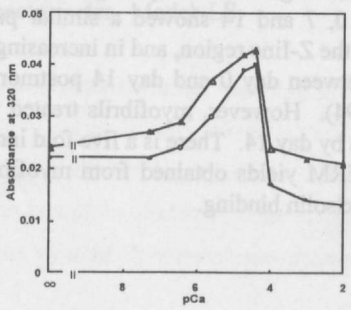

Fig. 3. Effect of Calcium Ion Concentration on Turbidities of the Mixtures of Paratropomyosin and Connectin Fragments
Various amounts of $CaCl_2$ were added to the mixtures of paratropomyosin (50 μg/ml) and connectin fragments (50 μg/ml) in 0.1 M NaCl, 50 mM MOPS buffer (pH 7.0) as described in "**Methods**". ○, β-Connectin; △, 400 kDa fragment.

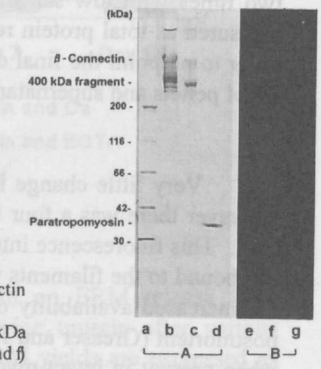

Fig. 4. Binding of Calcium ions to Connectin Fragments.
β-Connectin preparation (b, e), 400 kDa fragment (c, f) and paratropomyosin (d, g) separated by SDS-PAGE were transferred onto a PVDF membrane. The membrane was stained with naphthol blue black (A). Another membrane was incubated with 1 mM $CaCl_2$ overnight and then with 1 mM quin2 for 1 h (B). Fluorescent patterns were visualized with UV light at 365 nm. a, Molecular weight marker.