Demonstration of Calcium-Dependent Proteases (Calpains) and Thyroglobulin Proteolysis in Hog Thyroid Cytosol

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Abstract

Ca²⁺-dependent neutral proteases in hog thyroid cytosol were found to digest thyroglobulin. The protease activity was divided into two peaks by DEAE-cellulose column chromatography. Peak I was eluted at 0.2 M NaCl and required only a micromolar range of Ca²⁺ for its 50% activation, while peak II, which was eluted at about 0.4 M NaCl, displayed little activity until the Ca²⁺ concentration was increased at more than 10⁻⁴ M. Among various inhibitors used, thiol protease inhibitors (leupeptin, E-64 and moniodoacetic acid) were the most effective, whereas a calmodulin antagonist (trifluoperazine) and serine protease inhibitors (phenylmethyl-sulfony-fluoride and pepstatin A) were not effective, indicating that these Ca²⁺-dependent proteases corresponded to calpains 1 and 2. Among the substrates tested, casein was the best and thyroglobulin was also a good for calpain 2. By using immunoblotting procedure with anti-thyroglobulin antibody, it has been found that calpain 2 degrades thyroglobulin to yield 67 K and 46 K thyroglobulin and further that it also degrades 40 K thyroglobulin.

Evidence has been accumulating to indicate the ubiquitous distribution of Ca²⁺-dependent proteases (calpains) in a wide variety of tissues and cells (Mollgrem, 1980; DeMartino, 1981). There are two types of enzymes called calpain 1 and calpain 2 which are eluted in this order from a DEAE-cellulose column. The former has high and the latter has low Ca²⁺ sensitivities. Their physiological roles are diverse, but they are known to be closely linked to cellular Ca²⁺-related phenomena. It is known that filamin (Fox *et al.*, 1983) and alpha-actinin (Reddy *et al.*, 1975) are the

endogenous substrates of calpains and that they are closely linked to morphological changes and serotonin secretion in platelets. Calpains are also known to activate protein kinase C by rendering it into its phospholipid-independent form of M-kinase (Meyer *et al.*, 1964).

Many investigations on thyroidal proteases dealt mainly with lysosomes. However, acidic pH optimum, defined existence in lysosomes and broad substrate specificity make it difficult for lysosomal enzymes to be involved in many physiological events occurring in cytosol.

In this study, we have demonstrated Ca²⁺-dependent proteases in hog thyroid.

Material and Method

Materials

Alpha-casein was obtained from E. Merk (Darmstadt, Hessen). Porcine thyroglobulin, ovalbumin, bovine hemoglobin, phenyl-methylsulfonyl-fluoride, pepstatin A and leupeptin were purchased from Sigma Chemical Co. (USA). DEAE-cellulose (DE-52) was obtained from Whatman Chemical Separation, Ltd. (USA). E-64 (N-(N-(L-3-trans-carboxyran-2-carbonyl)-L-leucyl) agmatine) was a generous gift from Dr. Masaharu Tamai, Taisho Pharmaceutical Co., Japan. Normal goat serum, goat anti-rabbit IgG and peroxidase-anti-peroxidase complex were obtained from Miles (USA). Goat anti-rabbit IgG-peroxidase conjugate was obtained from Kappel (USA).

Preparation and assays of thyroidal calpains

All subsequent procedures were carried out at 0–4°C. Thyroid glands from 4–6 pigs (40 g) were homogenized in 3 vol of homogenizing buffer (0.25 M sucrose, 10 mM Tris-HCl (pH 7.6), 5 mM mercaptoethanol, 2 mM EDTA) with a Waring blender at the top speed for 30 sec. Cytosolic fraction was obtained by centrifuging the homogenate at 105,000×g for 90 min. The cytosolic fraction was applied on a DEAE-cellulose column (1.4×10.0 cm) previously equilibrated with elution buffer (0.1 M sucrose, 10 mM Tris-HCl (pH 7.5), 5 mM mercaptoethanol and 1 mM EGTA). After the column was washed with 300 ml of the elution buffer, the elution was carried out with a linear gradient (0–0.7 M) of NaCl in the same buffer.

Proteolytic activity was assayed with casein as the substrate by the method of Nishiura *et al.* (1978) with some modifications. The activity was determined using 200 µl aliquots of each fraction of eluates from the DEAE-cellulose column in 50 mM Tris-HCl buffer (pH 7.6), 10 mM 2-mercaptoethanol, 0.5 mM EGTA and 20 mM KCl (final volume 500 µl) with or without adding Ca²⁺. After incubation for 30 min at 37°C, the reaction was stopped by adding 200 µl of 20% trichloroacetic acid (TCA). The TCA soluble products formed were colorimetrically determined at 750 nm by the method of Lowry *et al.* (1951). One unit of the protease activity was defined as an increase in 1.0 absorbance unit at 750 nm in the presence of Ca²⁺ after

subtraction of the result obtained in the absence of Ca²⁺.

Free Ca²⁺ concentration

The free Ca²⁺ concentration was adjusted by means of a metal-metal chelator buffer system using EGTA as the chelator. Composition of a metal-metal chelator buffer system was determined by calculation using the stability constant as described by Pershadsigh and McDonald (1980).

Antiserum

Antiserum to porcine thyroglobulin was made as follows: The emulsion of porcine thyroglobulin (0.2 mg) and Freund's complete adjuvant (0.2 ml) were injected at 6 sites in rabbit back and the same amount of antigen was boosted once a week. After 2 months, the rabbits were bled and the antiserum was tested by double immunodiffusion. The antiserum produced a precipitation line against porcine thyroglobulin.

SDS-polyacrylamide gel electrophoresis and immunoblotting

Aliquots of each fraction eluted from the DEAE-cellulose column were tested as the substrates of calpain 2 in place of casein. Incubations were for 30 min and stopped by treating the samples with 1% SDS and 2% mercaptoethanol for 5 min in boiling water. Samples were applied to SDS-polyacrylamide gel electrophoresis by the method of Laemmli (1970). The proteins and peptides in the gels were transferred to nitrocellulose membranes and then subjected to immunolabelling as described by Towbin *et al.* (1979) with some modifications: Nitrocellulose membranes were incubated with goat anti-rabbit IgG-peroxidase conjugate (1:200). Enzyme reaction was developed in 50 mM Tris-HCl buffer (pH 7.5) containing diaminobenzidine (50 mg/100 ml) by adding H₂O₂ (0.05%).

Results

Resolution of peak I and peak II calpain activities

Protease assay was carried out either with or without adding 1 mM CaCl₂ to the incubation medium. Fig. 1 shows the re-

Fig. 1. Isolation of Ca^{2+} -activated protease activity from hog thyroid cytosol. After washing the column with elution buffer, elution was carried out with a linear gradient of NaCl (0–0.7 M) added to the buffer. Aliquots from the eluates were assayed for protease activity. Aliquots were also tested as the substrate of calpain 2 by using an immunoblotting procedure. Fractions 10, 20 and 26 were called F-1, F-2 and F-3, respectively, and used for the studies described in Fig. 5.

presentative chromatographic pattern of Ca^{2+} -activated protease activity eluted on DEAE-cellulose column chromatography. An aliquot of each fraction was used for calpain assay. Two major peaks of protease activities were eluted. The first small peak was eluted at 0.2 M NaCl and the second peak was eluted at 0.4 M NaCl. The calpain activities of peak I and peak II were 0.112 U and 0.210 U, respectively.

Ca^{2+} dependency

Both peaks of protease activities were dependent on Ca^{2+} , although there was a difference between the degrees of Ca^{2+} sensitivity of the two peaks. As shown in Fig. 2, the free Ca^{2+} concentration which maximally stimulated both protease activities was about 1.0 mM, but the 50% activation was obtained at 5 μM Ca^{2+} for peak I and 0.5 mM Ca^{2+} for peak II. At 3.0 mM Ca^{2+} ,

both peak I and peak II activities were slightly suppressed by an unknown cause.

Peak I had rather little activity compared with peak II possibly because of coelution of an endogenous inhibitor (Inomata *et al.*, 1983) (data not shown). Fractions 24, 25 and 26 (peak II) were collected and used mainly for further characterization of Ca^{2+} -activated protease.

The enzyme was demonstrated to have a broad neutral pH optimum between 6.0 to 8.0 when assayed by caseinolysis (data not shown). In subsequent experiments, Tris-ECI buffer (pH 7.5) was used.

Inactivation of calpain peak II by preincubation with Ca^{2+}

Preincubation of peak II was started in the same assay mixture as used for calpains, but without casein. After this preincubation, casein was added to the medium and

Fig. 2. Effects of increasing Ca^{2+} concentrations on the activities of proteases from hog thyroid cytosol. Ca^{2+} concentrations were fixed by a EGTA- Ca^{2+} buffer method. Peak I and peak II activities were expressed as a percent of maximal activity measured in the presence of 1 mM Ca^{2+} .

the incubation was started.

After 3 min, incubation was stopped by adding 200 μl of 20% TCA. Peak II activity was decreased by 50% after preincubation with 1.0 mM Ca^{2+} for less than 2.5 min (Fig. 3). In contrast, peak II activity was fairly well maintained without Ca^{2+} .

Enhancement of Ca^{2+} sensitivity of calpain peak II

To know the effect of preincubation with Ca^{2+} on Ca^{2+} sensitivity, peak II was preincubated with 1.0 mM Ca^{2+} for 3 min and then dialyzed in the elution buffer at 4°C. After dialyzing with the elution buffer for at least 5 h, protease activity was mea-

Fig. 3. Effect of preincubation of peak II with Ca^{2+} . Preincubation was performed at 1.0 mM Ca^{2+} . After the preincubation, incubation was started by adding casein to the medium. The activities measured after each preincubation time was plotted.

Fig. 4. Enhancement of Ca^{2+} sensitivity of peak II. Preincubation was performed at 1.0 mM Ca^{2+} . After preincubation for 2 min, peak II was dialyzed against elution buffer for at least 5 h. Calpain activities of dialyzed samples were measured at various concentrations of Ca^{2+} .

Table 1. Effect of various protease inhibitors on protease activity

Inhibitors	Concentrations	Remaining Activity (%)	
		Calpain 1	Calpain 2
None		100	100
Leupeptin	5 μ g/ml	N. D.	8
	20 μ g/ml	N. D.	0
E-64	5 μ g/ml	32	15
	20 μ g/ml	0	11
Pepstatin A	10 μ g/ml	N. D.	95
Soybean trypsin inhibitor	100 μ g/ml	100	100
Phenylmethyl-sulfonyl fluoride	1 mM	100	100
Monoiodoacetic acid	0.01 mM	90	70
	0.1 mM	26	56
Trifluoperazine	0.1 mM	N. D.	100

N. D.: not determined.

Remaining activities in the presence of inhibitors listed above were expressed as a percent of control using casein as the substrate. Each inhibitor was preincubated at 37°C for 10 min without adding calcium in the assay medium.

sured at the indicated Ca^{2+} concentrations (Fig. 4). There was an enhancement of Ca^{2+} sensitivity of peak II. Fifty percent activation by Ca^{2+} was observed at 10^{-7} M. During the preincubation and the succeeding dialysis, 83% of the calpain activity was lost.

Effects of various protease inhibitors on calpain activities

Table 1 shows the effects of various inhibitors on peak I and peak II. Leupeptin and E-64 were the most potent inhibitors used. E-64, one of the synthetic thiol protease inhibitors, is known to be as potent as leupeptin (Handa *et al.*, 1978). Concentrations of leupeptin and E-64 for 50% inhibition of peak II were 0.5 μ g/ml and 2.0 μ g/ml, respectively. Monoiodoacetic acid suppressed the peak II activity by 44% at 0.1 mM. Pepstatin A, soybean trypsin inhibitor, penylmethylsulfonyl-fluoride and trifluoperazine were not inhibitory at all. As mentioned above, peak II activity seems to share the same characteristics with the thiol proteases.

Substrate specificity

Various substrates at a concentration of

1.4 mg/ml were tested in addition to casein. Bovine serum albumin, ovalbumin, native hemoglobin and porcine thyroglobulin were used. The magnitude of proteolytic activity attributable to thyroglobulin or other proteins contaminated with peak II was estimated by incubation of peak II with or without Ca^{2+} in the absence of exogenous substrate. The Ca^{2+} -dependent proteolysis in the absence of exogenous substrate was subtracted from the value of Ca^{2+} -dependent proteolysis obtained with each exogenous substrate. The value thus obtained for casein was defined as 100%. Casein was the best substrate, followed in succession by ovalbumin (52%), porcine thyroglobulin (37%), bovine serum albumin (14%) and native hemoglobin (0%).

Thyroglobulin proteolysis by peak II

All the fractions eluted from DEAE-cellulose column were tested as the substrate of peak II with the immunoblotting technique. When the F-1 and F-2 were incubated with peak II, thyroglobulin immunoreactivities were detected at mol wt in the 67,000 and 46,000 bands (Fig. 5; F-1, F-2), respectively. Thus, the 67 K and 46 K proteins were confirmed to be the products

Fig. 5. Immunoblotting of thyroglobulin treated with calpain 2. F-1, F-2, and F-3 treated with calpain 2 were subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. Thyroglobulin-immunoreactivity was detected by using anti-porcine thyroglobulin antiserum (1 : 400).

of thyroglobulin proteolysis by calpain 2. These products were not clearly seen in the absence of Ca^{2+} in the medium.

When the F-3 was incubated with calpain 2, thyroglobulin immunoreactive 40 K protein decreased in the presence of calcium, indicating that 40 K protein was also the substrate for calpain 2.

Discussion

Among the cytosolic proteases, calpains are of interest since many physiological events induced by TSH are linked to Ca^{2+} in the thyroid gland (Zor *et al.*, 1968; Hashizume *et al.*, 1984). Two Ca^{2+} -depen-

dent proteases are separated from each other on DEAE-cellulose column chromatography of hog thyroid cytosol. Peak I showed high Ca^{2+} sensitivity and peak II had low Ca^{2+} sensitivity. Peak II digested casein in the neutral pH range. Peak II was sensitive to thiol protease inhibitors and monoiodoacetic acid, and was insensitive to a calmodulin inhibitor and serine protease inhibitors. This protease is classified in thiol protease and metal-activated protease. All the characteristics described above indicate that peak I and peak II resemble calpain 1 and calpain 2, respectively (Mollgrem, 1980; DeMartino, 1981).

In porcine thyroid, a few proteases in lysosomes have been known to be inhibited

by thiol protease inhibitors (Nakagawa *et al.*, 1981; Nakagawa and Ohtaki, 1984). They are thought to be cathepsin B and cathepsin H reported in other tissues (Tawatari *et al.*, 1978). Although they have some characteristics in common with calpains, calpains are different from them in their Ca^{2+} sensitivity and existence in cytosol.

Alteration of Ca^{2+} sensitivity from a low sensitive form with a millimolar range of K_a to a highly sensitive form with a micromolar range of K_a was induced by preincubation with Ca^{2+} . The possibility that the enhancement of Ca^{2+} sensitivity (Fig. 4) with partial inactivation (Fig. 3) was elicited by the autolysis of calpain itself might be considered. Such great enhancement of the sensitivity of calpain to Ca^{2+} was also observed in chicken skeletal muscle (Suzuki *et al.*, 1981). This suggests that once the local concentration of Ca^{2+} in the cytosol is raised to the mM range, the enhancement of Ca^{2+} sensitivity may occur. Localization studies have demonstrated that plasma membrane, nucleus, and mitochondria are possible candidates for Ca^{2+} storage (Popescu *et al.*, 1974; Debbas *et al.*, 1975). Although TSH has been shown to increase the intracellular free Ca^{2+} in thyroid cells (Chorda *et al.*, 1985), it is not clear whether Ca^{2+} ion increases to the mM order concentration. The highly Ca^{2+} -sensitive form may be able to work at a physiological Ca^{2+} concentration. The small activity observed in peak I can be attributed to coelution of the endogenous inhibitor. It is also likely that the activity of peak I is better expressed in intact cells. Whether these highly sensitive forms of peak II and peak I are exactly the same is not clear from the data at hand.

The observation that thyroglobulin was also hydrolysed by calpain 2 is of interest since it is noted that thyroglobulin is released from the thyroid via the lymphatic circulation to some extent (Daniel *et al.*, 1967), and other investigators have shown that thyroglobulin released in the circulation is

TSH-dependent (Unger *et al.*, 1980) and poorly iodinated (Ikekubo *et al.*, 1981). Kohn *et al.* (1985) demonstrated the existence of a thyroglobulin secretion path which bypasses the Golgi without lysosomal degradation using the rat clonal thyroidal cells (FRTL-5). This may imply that thyroglobulin can be released by passing the normal site of iodination and lysosomal proteolytic degradation as well. Our data further suggest that thyroglobulin is a candidate for an endogenous substrate of calpain 2 in hog thyroid cytosol. Furthermore, it appears to be possible that intra-thyroidal Ca^{2+} -dependent proteolysis is, at least in part, controlled by cytosolic distribution of calpains, its inactivation by Ca^{2+} and the enhancement of Ca^{2+} sensitivity.

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