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INHIBITION OF PROTEOLYTIC ACTIVITY OF CALCIUM ACTIVATED NEUTRAL PROTEASE BY LEUPEPTIN AND ANTIPAIN

Teruhiko TOYO-OKA[1], Teruo SHIMIZU[2] and Tomoh MASAKI

Research section of Muscle Contraction, Tokyo Metropolitan Institute of Medical Science, Honkomagome, Bunkyo-ku, Tokyo, Japan.

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SUMMARY

The calcium activated neutral protease from bovine ventricular muscle requires milli-molar concentration of Ca ions for the activation of the proteolysis of troponin-T, troponin-I and tropomyosin. The exogenous protease inhibitors were examined concerning the blocking action of this enzyme. Both leupeptin and antipain were effective for the inhibition at the nearly same molar concentration as the protease. Lineweaver plot for both the protease alone and protease with leupeptin showed straight lines, and the mode of the inhibition was non-competitive type. Natural actomyosin, pretreated with this protease showed markedly reduced sensitivity to Ca ions. With the addition of leupeptin to the pretreatment, however, the Ca sensitivity was well preserved.

INTRODUCTION

Since the discovery of the neutral protease in skeletal muscle, which requires milli-molar concentrations of Ca^{2+} ions (1-3), this protease has recieved attention because of its probable rôle for protein turn-over (4, 5) or muscle necrosis in muscular dystrophy (6-8). Dayton et al. have purified this enzyme, named calcium activated factor and reported its properties (4, 5). The name of this enzyme might be inadequate for its proteolytic activity, and we call this enzyme "Calcium Activated Neutral Protease (CANP)".

We have purified this enzyme by another method from both rabbit skeletal muscle and bovine cardiac muscle (9), and obtained some results differing from the previous reports (9). Then we searched for the exogenous inhibitors of CANP, which might be used in living animal. The previously

Present address: 1- The Second Department of Internal Medicine, 2- Department of Neurology, University of Tokyo, Tokyo, Japan.

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reported inhibitors, such as EDTA and monoiodoacetate are harmful for use in vivo.

MATERIALS and METHODS

Preparation of CANP and proteins

Bovine ventricular CANP was isolated as described elsewhere (9). Natural actomyosin (NAM), tropomyosin (TM) and troponin (TN) were prepared from chicken breast muscle (10, 11).

Assay of proteolytic activity of CANP

The activity was measured by caseinolytic activity, following the method of Dayton et al. (5). Casein (Hammersten grade, 5 mg) was preincubated in 1 ml of reaction mixture, containing 0.1 M KCl, 10 mM 2-mercaptoethanol, 20 mM Tris-acetate (pH 7.5) and 5 mM $CaCl_2$ at 25 °C for 5 min. Reaction was initiated with the addition of CANP and was stopped with the addition of 2 ml of 4 % ice-cold trichloroacetic acid. After the centrifugation at 3,000 g for 20 min., the absorbancy of the supernatant was measured with spectrophotometer. One unit of caseinolytic activity was defined as the increase of 1.0 absorbancy in 1 min. by 1 gr. of the enzyme.

Pretreatment of NAM by CANP

Skeletal NAM (5 mg) was incubated in 0.1 M KCl, 10 mM 2-mercaptoethanol, 20 mM Tris-acetate (pH 7.5), 20 μg of CANP, either in the presence of 2 mM $CaCl_2$ or 1 mM EDTA for 2 hours at 25 °C. The reaction was terminated with the addition of EDTA 5 mM in final concentration. In some experiments, leupeptin (LP 7 μg) was added to the reaction mixtures. After these pretreatment, NAM was precipitated by centrifugation at 3,000 g for 20 min., and the precipitates were washed with 30 vols of 0.03 M KCl, 10 mM K-phosphate buffer (pH 7.0), and were centrifuged as before. This washing procedure was repeated 3 times. In these steps, CANP, leupeptin and EDTA were considered to be washed out, preserving regulatory proteins, if any, on NAM.

Superprecipitation measurement

The turbidity of NAM gel was measured in a Beckman spectrophotometer ACTA C III, following the method of Ebashi (12) at 25 °C.

Electrophoresis in sodium dodecylsulfate polyacrylamide gel

Electrophoresis in 0.1 % sodium dodecylsulfate (SDS)-10 % polyacrylamide gel was performed according to the method of Weber and Osborn (14), with the modification to slab form.

Protein concentration measurement

The concentration of proteins was determined by the biuret method.

Water and Reagents

Deionized and distilled water was redistilled prior to use in a glass apparatus. All reagents used were of analytical grade. Antipain was presented from Dr. H. Umezawa, the Institute of Microbial Chemistry, Tokyo, Japan. Leupeptin was purchased from Protein Research Foundation, Osaka, Japan.

RESULTS

As listed in Table 1, both leupeptin (LP) and antipain (AP) were shown to be effective for the inhibition of the activity of CANP. These inhibitors

Table 1: CANP activity with protease inhibitors

Inhibitors	dose	Caseinolytic activity (%)
None		100
Soy bean trypsin inhibitor	0.05 mg	96.5
alpha-1 antitrypsin	0.05 mg	100
Trasylol®	0.1 mg	85.9
leupeptin	0.1 mg	0.00
antipain	0.1 mg	6.72
pepstatin	0.1 mg	63.6*

* In the final mixture, 5 % ethyl alcohol is contained to solubilize inhibitor.

are antibiotics and have been considered to block the action of cathepsin B and papain, respectively (14). When the caseinolytic activity in the absence of inhibitors is taken as 100 %, dose-inhibition curves both for LP and AP were sigmoidal, parallel each other, and showed 50 % inhibition ($ID_{50\ \%}$) at 0.31 and 1.2 ug/ml, respectively (Figure 1). Calculating from molecular weights of CANP and inhibitors, these inhibitors were considered to block the caseinolytic activity at the same inhibitory site and at the same molar concentration as that of the protease.

Lineweaver-Burk plot for the CANP showed a linear relationship, indicating a Michaelis-Menten type of enzyme kinetics, giving the values for K_m 3.03×10^{-5} M and V_{max} 272 unit. With the addition of LP to this system, clear non-competitive type of inhibition was demonstrated (Figure 2), showing the value for K_i 9.82×10^{-7} M, when the molecular weights of casein and LP are assumed to be 23,600 (15) and 490 (14), respectively.

CANP of skeletal muscle was said to destroy the regulatory proteins, i.e. tropomyosin (TM) and troponin (TN), rather than contractile proteins, i.e. myosin and actin (4). These results were confirmed in the CANP from bovine ventricular muscle (9). As is shown in SDS-polyacryamide gel electrophoretic pattern in

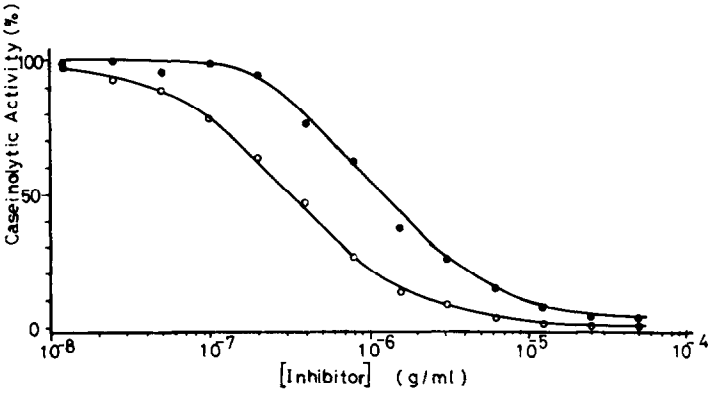

Figure 1. The dose-inhibition curves both for LP (o--o) and for AP (•--•). The activity without addition of inhibitor is taken as 100 % caseinolytic activity, following the method of Dayton et al. (4, 5).

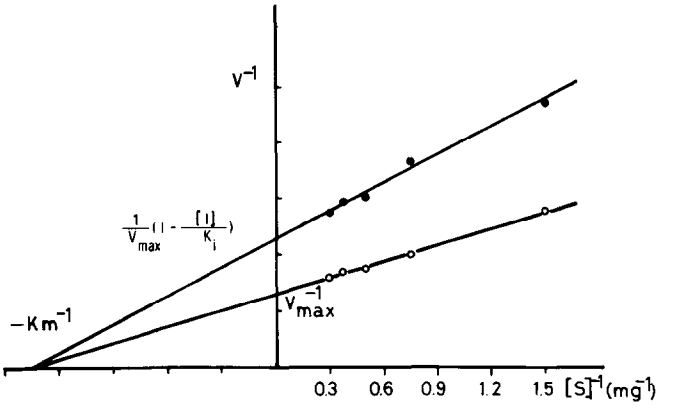

Figure 2. Lineweaver-Burk plot for the activity of CANP alone (o--o) and CANP with LP (•--•, 0.3 μg/ml). The assay conditions are described in the MATERIALS and METHODS section.

Figure 3, chicken skeletal TM, TN-T and TN-I were degraded in the presence of both 2 mM $CaCl_2$ and CANP. The digested fragments are shown by bars in Figure 3.

In the absence of Ca^{2+} ions, however, no degradation was detected. With the addition of LP to this system at the enzyme to inhibitor ratio 3:1 (w/w), this degradation was inhibited, even in the presence of 2 mM $CaCl_2$.

In both the skeletal and cardiac muscles of vertebrates, it is well known that small amounts of free Ca^{2+} ions (ex. 10^{-5} - 10^{-6} M) regulate the

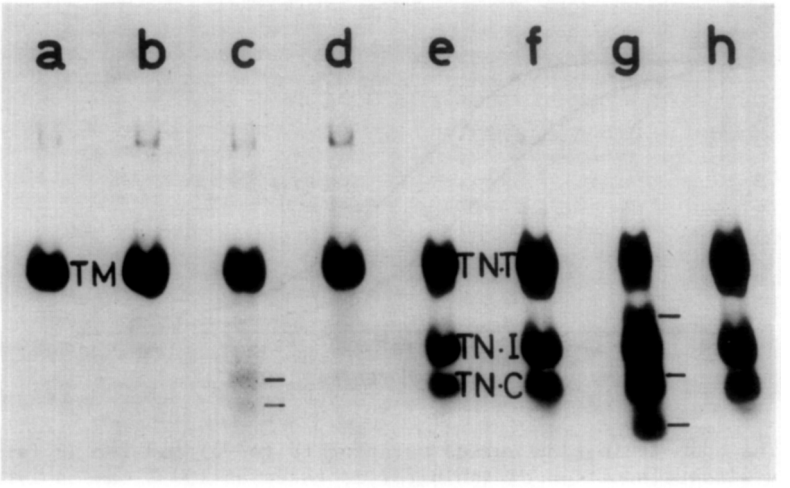

Figure 3. The SDS-polyacrylamide slab gel electrophoretic pattern of regulatory proteins with or without treatment by CANP and LP. All reaction mixtures containing 1 mg of purified TM or TN, 20 mM Tris-acetate (pH 7.5), 10 mM 2-mercaptoethanol supplemented with purified CANP (20 μg/ml) or CANP and LP (7 μg/ml), where indicated. Incubation was for 20 min. at 25 °C, and was stopped with the addition of TCA to a final concentration of 5 %. Precipitates (50 μg) were applied to the gel. a. Control TM alone, b. TM + CANP, Ca free, c. TM + CANP, $CaCl_2$ 2 mM, d. TM + CANP + LP, $CaCl_2$ 2 mM, e. Control TN alone, f. TN + CANP, Ca free, g. TN + CANP, $CaCl_2$ 2 mM, h. TN + CANP + LP, $CaCl_2$ 2 mM

muscle contraction via troponin-tropomyosin system (16, 17). So, when both tropomyosin and troponin are degraded by CANP, the regulatory action of small amounts of Ca^{2+} ions in the contraction of muscle is expected to be abolished. Therefore, we examined the effect of CANP on natural actomyosin with or without the addition of inhibitor.

After natural actomyosin is pretreated with CANP in the absence of Ca^{2+} ions, normal sensitivity to Ca^{2+} ions was shown (data, not shown). Natural actomyosin, pretreated with CANP in the presence of 2 mM $CaCl_2$, showed super-precipitation response after the addition of MgATP, indicating that functions of both myosin and actin were preserved, but the sensitivity to Ca^{2+} ions reduced markedly (Figure 4). When leupeptin is added to the pretreatment procedure in the presence of 2 mM $CaCl_2$, that sensitivity was well preserved (Figure 4).

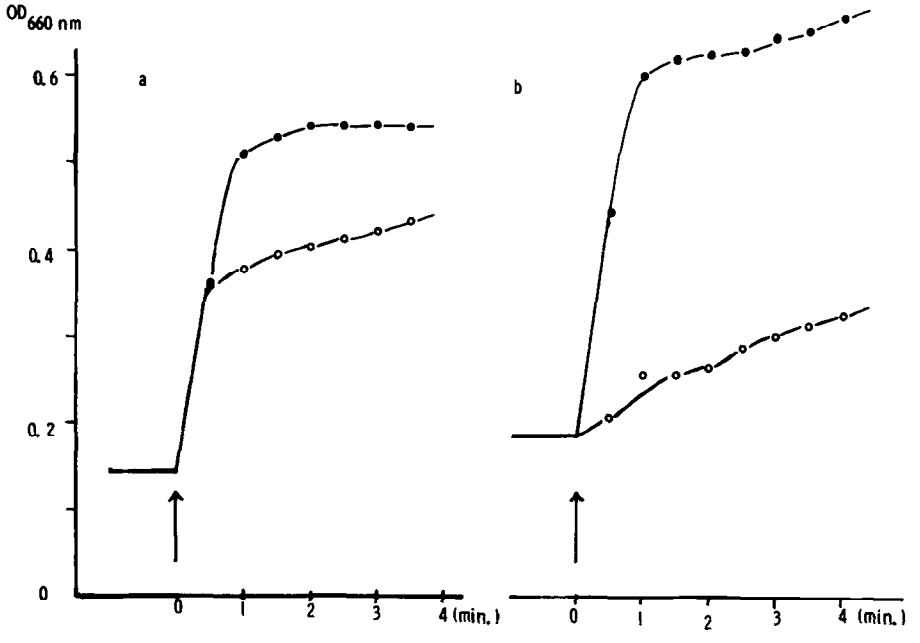

Figure 4. The superprecipitation responses of NAM, pretreated with CANP in the absence (a) or in the presence (b) of LP (see MATERIALS and METHODS). Reaction mixtures in the superprecipitation contained 0.1 M KCl, 1 mM $MgCl_2$, 0.2 mg/ml pretreated NAM and 20 mM Tris-maleate buffer (pH 6.8), with free Ca^{2+} ions at 1.3×10^{-5} M (•--•) or at 1.0×10^{-7} M (o--o). Arrow denotes the addition of MgATP to 0.1 mM final concentration.

DISCUSSION

At first, it must be mentioned that the concentration of Ca^{2+} ions, necessary for the activation of the proteolytic activity of CANP is millimolar concentration, while the concentration required for the regulation of muscle contraction is micro-molar level (9, 16). Thus, Ca^{2+} ions might have a dual actions concerning both the proteolysis of the regulatory proteins and the regulation of muscle contraction. The intracellular concentration of Ca^{2+} ions is considered to be too low to activate CANP.

In pathological state, however, the intracellular concentration of Ca^{2+} ions reached upto serum level, probably by the leakage of Ca^{2+} ions, sequestered in the sarcoplasmic reticulum or mitochondria, and by the enhancement of the permeability of the injured sarcolemma. Therefore, CANP might act as one of

the aggraving factors in cell necrosis, including muscular dystrophy, myocardial infarction and some kinds of myopathies.

In fact, Sugita and Toyokura have demonstrated that the SDS-polyacryl amide gel electrophoretic pattern of myofibrils from a patient with progressive muscular dystrophy was similar to that of the normal monkey myofibrils, pretreated with CANP (6, 7). In the experimentally induced myocardial infarction made by the coronary ligation, NAM, derived from necrotic lesion, showed reduced sensitivity to Ca^{2+} ions in the superprecipitation response, suggesting a dysfunction of either tropomyosin or troponin (N. Shibata, personal communication).

Protease inhibitors, including AP, LP or its derivatives, might be used for the therapeutics in muscle necrosis. Mc Gowan et al. have shown that the pathological aspects of dystrophic chicken muscle were improved, when LP, AP and pepstatin were simultaneously given to either the medium of cultured muscle cells or to the whole animal (8). For the trial of clinical application, however, the side effects, such as the induction of hemorrhagic diathesis, must be overcome.

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