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[COMMUNICATION]

Calcium-Activated Neutral Protease Quickly Converts α-Connectin to β-Connectin in Chicken Breast Muscle Myofibrils

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ABSTRACT—Calcium activated neutral protease (CANP) quickly hydrolyzed α-connectin (2800 kDa) to β-connectin (2100 kDa) in chicken skeletal muscle myofibrils and the latter was slowly degraded to 1700 kDa and 400 kDa peptides. The 1700 kDa peptide was more slowly hydrolyzed to 1400 kDa peptide. The hydrolysis of nebulin by CANP was slower than that of α-connectin to β-connectin. The action of CANP on the isolated β-connectin was similar to that on β-connectin in myofibrils, but much less effective. E64c, a thiol protease inhibitor, completely inhibited the actions of CANP.

INTRODUCTION

Connectin (also called titin) is the largest protein (MW, 2800 k) in vertebrate skeletal muscle myofibrils (see [1] for a review) and nebulin (MW, 700 k) is the second largest protein [2]. These two proteins are more or less degraded in Duchenne dystrophy muscles [3, 4]. In normal skeletal muscles of the chicken or of the rabbit, there are always a small amount of β-connectin (also called T_2; MW, 2100 k) in addition to the mother molecule, α-connectin (also called T_1) (cf. [1]). In Duchenne dystrophic muscles, α-connectin was largely degraded to β-connectin [4]. It has been claimed that calcium-activated neutral protease (CANP; also called calpain) activity is increased in Duchenne dystrophic muscles [5]. Previously, however, it was reported that CANP rapidly degraded nebulin, but not connectin [6].

In the present study, it was clearly shown that CANP quickly converted α-connectin to β-connectin that had been missed in the earlier investigation [6]. Nebulin was also hydrolyzed by CANP in agreement with the previous results [6], but not so effectively as α-connectin.

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MATERIALS AND METHODS

Myofibrils were prepared from chicken breast muscle using a solution containing 50 mM KCl, 1 mM $NaHCO_3$ and 5 mM EGTA (pH 8.2). Finally, myofibrils were washed several times with 50 mM KCl. β-Connectin was isolated from chicken breast muscle as described before [7]. Calcium-activated neutral protease (CANP) was purified from rabbit skeletal muscle and required millimolar calcium ions for its full activity (mCANP; calpain II) [8]. The action of CANP was tested at 25°C in a solution containing myofibrils (approximately 5 mg/ml) or β-connectin (approximately 0.5 mg/ml), 3 mM $CaCl_2$, 50 mM NaCl and 20 mM Tris-HCl buffer, pH 7.5. The reaction was stopped by the addition of 1mM E64c and 5% SDS (final concentration). After boiled for 3 min followed by centrifugation for 10 min at ×15,000*g*, the supernatant was subjected to SDS gel electrophoresis according to Weber and Osborn [9] using 1.8% polyacrylamide gels. E64c was kindly supplied from Taisho Pharm. Co., Ltd., Tokyo.

RESULTS AND DISCUSSION

When chicken breast muscle myofibrils were treated with CANP, 1: 100 by weight ratio, all the α-connectin was converted to β-connectin within 1 min at 25°C, and the β-connectin was slowly degraded to 1700 kDa peptide (Fig. 1a). After 1 hr, some β-connectin remained, 1700 kDa peptide was most abundant, and some 1400 kDa peptide was formed. Also, nebulin was rapidly degraded by CANP, disappeared completely within a few minutes of incubation, and smaller peptides were formed. After 1 hr, a distinct band of approximately 400 kDa appeared, and this 400 kDa peptide was one of the main hydrolytic products of β-connectin by trypsin or chymotrypsin ([10] cf. also, Fig. 3). On the other hand, myosin heavy chain was not appreciably hydrolyzed within 10 min (Fig. 1a).

It was not clear in Figure 1a whether the conversion of α-connectin to β-connectin was faster than the hydrolysis of nebulin or not. Under the conditions where a smaller amount of CANP was added to chicken breast muscle myofibrils (1:1000), it was revealed that α-connectin was more rapidly hydrolyzed to β-connectin than the proteolysis of nebulin (Fig. 1b). α-Connectin was largely degraded toβ-connectin within 5 min, while nebulin was practically kept intact. The latter was hydrolyzed about 10 min after incubation when almost all the α-connectin was already gone.

The action of CANP was reduced in the absence of added Ca^{2+} (3 mM), as shown in Figure 2. With EGTA, the activity was to a small extent. However, it was completely inhibited by the addition of 1 mM E64c that specifically inhibits SH proteolytic enzymes [11], including CANP [12].

The action of CANP on the isolated β-connectin was examined. As seen in Figure 3a, CANP, 1:20 by weight ratio, slowly hydrolyzed β-connectin to 1700 kDa and 400 kDa peptides. The former was even more slowly converted to 1400 kDa peptide that appeared 30 min after incubation. These processes were also observed in the hydrolysis of β-connectin in myofibrils (Fig. 1). One of the reasons why isolated β-connectin was difficult to be attacked by CANP might be due to lateral association of β-connectin filaments *in vitro* [13]. The

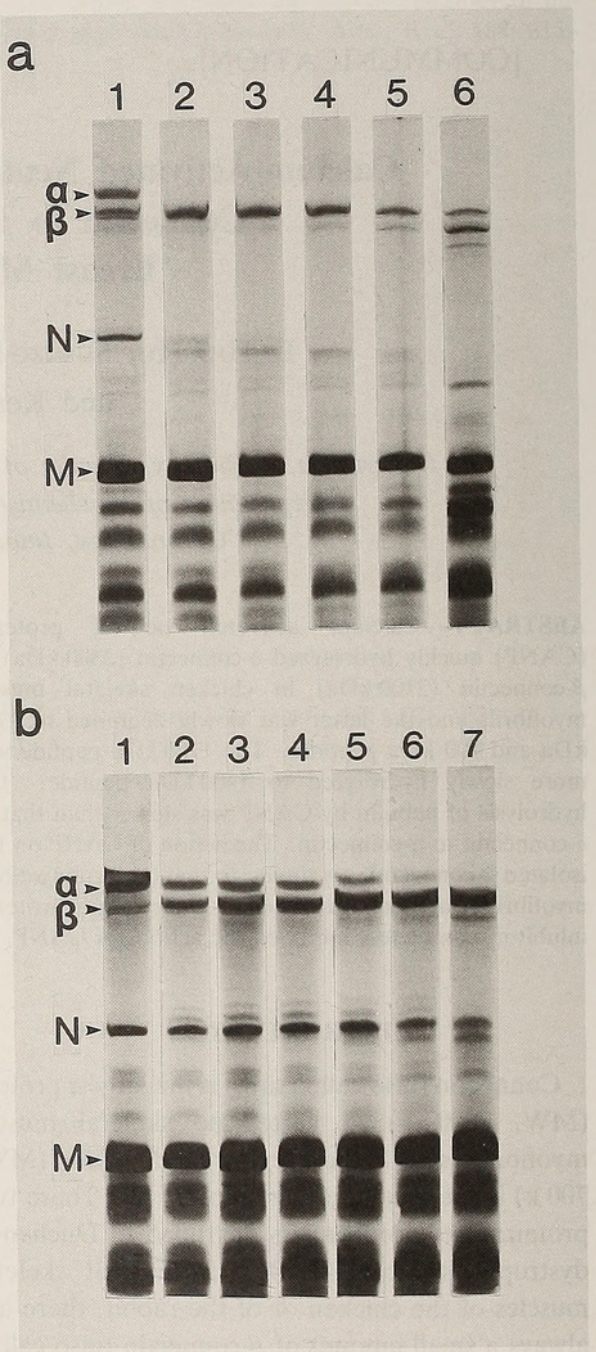

Fig. 1. Effects of calcium-activated neutral protease on the myofibrillar proteins of chicken breast muscle. Myofibrils, 5 mg/ml, were incubated with CANP (a, 1: 100 and b, 1: 1000 by weight ratio) in 50 mM NaCl, 3 mM $CaCl_2$ and 20 mM Tris-HCl buffer, pH 7.5 at 25°C. Reaction was stopped by adding 1 mM E64c and 5% SDS. SDS gel electrophoresis was carried out using 1.8% polyacrylamide gels [9]. The incubation time: a, 1, 0 time; 2, 1 min; 3, 2 min; 4, 5 min; 5, 10 min; 6, 60 min; b, 1, 0 time; 2, 30 sec; 3, 1 min; 4, 2 min; 5, 5 min; 6, 10 min; 7, 30 min. α, α-connectin; β, β-connectin; N, nebulin; M, myosin heavy chain.

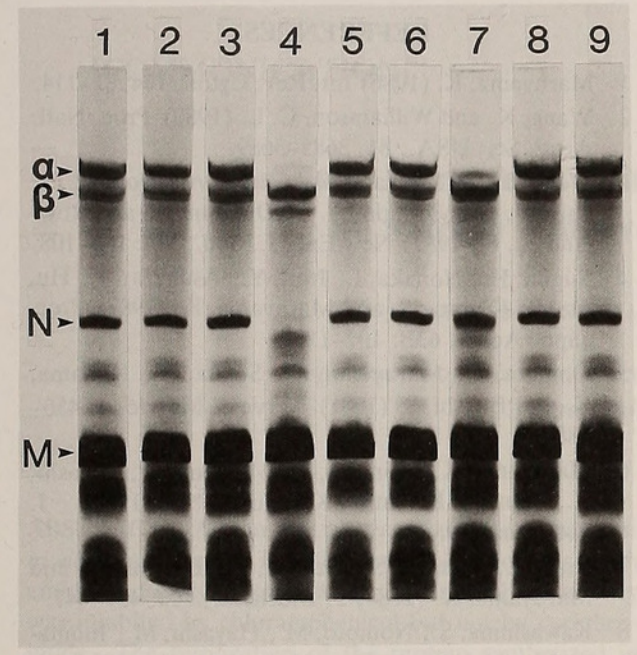

FIG. 2. Effects of various agents on the proteolysis of chicken breast muscle myofibrillar proteins by calcium-activated neutral protease. Conditions as in Fig. 1 except for the reaction time, 30 min in the presence of CANP, 1: 500 by weight ratio. 1, before incubation; 2, control after incubation; 3, CANP; 4, CANP+3 mM $CaCl_2$; 5, CANP+3 mM $CaCl_2$+1 mM E64c; 6, 3 mM $CaCl_2$+1 mM E64c; 7, 3 mM $CaCl_2$; 8, 5 mM EGTA; 9, 1 mM E64c.

action of CANP on β-connectin also required the presence of Ca^{2+} and was inhibited by E64c (Fig. 3b).

Chicken breast myofibrils tended to be degraded even when stored in 50 mM KCl and 1 mM $NaHCO_3$ at 0°C. Thus after 24 hr, α-connectin was considerably degraded to β-connectin while nebulin was hardly hydrolyzed (Fig. 4). Addtion of 5 mM EGTA retarded this spontaneous breakdown of α-connectin, and that of 1 mM $CaCl_2$ greatly enhanced the hydrolysis. Also, E64c completely inhibited the degradation. These observations support that CANP may be mainly, if not solely, responsible for the spontaneous breakdown of α-connectin in isolated myofibrils.

The present work suggests that calcium-activated neutral protease play a role in the degradation of α-connectin and nebulin in Duchenne muscular dystrophy [3, 4]. However, since an endogeneous CANP inhibitor exists in muscle cells [14, 15], the control mechanism of CANP activity

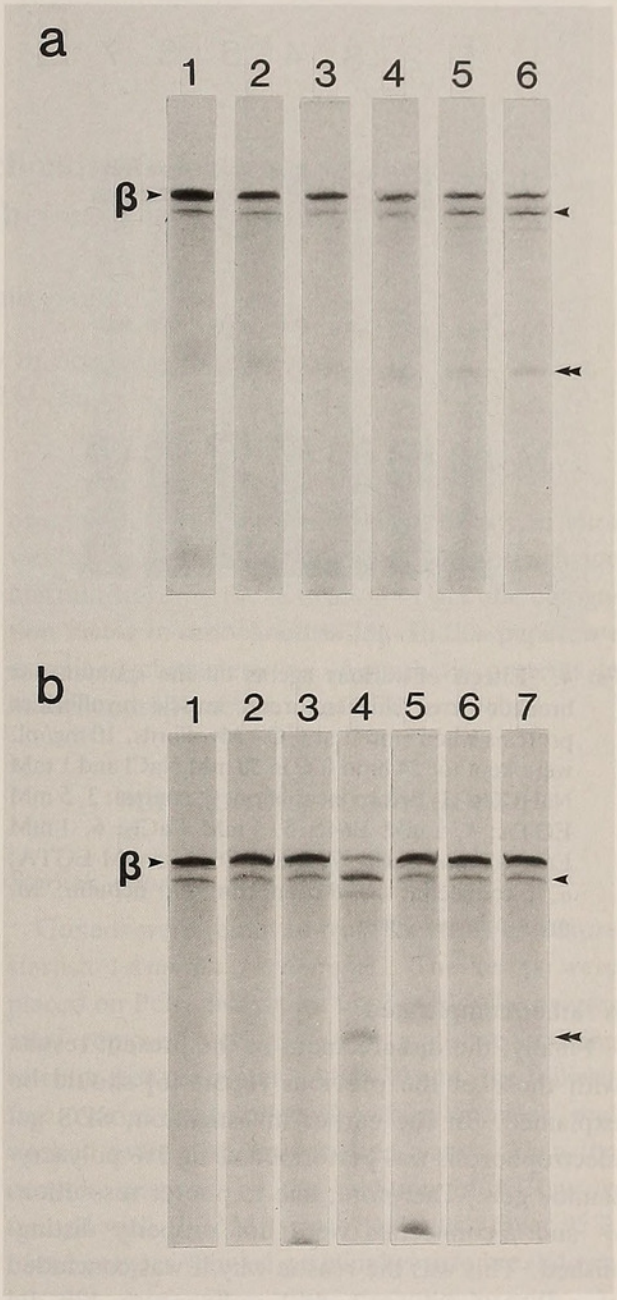

FIG. 3. Hydrolysis of isolated β-connectin by calcium-activated neutral protease. β-Connectin, 0.5 mg/ml was incubated with CANP, 1: 20 by weight ratio, in 0.3 M NaCl and 20 mM Tris buffer, pH 7.5, at 25°C. a, time course in the presence of 3 mM $CaCl_2$. 1, 0 time; 2, 1 min; 3, 2 min; 4, 10 min; 5, 30 min; 6, 60 min. b, effects of various agents. Incubated for 30 min. 1, initial; 2, control after incubation; 3, CANP; 4, CANP+3 mM $CaCl_2$; 5, CANP+3 mM $CaCl_2$+1 mM E64c; 6, 3 mM $CaCl_2$+1 mM E64c; 7, 3 mM $CaCl_2$. β, β-connectin; arrowheads, 1700 kDa peptide; double arrowheads, 400 kDa peptide.

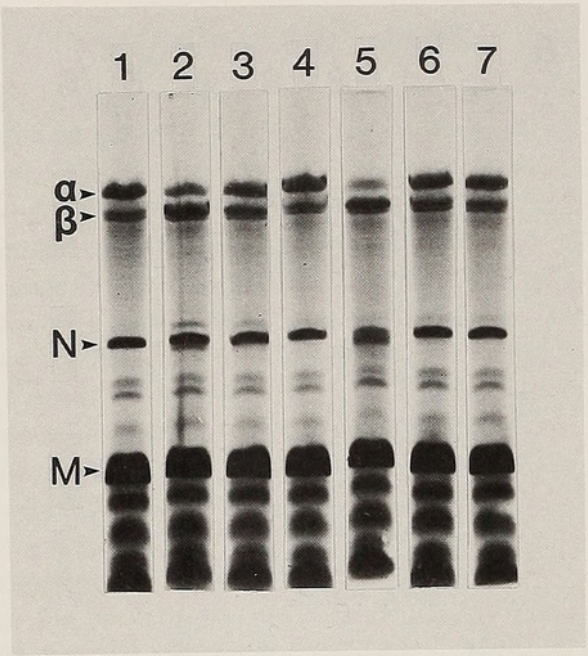

Fig. 4. Effects of various agents on the spontaneous breakdown of chicken breast muscle myofibrillar proteins when stored at 0°C. Myofibrils, 10 mg/ml, were kept for 24 hr at 0°C in 50 mM NaCl and 1 mM $NaHCO_3$. 1, before incubation; 2, control; 3, 5 mM EGTA; 4, 1 mM E64c; 5, 1 mM $CaCl_2$; 6, 1 mM E64c+1 mM $CaCl_2$; 7, 1 mM E64c+5 mM EGTA; α, α-connectin; β, β-connectin; N, nebulin; M, myosin heavy chain.

is rather complicated.

Finally, the disagreement of the present results with those of the previous report [6] should be explained. In the earlier investigation, SDS gel electrophoresis was performed using 3% polyacrylamide gels. Therefore, due to poorer resolution, α- and β-connectins were not properly distinguished. This was the reason why it was concluded that CANP did not hydrolyze connectin but did degrade nebulin in the previous study [6].

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REFERENCES

1 Maruyama, K. (1986) Int. Rev. Cytol., **104**: 81–114.
2 Wang, K. and Williamson, C. L. (1980) Proc. Natl. Acad. Sci. USA., **81**: 3685–3689.
3 Wood, D. S., Zeviani, M., Prelle, A., Bonilla, E., Salviati, G., Miranda, A. F., Dimauro, S. and Rowland, L. P. (1987) New Eng. J. Med., **316**: 106–108.
4 Sugita, H., Nonaka, I., Itoh, Y., Asakura, A., Hu, D. H., Kimura, S. and Maruyama, K. (1987) Proc. Japn. Acad., **63B**: 107–110.
5 Obinata, T., Maruyama, K., Sugita, H., Kohama, K. and Ebashi, S. (1981) Nerve & Muscle, **4**: 456–488.
6 Maruyama, K., Kimura, M., Kimura, S., Ohashi, K., Suzuki, K. and Katunuma, N. (1981) J. Biochem., **89**: 711–715.
7 Itoh, Y., Kimura, S., Suzuki, T., Ohashi, K. and Maruyama, K. (1986) J. Biochem., **100**: 439–447.
8 Kawashima, S., Nomoto, M., Hayashi, M., Inomata, M., Nakamura, M. and Imahori, K. (1984) J. Biochem., **95**: 95–101.
9 Weber, K. and Osborn, M. (1969) J. Biol. Chem., **244**: 4406–4412.
10 Yoshidomi, H., Kimura, S. and Maruyama, K. (1984) J. Biochem., **96**: 1947–1950.
11 Hanada, K., Tamai, M., Adachi, Oguma, K., Kashiwagi, K., Ohmura, S., Kominami, E., Towatari, T. and Katsunuma, N. (1983) In "Proteinase Inhibitors". Ed. by N. Katsunuma, H. Umezawa and H. Holzer, Jap. Sci. Soc. Press, Tokyo and Springer-Verlag, Berlin, pp. 25–36.
12 Sugita, H., Ishiura, S., Suzuki, K. and Imahori, K. (1980) J. Biochem., **87**: 339–341.
13 Maruyama, K., Kimura, S., Yoshidomi, H., Sawada, H. and Kikuchi, M. (1984) J. Biochem., **95**: 1424–1433.
14 Imahori, K., Suzuki, K. and Kawashima, S. (1983) In "Proteinase Inhibitors". Ed. by N. Katsunuma, H. Umezawa and H. Holzer, Jap. Sci. Soc. Press, Tokyo and Springer-Verlag, Berlin, pp. 173–180.
15 Murachi, T. (1983) Trends in Biochem. Sci., **8**: 167–169.