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Insulin-like Activity of Proteases. V.[1] Stimulation of Cyclic Adenosine 3′,5′-Monophosphate Phosphodiesterase by an *N*-Succinyl-L-alanyl-L-alanyl-L-alanine *p*-Nitroanilide-hydrolyzing Protease

Hiroshi Ueki,* Keiji Furuta, Takayuki Funakoshi, Yuko Matsunami, and Yukiho Kubota

Faculty of Pharmaceutical Sciences, Kumamoto University, 5-1, Oe-honmachi, Kumamoto 862, Japan

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The stimulatory effect of an *N*-succinyl-L-alanyl-L-alanyl-L-alanine *p*-nitroanilide-hydrolyzing protease (STA-protease), which was partially purified from Pronase, on cyclic adenosine 3′,5′-monophosphate (cyclic AMP) phosphodiesterase of bovine heart was investigated by measuring the changes in kinetic constants. The phosphodiesterase used showed hydrolytic activity towards cyclic AMP at a concentration of 1 or 100 μM. STA-protease stimulated low K_m phosphodiesterase but not high K_m phosphodiesterase activity, in the presence of ethylene glycol bis(β-aminoethylether)-*N*,*N*′-tetraacetic acid (EGTA). No stimulation of the low K_m enzyme was observed when theophylline was used instead of EGTA. Proteases such as Pronase, trypsin, chymotrypsin, elastase, and substilisin BPN′ stimulated the low K_m enzyme to various extents. Although STA-protease showed the lowest caseinolytic activity among proteases tested, its stimulatory activity towards the low K_m enzyme was rather high. STA-protease increased the K_m and V_{max} values of the low K_m enzyme, whereas trypsin decreased the K_m value without causing significant change in V_{max}. The stimulatory mechanism of STA-protease probably differs from that of trypsin.

Keywords——protease; insulin-like activity; phosphodiesterase; stimulation of phosphodiesterase; cyclic AMP; proteolytic stimulation

Phosphodiesterases hydrolyze cyclic 3′,5′-nucleotide, and the activities of some of them are regulated by a calcium-dependent modulator protein (calmodulin). Kono *et al.*[2] reported that a low K_m phosphodiesterase of rat epididymal fat cells, having a K_m value of 0.2 to 0.3 μM for the hydrolysis of cyclic adenosine 3′,5′-monophosphate (cyclic AMP), was stimulated when intact cells were treated with insulin. A similar result was reported by Loten *et al.*[3] on a low K_m phosphodiesterase of rat liver cells treated with insulin. This stimulation of phosphodiesterase was observed after the treatment of intact cells with insulin but not when cell homogenates or isolated enzymes were treated with insulin. On the other hand, phosphodiesterases which were partially purified from rat brain and bovine heart were stimulated by proteases when directly treated in the absence of calcium ion.[4,5] We previously reported that a protease which was partially purified from Pronase showed a glycogen-increasing effect on isolated mouse diaphragm[6] and we suggested that it may digest the surface of the diaphragm at a limited region other than epinephrine receptor.[1]

The present paper describes the stimulatory effect of the protease obtained from Pronase on the phosphodiesterase of bovine heart.

Experimental

Materials——Phosphodiesterase (cyclic adenosine 3′,5′-monophosphate-nucleotidohydrolase, from bovine heart, about 0.25 U/mg with cyclic AMP as a substrate) was purchased from Boehringer Mannheim Yamanouchi Co., Tokyo. This enzyme preparation was reported to contain at least two forms of enzyme, possessing a low or high K_m value for the hydrolysis of cyclic AMP,[7] and as reported by Mohindru *et al.*,[5] the low K_m phosphodiesterase from bovine heart may consist of two forms, *i.e.*, calmodulin-dependent and

independent enzymes. [^{3}H(G)] cyclic adenosine 3′,5′-monophosphate, ammonium salt, ([^{3}H] cyclic AMP, 42.5 Ci/mmol) was obtained from New England Nuclear Co., Boston, Mass., U.S.A. Cyclic AMP was purchased from P-L Biochemicals Co., Milwaukee, Wis., U.S.A. Trypsin (from bovine pancreas) and soybean trypsin inhibitor (SBTI) were obtained from Miles Research Products Co., Elkhart, Ind., U.S.A. Subtilisin BPN′ (*Bacilus subtilis* alkaline protease) was purchased from Nagase Sangyo Co., Osaka. Ethylene glycol bis(β-aminoethylether)-*N*,*N*′-tetraacetic acid (EGTA) and theophylline were purchased from Nakarai Chemicals Co., Kyoto. Pronase (from *Streptomyces griseus*, type E, 70 PUK/mg) was a gift from Kaken Chemical Co., Tokyo. Alkaline protease inhibitor (API, from *Streptomyces pseudogriseolus*) was donated by Professor M. Shibata and Dr. M. Uyeda of this University. Elastase and α-chymotrypsin (from bovine pancrease) were gifts from Eisai Co., Tokyo.

Preparation of the Protease from Pronase——The protease, which was called an insulin-like activity-possessing protease (ILAPP), was partially purified from Pronase, as described in a previous paper.[2] Hereafter, this protease is called the *N*-succinyl-L-alanyl-L-alanyl-L-alanine *p*-nitroanilide-hydrolyzing protease (STA-protease).

Determination of High K_m Phosphodiesterase——The hydrolytic activity towards cyclic AMP was determined in 50 mM Tris–HCl buffer, pH 7.4, containing 7.7 mM $MgCl_2$ at a substrate concentration of 100 μM. Subsequent procedures were carried out according to the method of Butcher and Sutherland[8] and Kakiuchi *et al.*[9] Inorganic phosphate produced was measured by the method of Ames.[10]

Determination of Low K_m Phosphodiesterase——The hydrolytic activity towards cyclic AMP was determined in 33 mM Tris–HCl buffer, pH 7.4, containing 4 mM $MgCl_2$ at a substrate concentration of 1 μM. An aliquot of 0.05 ml of phosphodiesterase solution (250 μg/ml) was added to 0.2 ml of 1.25 μM cyclic AMP containing 125 nCi [^{3}H]cyclic AMP and the mixture was incubated at 30°C for 5 min. Subsequent procedures were carried out according to the method of Kono *et al.*[11] The radioactivity in adenosine fractions obtained was determined in a Triton-based scintillation mixture[12] with an Aloka LSC-502 liquid scintillation counter.

Protease Treatment——For treatment of the high K_m phosphodiesterase, STA-protease or trypsin was added to the phosphodiesterase solution after preincubation with 2 mM EGTA at 4°C for 30 min. The incubation was carried out in 50 mM Tris–HCl buffer, pH 7.4, containing 7.7 mM $MgCl_2$ at 30°C for 10 min and stopped by the addition of a two-fold molar excess of proteinase inhibitor (API for STA-protease and SBTI for trypsin). The hydrolytic activity towards cyclic AMP was determined as described previously. For treatment of the low K_m phosphodiesterase, protease was added to the phosphodiesterase solution in the presence of 0.1 mM EGTA or 0.5 mM theophylline. The incubation was carried out in 33 mM Tris–HCl buffer, pH 7.4, containing 4 mM $MgCl_2$ at 30°C for 3 min and stopped by the addition of the proteinase inhibitor. In the case of treatment with proteases other than STA-protease and trypsin, the hydrolytic activities towards cyclic AMP were determined immediately after incubation without stopping the proteolytic action.

Determination of Caseinolytic Activity——Caseinolytic activity was determined with 1.7% casein in 0.07 M phosphate buffer, pH 8, at 37°C for 30 min.[13]

Results

Effects of Proteases on High K_m Phosphodiesterase

First, the inhibitory effect of EGTA on the high K_m phosphodiesterase was investigated at different concentrations. EGTA inhibited the phosphodiesterase and decreased its activity to 50% of the original level at a concentration of 2 mM. The effects of STA-protease and trypsin on the phosphodiesterase were then tested in the range of concentration from 0.01 to 100 μg/ml after pretreatment with 2 mM EGTA. The proteases exerted no stimulatory effect either on the EGTA-pretreated phosphodiesterase or on the non-pretreated enzyme. A decrease in phosphodiesterase activity was observed after incubation with proteases concentrations of 5 μg/ml or more.

Effects of Proteases on Low K_m Phosphodiesterase

The activity of low K_m phosphodiesterase was decreased by EGTA to 40% of the original level at a concentration of 0.1 mM and to 15% at 2 mM. The low K_m phosphodiesterase appeared to be more susceptible to EGTA than the high K_m enzyme.

As shown in Fig. 1, stimulation of the low K_m phosphodiesterase by STA-protease or trypsin was observed in the presence of 0.1 mM EGTA. The maximum values of the stimulation were found to be 188% of the original level at 1 μg/ml of STA-protease and 207% at 2.5 μg/ml of trypsin. No stimulation was observed in the absence of EGTA. The inhibitory effect of theophylline on the phosphodiesterase was investigated in the range of concentration

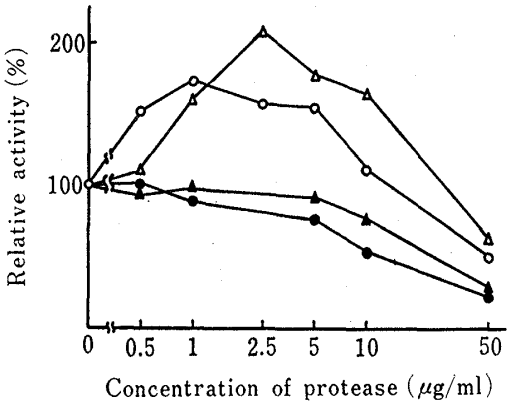

Fig. 1. Effects of Proteases on Low K_m Phosphodiesterase in the Presence of EGTA

○ : incubated with STA-protease in the presence of 0.1 mM EGTA.
● : incubated with STA-protease in the absence of EGTA.
△ : incubated with trypsin in the presence of 0.1 mM EGTA.
▲ : incubated with trypsin in the absence of EGTA.

Table I. Caseinolytic and Low K_m Phosphodiesterase stimulatory Activities of Various Proteases

	Caseinolytic activity[a]	Low K_m phosphodiesterase-stimulatory activity[b]	
		1	2.5 (μg/ml)[c]
STA-protease	2.1	188	156
Pronase	2.9	122	171
Chymotrypsin	4.5	124	128
Trypsin	3.1	175	207
Elastase	2.7	115	119
Subtilisin BPN'	3.4	209	131

a) μmol of tyrosine released per min per mg.
b) Relative activity (%) with respect to the control.
c) Concentrations of proteases.

from 0.1 to 10 mM. After preincubation at 30°C for 3 min, the activity of phosphodiesterase decreased to 85% of the original level at a concentration of 0.1 mM, 50% at 0.5 mM, and 5% at 10 mM. The inhibition was not released by STA-protease or trypsin at different concentrations (0.5—10 μg/ml). The caseinolytic activities of STA-protease and trypsin were not inhibited by theophylline. These results distinctly differ from those obtained in the presence of EGTA.

As shown in Table I, Pronase, chymotrypsin, trypsin, elastase, and subtilisin, BPN' stimulated the low K_m phosphodiesterase to various extents. Although STA-protease showed the lowest caseinolytic activity among proteases tested, its stimulatory effect on the phosphodiesterase was rather high.

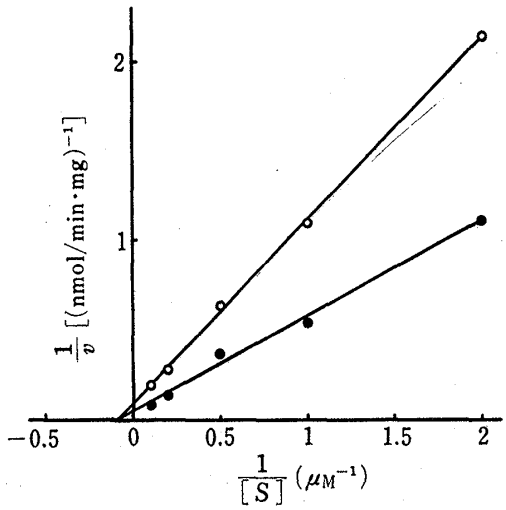

Fig. 2. Lineweaver-Burk Plots for Low K_m Phosphodiesterase

○, in the presence of 0.1 mM EGTA.
●, in the absence of EGTA.
The enzymatic activity was determined with cyclic AMP in the range of concentration from 0.5 to 10 μM.

Table II. Kinetic Constants of Phosphodiestrase treated with STA-protease or Trypsin

	Concentration of protease (μg/ml)	K_m (μM)	V_{max} (nmol/min·mg)
Control I[a]	0	10.1	19.2
Control II[b]	0	10.7	10.5
STA-protease-treated	1	16.4	18.6
	5	17.5	15.8
	10	17.7	13.4
Trypsin-treated	1	8.0	9.6
	5	6.9	9.5
	10	3.2	5.7

a) In the absence of EGTA.
b) In the presence of EGTA.
Determinations were carried out in the range of concentration from 0.5 to 10 μM of cyclic AMP as a substrate, in the presence of 0.1 mM EGTA.

Kinetic Properties of Protease-treated Low K_m Phosphodiesterase

Figure 2 shows Lineweaver–Burk plots for the low K_m phosphodiesterase in the presence or absence of 0.1 mM EGTA. Typical straight lines were obtained with cyclic AMP in the range of concentration from 0.5 to 10 μM by the least-squares method. The K_m and V_{max} values obtained with the phosphodiesterase in the absence of EGTA were 10.1 μM and 19.2 nmol/min·mg, respectively. In the presence of 0.1 mM EGTA, the V_{max} value decreased to 10.5 nmol/min·mg, whereas the K_m value, 10.7 μM, was close to that obtained above, suggesting that EGTA inhibits the phosphodiesterase non-competitively.

Table II compares the kinetic constants of the phosphodiesterase incubated with STA-protease or trypsin at concentrations of 1, 5, and 10 μg/ml in the presence of 0.1 mM EGTA. STA-protease increased the values of both K_m and V_{max} of the phosphodiesterase at any concentration tested. On the other hand, trypsin decreased the K_m value without causing significant change in the V_{max} value at concentrations of 1 and 5 μg/ml, whereas it markedly lowered both K_m and V_{max} at 10 μg/ml.

Discussion

Several workers have reported on the kinetic characteristics of bovine heart phosphodiesterases. Beavo *et al.*[14] reported first that two different phosphodiesterase activities were found in a crude particulate fraction, the K_m values for cyclic AMP being 0.8 and 25 μM. Hrapchak and Rasmussen[15] reported that the K_m values of phosphodiesterases separated from a 13500×g supernatant fraction of the homogenate were 36 and 69 μM. Ho *et al.*[16] studied the regulation of two forms of phosphodiesterases, and found that one enzyme was stimulated by a calmodulin and the other was insensitive to it or to EGTA, in the presence of Ca^{2+}. A calmodulin-dependent phosphodiesterase showed a 1.7-fold increase in the V_{max} value for cyclic AMP without significant change in the K_m value upon addition of calmodulin obtained from bovine liver.[17] Mohindru *et al.*[5] reported recently that two active peaks of phosphodiesterases were obtained by affinity chromatography on succinylated trimethyl-papaveroline-conjugated Sepharose 4B when the activity towards cyclic AMP was measured at concentrations of 1 and 100 μM in the presence of EGTA. The first peak enzyme was stimulated several fold by the addition of the calmodulin obtained from bovine brain cortex. This calmodulin-dependent enzyme was stimulated by trypsin treatment. In contrast, the second peak enzyme was insensitive to calmodulin or trypsin treatment.

We found that STA-protease and trypsin stimulated the low K_m phosphodiesterase only in the presence of EGTA, which rather selectively chelates Ca^{2+}. EGTA thus eliminates Ca^{2+} and may cause the dissociation of calmodulin from the enzyme molecule, leading to the marked decrease of the enzymatic activity. In the presence of 0.5 mM theophylline, STA-protease and trypsin did not stimulate the phosphodiesterase, the activity of which decreased to 50% of the original level. This is presumably because theophylline competitively inhibits the phosphodiesterase.[8] STA-protease increased the values of both K_m and V_{max}, whereas trypsin decreased the K_m value without significant change in V_{max}. These results suggest that the stimulatory mechanism of STA-protease differs from that of trypsin or calmodulin. The ratio of V_{max} to K_m was not much different between STA-protease- or trypsin-treated and non-treated phosphodiesterases. This may be because the phosphodiesterase consists of two forms, calmodulin-dependent and independent enzymes,[5] so that the stimulatory effect of STA-protease is produced as a composite result of the partial inhibition (increase in K_m value) and stimulation (increase in V_{max}) of the phosphodiesterase.[18] It is difficult to explain how STA-protease stimulates the phosphodiesterase in the presence of EGTA, but one possible explanation is that the protease produces active units by partial proteolytic digestion of the enzyme molecule. It is not clear at present whether or not the phosphodiesterase of mouse

diaphragm is stimulated when the intact tissue is treated with STA-protease. Further work is required to clarify this point.

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