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PHOSPHORYLATION WITH CYCLIC ADENOSINE 3': 5' MONOPHOSPHATE-DEPENDENT PROTEIN KINASE RENDERS BOVINE CARDIAC TROPONIN SENSITIVE TO THE DEGRADATION BY CALCIUM-ACTIVATED NEUTRAL PROTEASE.

Teruhiko TOYO-OKA

Department of Cardiology, Jichi Medical School, Kawauchi, Tochigi 329-04, Japan and The Second Department of Physiology, University of Heidelberg, Im Neuenheimer Feld 326, 6900 Heidelberg 1, F.R.G.

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SUMMARY Phosphorylation of the inhibitory subunit of cardiac troponin (TN-I) occurs *in vivo* after catecholamine intervention through adenylcyclase, cyclic AMP and cyclic AMP dependent protein kinase system. Also, TN-I and tropomyosin binding subunit of troponin (TN-T) are specifically hydrolyzed by calcium-activated neutral protease (CANP). In this study, we compared proteolysis of a set of TNs before and after phosphorylation by cyclic AMP dependent protein kinase plus cyclic AMP, using CANP from cardiac muscle. The initial rate of peptide release from both TNs was the same. After prolonged incubation, however, unphosphorylated TN degradation retarded, while phosphorylated TN proteolysis still continued. The amount of peptide release at the latter phase was dependent on the degree of phosphorylation. These results were confirmed by SDS polyacrylamide gel electrophoresis, and they suggest that a conformational change occurred in the whole TN molecule after phosphorylation of TN-I.

INTRODUCTION

The mechanism of protein degradation in muscle is very obscure (1). Recently, calcium ions have been shown to be involved in protein catabolism under physiological (2) and pathological conditions (3, 4). Several groups isolated a new intracellular protease, calcium-activated neutral protease (CANP) from both skeletal (5-7) and cardiac muscle (7,8).

This protease specifically degrades troponin (TN) (6-8), tropomyosin (6-8), alpha-actinin (5) and euactinin (9, 10) among myofibrillar proteins, and is inhibited by leupeptin and antipain (10, 11). It is also speculated that CANP might be involved in the pathological destruction of TN (12) and z-discs (10, 13) in ischemic myocardium.

The activity of intramuscular proteases, including CANP, is increased, when adrenergic tone is continuously increased, as is the case in hyperthy-

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Address correspondence to T. Toyo-oka M.D., Department of Cardiology, Jichi Medical School, Kawauchi, Tochigi 329-04, Japan.

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roid state (14, 15). To determine whether protein degradation is dependent on catecholamine action, we compared the proteolytic effect of CANP, using the same substrate, cardiac TN, before and after phosphorylation with cyclic adenosine 3':5' monophosphate dependent protein kinase.

Cardiac TN is suitable for this study, because TN-I subunit is phosphorylated *in vitro* by cyclic AMP dependent protein kinase in the presence of cyclic AMP (16, 17) and also *in vivo* during catecholamine intervention (18, 19).

MATERIALS AND METHODS

Preparation of cardiac troponin and its phosphorylation

Cardiac TN was isolated from bovine heart (20), further purified (21), and then phosphorylated as described by Stull and Buss (17). The reaction mixture contained 5 mg/ml cardiac TN, 15 p units of cyclic AMP dependent protein kinase from bovine heart (Sigma), 10^{-5} M cyclic AMP (Sigma), 5 mM NaF, 5 mM theophylline, 5 mM Mg acetate, 5 mM (γ - ^{32}P) adenosine triphosphate (ATP, New England Nuclear), and 20 mM HEPES buffer (pH 7.5). To obtain different extent of phosphate incorporation into TN, the incubation time at 37°C was changed from 15 min to 3 hrs.

To stop enzyme reaction, samples were transferred into ice. Non-radioactive cold ATP (5 mM in final concentration) and then solid ammonium sulfate up to 80 % saturation were added. Precipitated TN was centrifuged at 8,000 g for 20 min, suspended in 0.5 ml of the buffer solution containing 0.1 M NaCl, 5 mM NaF and 20 mM HEPES buffer (pH 7.0), and applied to a Sephadex G 25 superfine (Pharmacia) column (0.6 x 25 cm), which had been equilibrated with the same buffer solution.

The first fraction that showed radioactivity was eluted in the void volume and used as a substrate for the proteolysis study. Unphosphorylated TN was obtained by the same method but without adding cyclic AMP to the reaction mixture. The phosphorylation of TN was verified by radioautography on X ray film (Agfa).

Isolation of calcium-activated neutral protease (CANP)

CANP was isolated from porcine ventricular muscle as described before (8) but with the following modification. Instead of antipain-Sepharose 4 B, p-aminophenylmercury acetate-Sepharose 4 B was used as the affinity column.

Assay of CANP activity

CANP activity was measured by a modified method of increased activity. The reaction mixture contained 1 mg/ml phosphorylated or unphosphorylated TN, 0.1 M NaCl, 2 mM $CaCl_2$, 5 mM dithiothreitol and 20 mM HEPES buffer (pH 7.0) in 0.2 ml, incubated for 5 min at 37 °C. The proteolytic enzyme reaction was started by addition of CANP (14 μg/ml in final concentration) and stopped by adding 0.2 ml of ice-cold trichloroacetic acid (TCA).

After spinning at 9,000 g for 15 min, TCA soluble peptides and amino acids were quantitated as fluorescamine reactive materials (22). In each assay, a calibration curve of fluorescence intensity was obtained using glycine as a standard. To confirm the proteolysis of TNs, polyacrylamide gel electrophoresis was performed in sodium dodecylsulfate (23).

Miscellaneous

Protein concentration was measured by Lowry 's method (24), using bovine serum albumin (Sigma) as a standard. The amount of phosphate incorporated into TN subunits was determined by counting ^{32}P in a liquid scintillation counter (Beckman) after slicing each protein band from the gel. The molecular weights of TN-T, TN-I and TN-C were assumed to be 41,000, 28,000 and 18,000, respectively (20), and the molar ratio of each TN subunit was 1:1:1 (20).

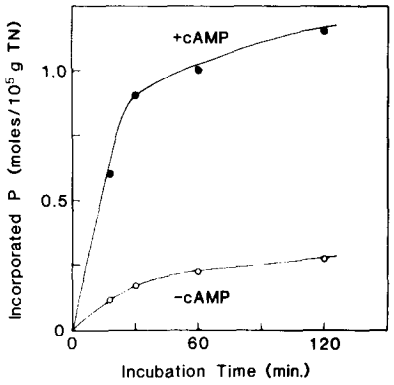

Figure 1. Phosphate incorporation from (γ - ^{32}P) ATP into cardiac TN in the presence of 10^{-5} M cyclic AMP (closed circle) or in its absence. For experimental details, see the MATERIALS AND METHODS section.

RESULTS

Phosphorylation of cardiac TN

Phosphate incorporation into TN was determined using [γ-^{32}P] ATP. In the absence of cyclic AMP, TN was weakly, but significantly phosphorylated (Figure 1), confirming the previous data (16, 17). In the presence of cyclic AMP, TN was phosphorylated linearly for up to 30 min (Figure 1).

The amount of phosphate incorporated was determined after separating each TN subunit by sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (23). As summarized in Table 1, a similar but smaller amount of phosphate was incorporated into both TN-T and TN-I subunits in the absence of cyclic AMP, while eight-fold more incorporation was found into TN-I subunit in the presence of cyclic AMP. Cyclic AMP did not cause incorporation of additional phosphate into TN-T and TN-C subunits.

Amino acids or peptides release from TNs with CANP

After addition of CANP, amino acids and/or peptides were quantitatively released from unphosphorylated, partially phosphorylated (0.6 moles P/10^5 g TN) or completely phosphorylated TN (1.1 moles P/10^5 g TN). For the first 5 min, the rate of peptide release was the same in all three

TABLE I

Phosphate incorporation from [γ-^{32}P] ATP into cardiac TN subunits.

	TN-T	TN-I	TN-C	
-cyclic AMP	414	485	55	c.p.m.
+cyclic AMP (10^{-5} M)	445	3817	64	

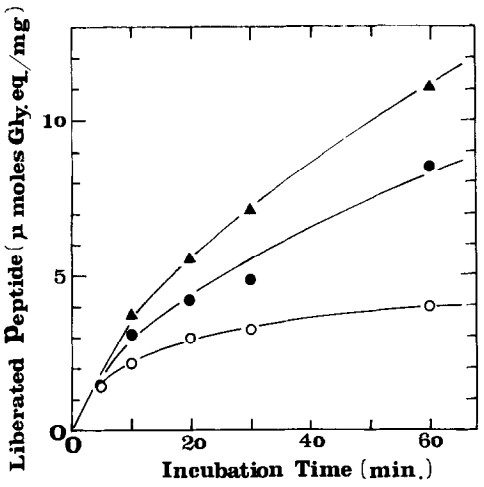

Figure 2. The time course of amino acids or peptides release from unphosphorylated TN (open circle), partially phosphorylated (0.6 mole P/10^5 g TN, closed circle) or completely phosphorylated TN (1.1 moles P/10^5 g TN, triangle) with CANP. For experimental details, see the MATERIALS AND METHODS section.

samples (Figure 2). After prolonged incubation, however, the amount of peptide release from unphosphorylated TN was retarded (Figure 2). From the partially phosphorylated TN and the completely phosphorylated TN, the peptide release still continued. The amount of peptide release at the latter phase was dependent on the degree of phosphorylation (Figure 2).

SDS gel electrophoresis of TNs before and after degradation by CANP

The above data were confirmed by SDS polyacrylamide gel electrophoresis (23). Unphosphorylated TN was incubated under the same conditions as the CANP degradation study, without adding CANP, and then, applied to the gel (Figure 3A). After incubation, a new protein band was formed just below TN-I (Figure 3A). This was probably due to the degradation of TN-T or TN-I with another contaminant protease in TN preparation (25).

Following the incubation of phosphorylated TN (1mg/ml) with CANP (14 μg/ml), both the TN-T and TN-I subunits were nearly completely split and the bands corresponding to each TN subunit disappeared (Figure 3C). In contrast, the proteolysis of the unphosphorylated TN was weak, and the intensity of degraded protein bands of the unphosphorylated TN was much more intense (Figure 3B) than that of the phosphorylated one (Figure 3C), forming newly degraded product bands around 10,000 and 16,000 daltons (Figure 3B and C). This means that TN destruction by CANP is more complete, when it is phosphorylated.

When the weight ratio of CANP to TNs was decreased 10 times, the degradation of TN subunits became less, but still showed a greater proteolysis of phosphorylated TN than that of unphosphorylated TN (Figure

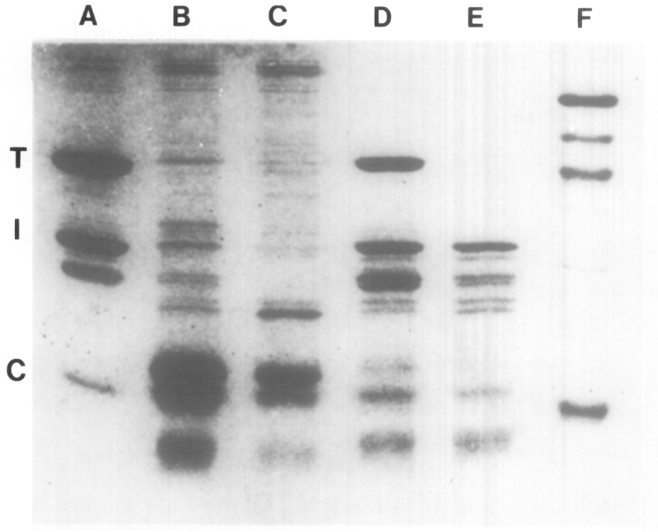

Figure 3. SDS polyacrylamide gel electrophoresis of the control TN before CANP treatment (A), the product of TN after CANP treatment (B-E) and marker proteins (F) for determination of molecular weights, using bovine serum albumin (68,000), catalase (58,000), aldolase (40,000), chymotrypsinogen (25,700) and cytochrome C (11,700). Cardiac TNs (1 mg/ml) of before or after phosphorylation were incubated with CANP (14 μg/ml) for 30 min at 37 °C and applied to the gel B or C, respectively. The amount of CANP was decreased to 1.4 μg/ml in the case of D (unphosphorylated TN) and E (phosphorylated TN). Trace bands around 80,000 daltons at B and C are CANP. The protein band just below TN-I is a degraded product of either TN-T (T) or TN-I (I) by another contaminant protease in TN preparation (25).

3D and E). In the unphosphorylated TN, both TN-T and TN-I subunits were equally hydrolyzed (Figure 3D). However, in the phosphorylated TN, TN-T was more easily hydrolyzed than TN-I (Figure 3E).

DISCUSSION

The decline of peptide release from the unphosphorylated TN is not due to the loss of CANP activity, because CANP retained its activity under the incubation condition, when partially or completely phosphorylated TN was used as substrate (Figure 2). The initial rate of peptide release was the same for all three types of TNs. This might indicate that the V_{max} value is the same, but the Km vlue is smaller in the phosphorylated TN than in the unphosphorylated TN. We could not obtain Km values, because proteolytic rate fluctuated greatly, when TN was hydrolyzed at dilute protein concentrations. Furthermore, these parameters might be meaningless, because TN subunits make a complex (20) and susceptibility is different among TN subunits (Figure 3), confirming the previous data (7, 8).

When the hydrolytic effect of CANP on TN-T and TN-I subunits were examined before and after phosphorylation, the degree of proteolysis was

larger in TN-T than TN-I, especially in the phosphorylated TN (Figure 3E). TN-I is mainly phosphorylated by cyclic AMP dependent protein kinase plus cyclic AMP (16, 17), as confirmed in the present study (Table 1). Therefore the conformational change of TN-I after phosphorylation might be transmitted from TN-I to TN-T. Subsequently, the higher structure of TN-T could be changed to a form attacked more easily by CANP.

Phosphorylation might be a recognition mechanism to identify old proteins during replacement by newly synthetized proteins, as was speculated by Goldberg and St John (1). We do not know whether only CANP is able to degrade phosphorylated TN more easily than unphosphorylated one, but are investigating other intracellular proteases to resolve that problem. Proteins susceptible to CANP are mostly those which are phosphorylated (6-8), and the present study confirmed partially that scheme.

The intracellular concentration of cyclic AMP has been reported to increase in ischemic myocardium (26). This might accelerate TN destruction in the region of myocardial infarction, as is the case in a study on dogs(12).

Glycogen metabolism is regulated by catecholamine through adenylcyclase, cyclic AMP and cyclic AMP dependent protein kinase system (27). The present study suggests that protein degradation in heart is also regulated by cyclic AMP dependent protein kinase plus cyclic AMP system, when CANP is working in the physiological or pathological protein catabolism. In fact, using hepatocyte monolayer culture, Hopgood et al. have reported that a close correlation between intracellular cyclic AMP concentration and the degree of proteolysis (28). Furthermore, they reported that the proteolysis is stimulated by added dibutyryl cyclic AMP, which penetrates cell membrane (28). Their data also confirms the present scheme.

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