Identification of μ-, m-Calpains and Calpastatin and Capture of μ-Calpain Activation in Endothelial Cells

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Abstract The presence of the calpain-calpastatin system in human umbilical vein endothelial cells (HUVEC) was investigated by means of ion exchange chromatography, Western blot analysis, and Northern blot analysis. On DEAE anion exchange chromatography, calpain and calpastatin activities were eluted at approximately 0.30 M and 0.15–0.25 M NaCl, respectively. For half-maximal activity, the protease required 800 μM Ca^{2+}, comparable to the Ca^{2+} requirement of m-calpain. By Western blot analysis, the large subunit of μ-calpain (80 kDa) was found to be eluted with calpastatin (110 kDa). Both the large subunit of m-calpain (80 kDa) and calpastatin were detected in the respective active fractions. By Northern blot analysis, mRNAs for large subunits of μ- and m-calpains were detected in single bands, each corresponding to approximately 3.5 Kb. Calpastatin mRNA was observed in two bands corresponding to approximately 3.8 and 2.6 Kb. Furthermore, the activation of μ-calpain in HUVEC by a calcium ionophore was examined, using an antibody specifically recognizing an autolytic intermediate form of μ-calpain large subunit (78 kDa). Both talin and filamin of HUVEC were proteolyzed in a calcium-dependent manner, and the reactions were inhibited by calpeptin, a cell-permeable calpain specific inhibitor. Proteolysis of the cytoskeleton was preceded by the appearance of the autolytic intermediate form of μ-calpain, while the fully autolyzed postautolysis form of μ-calpain (76 kDa) remained below detectable levels at all time points examined. These results indicate that the calpain-calpastatin system is present in human endothelial cells and that μ-calpain may be involved in endothelial cell function mediated by Ca^{2+} via the limited proteolysis of various proteins. J. Cell. Biochem. 66:197–209, 1997. © 1997 Wiley-Liss, Inc.

Key words: μ-calpain; m-calpain; calpastatin; μ-calpain activation in endothelial cells; autolytic intermediate form of μ-calpain; fully autolyzed postautolysis form of μ-calpain; calpeptin; talin; filamin; cytoskeletal proteolysis

Two forms of calpain (EC 3. 4. 22. 17.), an intracellular Ca^{2+}-activated neutral cysteine protease, have been identified with different Ca^{2+} requirements for their activities; μ-calpain and m-calpain, which require μM and mM levels of Ca^{2+}, respectively [Suzuki et al., 1984].

These two species of calpain are distributed in a wide variety of mammalian and avian tissues and cells [Murachi, 1983a; Kawashima et al., 1988; Murachi, 1989], together with calpastatin, a specific endogenous inhibitor of calpain [Murachi, 1983a,b]. A number of endogenous calpain substrates have been reported so far. These may be classified into three categories: (1) Cytoskeletal proteins such as actin binding protein [Truglia and Stracher, 1981], talin [Beckerle et al., 1986], filamin [Verhallen et al., 1987], and spectrin [Inomata et al., 1989]; (2) Enzyme proteins such as protein kinase C (PKC) [Kishimoto et al., 1983, 1989], cAMP-dependent protein kinase (A-Kinase) [Beer et al., 1984], myosin light chain kinase (MLCK) [Tsujinaka et al., 1988a], phospholipase C (PLC) [Low et al., 1984], and tyrosine kinase (pp60src) [Oda et

al., 1993]; and (3) Receptor proteins such as EGF receptor [Cassel and Glaser, 1982; King and Gates, 1985] and PDGF receptor [Ek and Heldin, 1986]. Thus, calpain may be involved in a variety of cellular processes mediated by Ca^{2+} via the limited proteolysis of various proteins.

One of the physiological roles of vascular endothelium is to function as a selective diffusion barrier between plasma and interstitial fluid. Breakdown of this barrier function results in increased vascular permeability to protein and fluid, leading to the development of edema. Increases in vascular permeability have been noted in response to a variety of inflammatory mediators such as histamine [Rotrosen and Gallin, 1986], α-thrombin [Garcia et al., 1986], and oxidants [Shasby et al., 1985]. Although the mechanisms involved in these events are not fully understood, an increase in intracellular Ca^{2+} ($[Ca^{2+}]i$) and cytoskeletal rearrangements are commonly observed, suggesting that calpain may be involved in these pathological conditions through degradation of cytoskeletal proteins. However, with respect to the existence of the calpain-calpastatin system in endothelial cells, there have been conflicting observations. Yoshimura et al. reported in their immunohistochemical studies that neither μ- nor m-calpain was detectable in vascular endothelium of rat liver [Yoshimura et al., 1984a] or of porcine kidney [Yoshimura et al., 1984b]. In contrast, Hayashi et al. reported immunohistochemical localization of calpain in various rabbit organs including interlobular artery and vein in liver, small vessels in skeletal muscle, blood vessels in kidney, and blood vessels in brain choroid plexus [Hayashi et al., 1987], although the exact identification of calpain in vascular endotelium was not documented. Rodgers et al. also reported the presence of Ca^{2+}-dependent proteinase in lysates of bovine aortic endothelial cells and of human umbilical vein endothelial cells using an enzyme-linked immunosorbent assay (ELISA) [Rodgers et al., 1987].

To solve this controversy, it is essential to determine whether calpains and calpastatin are expressed in endothelial cells not only at the protein level but also at the mRNA level. In this paper, we present several lines of evidence that human umbilical vein endothelial cells contain μ- and m-calpain, and their specific inhibitor calpastatin. In addition, we examined the activation of μ-calpain and breakdown of

cytoskeletal proteins in endothelial cells on calcium influx.

METHODS
Materials

The following materials were obtained from the sources shown: MCDB 131 medium, Chlorella Inc., Tokyo, Japan; fetal bovine serum, JRH Biosciences, Lenexa, KS; recombinant human b-FGF, DAINIPPON Pharmaceutical Inc., Tokyo, Japan; penicillin-streptomycin, GIBCO Life Technologies Inc, Grand Island, NY; dispase, Godo-shusei Inc., Tokyo, Japan. Collagen-coated flasks (T-75) were obtained from Corning Glassware, Inc., Corning, NY. DEAE-5PW anion exchange column (5.0 mmID × 5.0 cm) was purchased from TOSOH Inc., Tokyo, Japan. Casein (Hammarsten grade) was purchased from Merck, Darmstadt, Germany; bovine serum albumin, phenylmethylsulfonyl fluoride (PMSF) and calcium ionophore A23187 were from Sigma, St. Louis, MO; and leupeptin was from Peptide Institute, Osaka, Japan. Molecular weight standards for SDS-polyacrylamide gel electrophoresis and polyvinylidene difluoride (PVDF) membranes were obtained from Bio-Rad Laboratories, Richmond, CA. Mouse monoclonal IgG antibodies 1A8A2 and 1C6D1, specific for the large subunit of μ-calpain and m-calpain, respectively, were prepared as reported previously [Inomata et al., 1988; Kasai et al., 1986]. Mouse monoclonal IgG antibody CSL1-5, specific for domain III of calpastatin, was purchased from Takara Shuzo Co., Ltd., Tokyo, Japan [Yokota et al., 1991]. Mouse monoclonal anti-talin antibody (T-3287) was from Sigma, St. Louis, MO, and mouse monoclonal anti-filamin antibody (MCA 464S) was from Serotec Co., Oxford, UK. Peroxidase-conjugated goat anti-mouse IgG was from Cappel Laboratories Inc., Durham, NC. Alkaline phosphatase-conjugated goat anti-rabbit IgG, NBT (nitro blue tetrazolium) substrate and BCIP (5-bromo-4-chloro-3-indolyl-phosphate) substrate were purchased from SEIKAGAKU-KOGYO Inc., Tokyo, Japan. RNA Zol was from Biotecx Laboratories, Inc., Houston, TX. Biodyne nylon membranes were from Pall BioSupport, East Hills, NY. [α-^{32}P] dCTP (3,000 Ci/mmol) and megaprime DNA labeling system were purchased from Amersham, Buckinghamshire, UK., and RNA size markers were from GIBCO Life Technologies. All other chemicals were of the highest analytical grade available.

Preparation of Endothelial Cells

Human umbilical vein endothelial cells (HUVEC) were obtained by incubating surgically resected human umbilical cord for 90 min at room temperature in MCDB 131 medium containing 1,000 pu/ml of dispase. Cells were grown at 37°C in T-75 tissue culture flasks in MCDB 131 medium containing 10% fetal bovine serum, 10 ng/ml b-FGF, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified 5% CO_2 atmosphere. HUVEC were passed as a 1:5 split, fed every 3 days, and used at the 4th to 6th passage. These cells were characterized as endothelial cells by their typical cobblestone appearance and positive staining for factor VIII antigen.

Resolution of Calpain and Calpastatin Activities by Ion Exchange Chromatography

All the procedures were performed at 4°C or on ice. Confluent cultures were washed twice with PBS(−), pH 7.4, and scraped with a rubber policeman in 20 mM Tris-HCl buffer, pH 7.4, containing 250 mM sucrose, 5 mM EDTA, 1 mM DTT, 1 mM PMSF, and 100 μM leupeptin. Cells (1.0×10^8) were centrifuged at $1,000g$ for 10 min, resuspended in the same buffer, and then disrupted by sonication (Branson sonifier 250) with an index of 5 for 15 s × 8 cycles. The homogenate was centrifuged at $105,000g$ for 60 min and the resulting supernatant (30 mg of protein) was applied to a DEAE-5PW column equilibrated with buffer A (10 mM Tris-HCl, 250 mM sucrose, 5 mM EGTA, 1 mM DTT, pH 7.5). The adsorbed proteins were eluted with a linear gradient concentration of NaCl from 0 to 0.5 M in 12.5 ml of buffer A at a flow rate of 0.5 ml/min.

Assay of Calpain and Calpastatin

Calpain activity of the DEAE-5PW fraction was measured with casein as a substrate, according to our previous report [Kambayashi and Sakon, 1989]. An aliquot (10 μl) of each fraction (250 μl) was assayed in the presence of human platelet μ-calpain (10 u/ml), purified as described previously [Kambayashi and Sakon, 1989], for proteolytic and inhibitory activities. Calcium dependency of endothelial calpain was determined using a strictly adjusted Ca^{2+}-EGTA buffer [Harafuji and Ogawa, 1980].

Protein Determination

Protein concentration was determined by the method of Bradford using bovine serum albumin as a standard [Bradford, 1976].

Western Blot Analysis of Endothelial Calpains and Calpastatin

For Western blotting, 20 μg aliquots of protein from DEAE-5PW fractions (Fr.24–Fr.44) were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% polyacrylamide gels, according to the procedure of Laemmli [Laemmli, 1970]. Proteins were subsequently transferred from the gels onto 0.2 μm-pore size PVDF membranes by the method of Towbin et al. [Towbin et al., 1979]. PVDF membranes were incubated overnight at 4°C with monoclonal antibody against a large subunit of μ- or m-calpain or against calpastatin, and developed with horseradish peroxidase-conjugated goat anti-mouse IgG and 3,3′-diaminobenzidine tetrahydrochloride.

Northern Blot Analysis of Endothelial Calpains and Calpastatin

The following DNAs inserted into pUC8 plasmids and used as probes were kind gifts from Dr. Koichi Suzuki, Institute of Molecular and Cellular Biosciences, Tokyo University, Tokyo, Japan; p31 for the large subunit of human μ-calpain [Aoki et al., 1986] and p21 for the large subunit of human m-calpain [Imajoh et al., 1988]. The DNA used as a probe for human calpastatin, lambda cs19, inserted into pUC118 plasmid was kindly provided by Dr. Masatoshi Maki, Laboratory of Human Tumor Viruses, Institute for Virus Research, Kyoto University, Kyoto, Japan [Asada et al., 1989]. A human β-actin genomic DNA segment was purchased from Wako Pure Chemical, Osaka, Japan. Total RNA was extracted from cultured endothelial cells as well as from human placenta as a control using RNA Zol. Twenty micrograms of RNA was denatured and electrophoresed on a 1% agarose gel containing 2.2 M formaldehyde [Sambrook et al., 1989]. After electrophoresis, RNA was transferred onto a nylon membrane by capillary action, fixed under UV light, and then hybridized with ^{32}P-labeled DNA probes at 65°C in a solution containing 0.5M Na_2HPO_4, 1 mM EDTA, 7% SDS, and 1% bovine serum albumin. The membrane was washed in 0.2 × SSC/0.1% SDS (1 × SSC is 0.15 M NaCl/0.015

M sodium citrate, pH 7.0) at 65°C and then exposed to Kodak XAR film for 1 day with an intensifying screen at −80°C or to an imaging plate developed with BAS 2000 system (FUJIX, Japan).

Western Blot Analysis of Talin and Filamin in Endothelial Cells Stimulated With a Calcium Ionophore

Confluent endothelial cells in T-75 flasks were washed once with PBS(−), equilibrated in Krebs-Henseleit bicarbonate (KHB) buffer (118 mM NaCl, 4.74 mM KCl, 1.19 mM KH_2PO_4, 1.18 mM $MgSO_4$, 24.9 mM $NaHCO_3$, 25 mM HEPES, pH 7.4) containing either 2 mM $CaCl_2$ or 2 mM EGTA for 10 min at 37°C, and then incubated with 2 μM A23187 (in dimethyl sulfoxide, 0.2% final, v/v) for various time periods at 37°C. After terminating the reaction with an SDS-containing buffer (2% sodium dodecyl sulfate [SDS], 2% mercaptoethanol, 10% glycerol, 30 mM Tris-HCl, 5 mM EDTA, pH 6.8, 0.001% Bromophenol Blue), SDS-PAGE was performed on 4–20% exponential gradient polyacrylamide gels. Proteins were subsequently transferred from the gels onto PVDF membranes, incubated overnight at 4°C with monoclonal antibody against talin or filamin, and developed with horseradish peroxidase-conjugated goat anti-mouse IgG and 3,3′-diaminobenzidine tetrahydrochloride. Furthermore, the effect of calpeptin, a cell-permeable calpain specific inhibitor [Tsujinaka et al., 1988b; Ariyoshi et al., 1991], on hydrolysis of these cytoskeletal proteins was investigated. Endothelial cells were washed once with PBS(−), preincubated for 10 min at 37°C with various concentrations of calpeptin (0–20 μM) (in dimethyl sulfoxide, 0.1% final, v/v) in KHB buffer containing 2 mM $CaCl_2$, and then incubated with 2 μM A23187 for 15 min at 37°C. The subsequent Western blot analysis was performed as mentioned above.

Antibody Preparation for the Autolytic Intermediate Form of μ-Calpain Large Subunit and Western Blot Analysis of Activated μ-Calpain in Endothelial Cells Stimulated With a Calcium Ionophore

Peptides were synthesized on an Applied Biosystems 430A solid phase synthesizer, deprotected, and purified by HPLC. The following peptide was used as an immunogen: NH_2-AQVQKQC-COOH corresponding to the amino-terminal region of the autolytic intermediate form of μ-calpain large subunit (78 kDa). The peptide with a cysteine residue added onto the C-terminus was conjugated with keyhole lympet hemocyanin, and injected into rabbits as described previously [Kikuchi et al., 1993]. Purification of antibody and characterization of purified antibody using the dot blot assay were performed as described previously [Kikuchi et al., 1995b]. Rabbit monoclonal IgG antibody anti-ACTμ, specific for a fully autolyzed postautolysis form of μ-calpain large subunit (76 kDa), was also prepared as reported previously [Kikuchi et al., 1995a]. Using these antipeptidic antibodies specific to different activated forms of μ-calpain, autolytic intermediate form of μ-calpain (78 KDa) and fully autolyzed postautolysis form of μ-calpain (76 KDa), we examined the activation of μ-calpain in endothelial cells stimulated with A23187. Endothelial cells were washed once with PBS(−), equilibrated in KHB buffer containing 2 mM $CaCl_2$ for 10 min at 37°C, and then incubated with 2 μM A23187 for various periods at 37°C. After terminating the reaction with an SDS-containing buffer, SDS-PAGE was performed on 7.5% polyacrylamide gels. Proteins were subsequently transferred from the gels onto PVDF membranes, incubated overnight at 4°C with monoclonal antibodies against different activated forms of μ-calpain, and developed with alkaline phosphatase-conjugated goat anti-rabbit IgG, NBT substrate, and BCIP substrate.
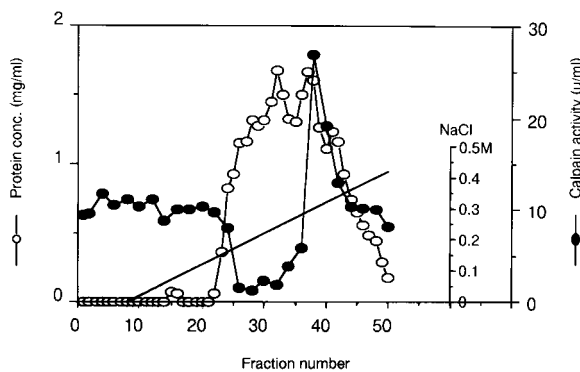
RESULTS

### Resolution of Calpain and Calpastatin Activities by DEAE-5PW Column Chromatography

A typical elution profile of calpain and calpastatin activities in endothelial cells is shown in Figure 1. Calpain activity was eluted at approximately 0.30 M NaCl as a single positive peak. Inhibitory activity of calpastatin was observed at 0.15–0.25 M NaCl as a broad negative peak of calpain activity. Endogenous calpastatin activity was found to be present in excess of total calpain activity because the crude endothelial lysate showed no calpain activity (data not shown).

Ca^{2+} Requirement of Calpain

Figure 2 shows the effects of Ca^{2+} concentration on the activity of the calpain fraction (Fr.38). For half-maximal activity, the protease

Fig. 1. Elution profile of calpain and calpastatin on DEAE-5PW column chromatography. The cytosolic fraction of cultured HUVEC (1.0×10^8) was applied to a DEAE-5PW column. Calpain and calpastatin activities were eluted and assayed as described in Methods. Fifty fractions of 250 µl each were collected. One unit of activity is defined as the amount of enzyme which liberates 1 µmol of α-amino group as leucine equivalent per hour.
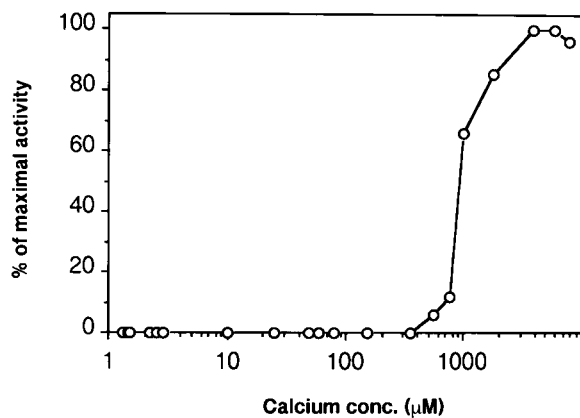
Fig. 2. Ca^{2+} requirement of endothelial calpain. The peak fraction of calpain activity (Fr.38) was assayed for its caseinolytic activity at various Ca^{2+} concentrations. Calpain activity is shown as % of the maximal activity.

required 800 µM Ca^{2+}. From the elution profile and Ca^{2+} requirement, the peak calpain activity was classified as m-calpain.

Detection of μ- and m-Calpains and Calpastatin by Western Blot Analysis

As μ-calpain is generally overlapped with calpastatin by DEAE anion exchange chromatography, its activity in the present preparation was considered to be masked in the broad inhibitory activity of calpastatin. To confirm this possibility, Western blotting with specific monoclonal antibodies for the respective protein was performed. DEAE-5PW fractions from 24 to 36 reacted with 1A8A2, a monoclonal antibody

against the large subunit of μ-calpain (Fig. 3A). Fractions 38 to 44 reacted with 1C6D1, a monoclonal antibody against the large subunit of m-calpain, in consort with its caseinolytic activity (Fig. 3B). The molecular weight of the large subunit of μ- and m-calpain in endothelial cells was estimated to be approximately 80 kDa. Fractions 24 to 32 reacted with CSL1-5, a monoclonal antibody against calpastatin (Fig. 3C). Calpastatin appeared in a fragmented pattern, ranging from 110 to 46 kDa. Thus, the activity of μ-calpain fractionated by DEAE anion exchange chromatography was masked by that of co-eluted calpastatin.

Expression of μ- and m-Calpains and Calpastatin mRNAs in Endothelial Cells

To further confirm the existence of the calpain-calpastatin system in endothelial cells, we examined the expression of their mRNAs by Northern blot analysis. As shown in Figure 4, mRNAs for both μ- (Fig. 4A) and m-calpain (Fig. 4B) large subunits were detected in single bands, each at positions corresponding to approximately 3.5 Kb. Calpastatin mRNA (Fig. 4C) was resolved in two bands corresponding to approximately 3.8 and 2.6 Kb.

Effects of Extracellular Ca^{2+} on Hydrolysis of Cytoskeletal Proteins

As shown in Figure 5, hydrolysis of talin (Fig. 5A) and filamin (Fig. 5B) was detected at 15 min after A23187 stimulation in the presence of extracellular Ca^{2+}, although it was totally inhibited by chelation of extracellular Ca^{2+} with 2 mM EGTA. The amount of intact talin molecule (225 kDa) was decreased with a concomitant increase in degraded talin molecule (190 kDa), followed by the further degradation of 190 kDa fragment (Fig. 5A). The amount of intact filamin molecule (250 kDa) was also decreased in a time-dependent manner, although baseline filamin degradation product (190 kDa) was observed at time 0 (Fig. 5B). These findings indicate that the hydrolysis of talin and filamin requires elevation of $[Ca^{2+}]i$. The possibility of cell lysis induced by A23187 was excluded, as cytoplasmic lactate dehydrogenase (LDH) was not detected in the suspending media as reported previously [Yano et al., 1993] (data not shown).
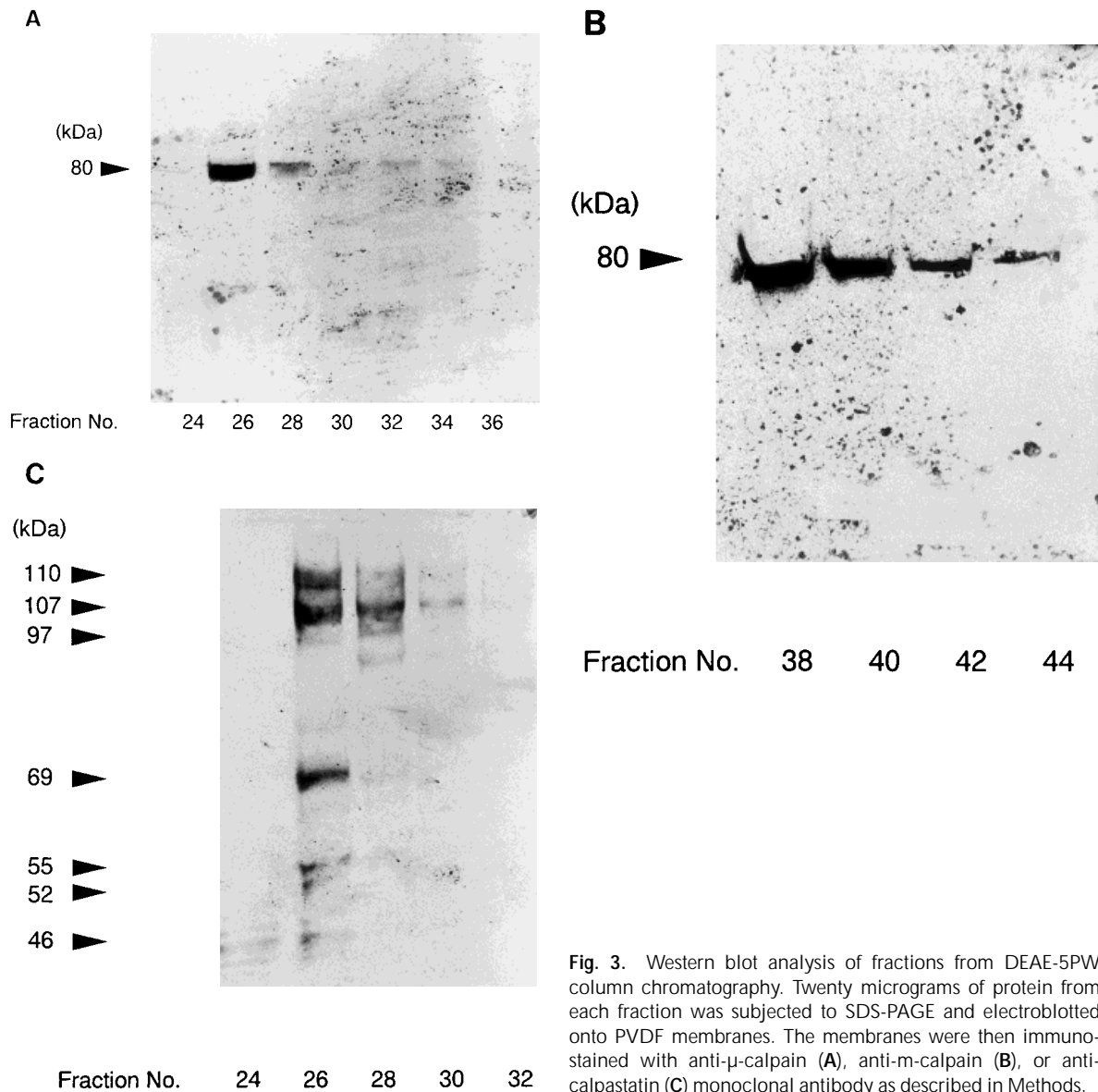
A

(kDa)

80 ▶

Fraction No.   24 26 28 30 32 34 36

B

(kDa)

80 ▶

Fraction No.   38   40   42   44

C

(kDa)

110 ▶
107 ▶
97 ▶

69 ▶

55 ▶
52 ▶

46 ▶

Fraction No.   24   26   28   30   32

Fig. 3. Western blot analysis of fractions from DEAE-5PW column chromatography. Twenty micrograms of protein from each fraction was subjected to SDS-PAGE and electroblotted onto PVDF membranes. The membranes were then immunostained with anti-μ-calpain (**A**), anti-m-calpain (**B**), or anti-calpastatin (**C**) monoclonal antibody as described in Methods.

Effects of Calpeptin on Hydrolysis of Cytoskeletal Proteins

To clarify whether calpain is involved in hydrolysis of these cytoskeletal proteins, we examined the effects of various concentrations of calpeptin on the cleavage of talin and filamin in endothelial cells incubated with A23187. As shown in Figure 6A, the degradation of intact talin molecule (225 kDa) was inhibited by calpeptin in a dose-dependent manner. Though there appears to be a difference in the amount of degraded talin molecule (190 kDa), compared the 190 kDa band in the PC (Fig. 6A) with that at 15 min in Figure 5A, the presence of this band was variable around the 15-min time

point. The degradation of intact filamin molecule (250 kDa) was also inhibited by calpeptin in a dose-dependent manner in Figure 6B. Several filamin degradation products other than 190 kDa band were observed in the PC (Fig. 6B). However, their presence was variable around the 15-min timepoint. These findings suggest the calpain involvement in these cytoskeletal proteolysis.

Specificity of the Anti-μ-Calpain Antibodies and Capture of μ-Calpain Activation in Endothelial Cells Stimulated With a Calcium Ionophore

The specificity of an antibody against an autolytic intermediate form of μ-calpain large sub-
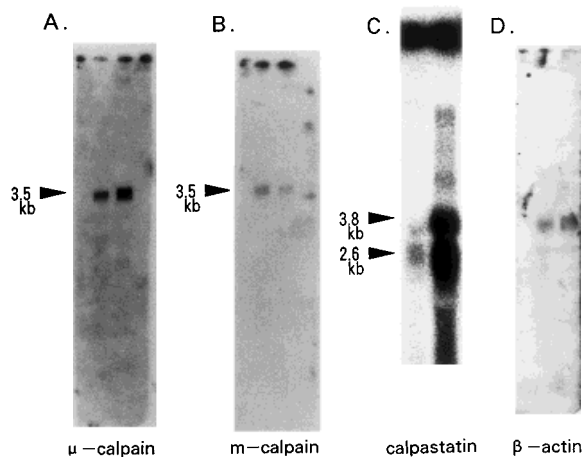
Fig. 4. Northern blot analysis of total RNA from HUVEC. Total cellular RNA (20 μg/lane) from HUVEC (left) or from the placenta (right) was electrophoresed on agarose gels under denaturing conditions and blotted onto nylon membranes as described in Methods. The membranes were then hybridized with ^{32}P-labeled DNA probes for the large subunits of μ- (**A**) and m- (**B**) calpains, calpastatin (**C**), and β-actin (**D**).

unit (78 kDa) was examined by Western blotting as shown in Figure 7, using purified human platelet μ-calpain. The antibody 1A8A2, specific to both pre- and post-autolysis forms of μ-calpain, reacted with both pre-autolytic (80 kDa) and fully autolyzed (76 kDa) forms of μ-calpain (Fig. 7A). Another antibody, anti-ACTμ, specific to a post-autolysis form of μ-calpain, reacted only with the fully autolyzed form of μ-calpain (Fig. 7B). The antibody raised against the N-terminal sequences of the autolytic intermediate form of μ-calpain reacted only with the corresponding form of μ-calpain (78 kDa), and not with either pre- or post-autolytic forms (Fig. 7C). Using these antibodies, we confirmed μ-calpain activation in endothelial cells stimulated with A23187. As shown in Figure 7D, the 78 kDa autolytic intermediate form of μ-calpain was observed at 3 min after A23187 stimulation and increased in concentration with time. The fully autolyzed post-autolysis form of μ-calpain (76 kDa) was not detected at any time point (data not shown).

DISCUSSION

In the present study, the presence of μ- and m-calpains and calpastatin was demonstrated in human endothelial cells by various approaches. Although it was difficult to separate calpastatin and μ-calpain by DEAE-5PW chromatography, both μ- and m-calpains and cal-

pastatin were clearly identified by Western blot analysis and μ-calpain activity was ascertained to be co-eluted with calpastatin activity. Two different molecular species of calpastatin, muscle-type and erythrocyte-type, have been reported so far, with respective molecular weights of 107 and 68 kDa as shown by SDS-PAGE [Takano et al., 1986]. Endothelial calpastatin was considered to be muscle-type and the molecular weight was 110 kDa on SDS-PAGE. By Western blot analysis, calpastatin appeared in a fragmented pattern. Nakamura et al. reported that 110 kDa calpastatin was degraded into many lower molecular weight species by unidentified endogenous proteases during purification steps, and that these fragmented species retained considerable inhibitory activity. Fragmentation of calpastatin can be avoided only with chemically drastic pretreatments including usage of leupeptin, PMSF, trichloroacetic acid precipitation, and heat treatment before chromatographic purification steps [Nakamura et al., 1984, 1985]. Fragmentation of calpastatin to lower molecular weight species such as 80, 70, 30, 25, 15 kDa by both μ- and m-calpain in the presence of Ca^{2+} has also been reported [Nakamura et al., 1989]. In this study, fragmentation of calpastatin was considered to be caused by endogenous proteases other than calpains because strict protection against calpain activation was enforced in our preparation. As the crude cell lysate showed no calpain activity, endogenous calpastatin was considered to be present in excess of total calpain activity in HUVEC.

It has been demonstrated in several cell types that a rise in $[Ca^{2+}]i$ causes the translocation of μ-calpain from the cytosol to the membrane, where it is activated by autolysis [Ariyoshi et al., 1993; Saido et al., 1993a; Nagao et al., 1994]. Acidic phospholipids, particularly phosphoinositides, assist μ-calpain activation in cells, reducing the calcium concentration required for μ-calpain activation to a level comparable to the physiological intracellular range reached upon stimulation of cells [Saido et al., 1992a]. These findings suggest that proteins associated with membranes serve as primary substrates for calpain. Therefore, in order to investigate the possibility of calpain involvement in endothelial cell function mediated by Ca^{2+}, we examined the hydrolysis of cytoskeletal proteins, especially talin and filamin, which are associated with membranes and are well

A

B
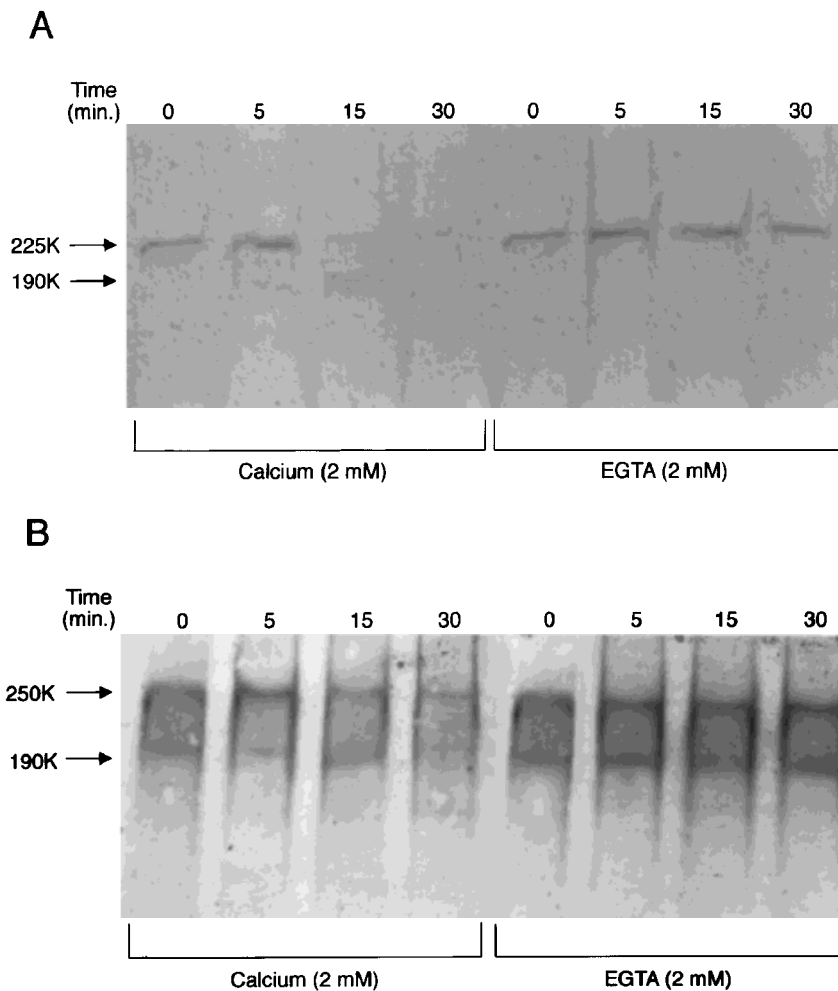
Fig. 5. Time courses of hydrolysis of talin and filamin. Conflu-ent endothelial cells in T-75 flasks (2.0×10^6) were incubated with A23187 (2 μM) in either the presence or absence of extracellular Ca^{2+} for the indicated time periods at 37°C. Then, endothelial cells solubilized in an SDS-containing buffer were subjected to SDS-PAGE and electroblotted onto PVDF mem-branes. The membranes were then immunostained with anti-talin (**A**) or anti-filamin (**B**) monoclonal antibody as described in Methods.

known substrates of calpain, in endothelial cells stimulated with A23187 in the presence or ab-sence of extracellular Ca^{2+}. Both talin and fila-min proteolysis were Ca^{2+}-dependent. To clarify the possible involvement of calpain in proteoly-sis of these cytoskeletal proteins, calpeptin, a cell-permeable calpain specific inhibitor, was employed. Proteolysis of the cytoskeleton was inhibited by calpeptin in a dose-dependent man-ner. From these observations, it was suggested that calpain was involved in the cytoskeletal proteolysis.

As the $[Ca^{2+}]i$ fluctuates normally at sub-micromolar levels under physiological condi-tions [Arslan et al., 1985], μ-calpain rather than m-calpain is more likely to function in cells, although there may exist some unknown mechanisms involving, for example, nuclear ma-terials for the activation of the latter [Mellgren et al., 1993]. μ-Calpain undergoes subunit au-tolysis on activation [Suzuki et al., 1987] and, therefore, we developed an anti-peptidic anti-body capable of distinguishing autolytic inter-mediate form of μ-calpain among other forms of the protease. In endothelial cells stimulated with A23187, the appearance of the autolytic intermediate form of μ-calpain preceded cyto-skeletal proteolysis, while the fully autolyzed form remained undetectable. This incompletely autolyzed form may have exerted the proteo-lytic activity. Autolysis of m-calpain was not detected under these experimental conditions

A

225K →
190K →

NC   PC   5   10   20

Calpeptin (μM)

B

250K →

190K →

NC   PC   5   20
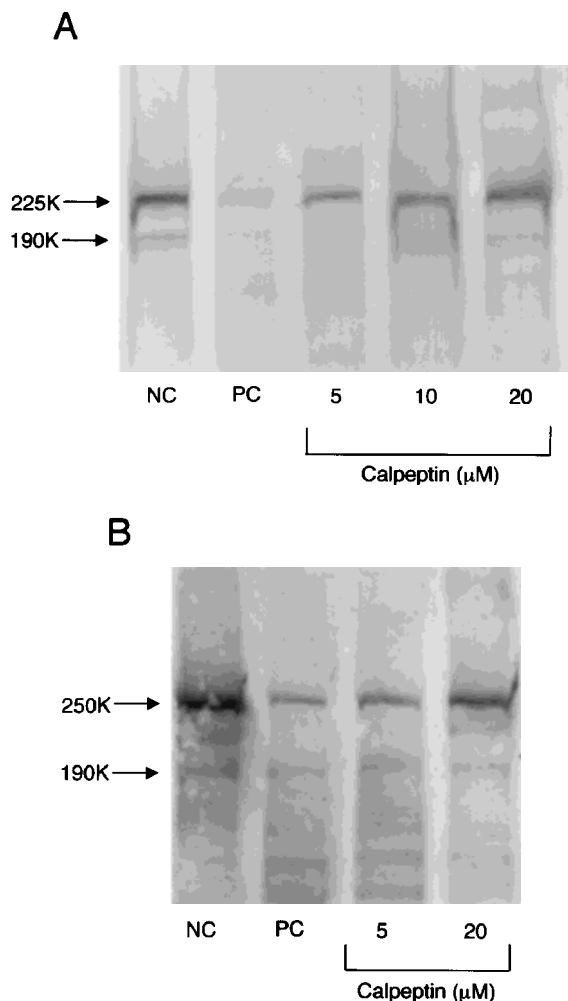
Calpeptin (μM)

Fig. 6. Effects of calpeptin on hydrolysis of talin and filamin. Endothelial cells were preincubated for 10 min at 37°C with the indicated concentration of calpeptin. Then, samples were incubated with A23187 (2 μM) for 15 min at 37°C in the presence of 2 mM $CaCl_2$ and solubilized in an SDS-containing buffer. Hydrolysis of talin (**A**) and filamin (**B**) was detected as described in the legend to Figure 5. NC (negative control) indicates cells preincubated with 0.1% dimethyl sulfoxide (calpeptin vehicle), and then stimulated with 0.2% dimethyl sulfoxide (A23187 vehicle). PC (positive control) indicates cells preincubated with 0.1% dimethyl sulfoxide, and then stimulated with A23187.

using an antibody specific to the amino-terminal portion of its post-autolysis form (data not shown).

In platelets, μ-calpain autolysis is completed within 1 min upon stimulation [Ariyoshi et al., 1993], whereas incubation for 3 min was necessary to observe the appearance of μ-calpain autolytic intermediate form in endothelial cells stimulated with A23187. It should be noted, however, that platelets are unique in that they possess calpain activity in large excess of cal-

pastatin activity [Kambayashi and Sakon, 1989] in contrast to most cells and tissues, in which calpastatin activity is usually comparable or greater [Murachi, 1983a]. This slow and incomplete progression of μ-calpain autolysis was also observed in other cell types such as epidermoid carcinoma KB cells [Nagao et al., 1994] and T-cell lymphoma Molt-4 cells [Saido et al., 1992b]. Calpain inhibitors in general decelerate the autolytic process in test tubes, resulting in the appearance of the autolytic intermediate form [Inomata et al., 1989]. Endothelial cells possess calpastatin activity in large excess of calpain activities. This slow and incomplete progression of μ-calpain autolysis was probably due, at least in part, to the excessive amount of calpastatin.

In the present study, we employed a calcium ionophore to raise $[Ca^{2+}]i$. This treatment is artificial. However, we believe that the use of ionophore certainly helped to enhance the detectability of the changes associated with calpain activation, which would otherwise proceed in a more subtle manner. $[Ca^{2+}]i$ has been shown to be significantly increased in endothelial cells stimulated by thrombin [Stasek and Garcia, 1992] and oxidants [Geeraerts et al., 1991]. Thus, efforts are being made to detect calpain activation coupled with more physiological stimuli such as oxidants.

Although the existence of the calpain-calpastatin system was clearly demonstrated and the possibility of μ-calpain involvement in endothelial cell function mediated by Ca^{2+} was suggested in the present study, its pathophysiological roles in endothelial cells remain to be clarified. However, its possible involvement in several cell functions has been postulated. For example, both m-calpain and talin are colocalized in focal contacts [Beckerle et al., 1987]. As talin is a major substrate for calpain, calpain may modulate the function of focal contacts via the cleavage of talin. In addition, calpain cleaves various adhesion molecules such as N-cadherin, NCAM (neural cell adhesion molecule) [Covault et al., 1991; Sheppard et al., 1991], α6β4 integrin [Giancotti et al., 1992], and CD 43 [Remold-O'Donnell et al., 1992]. Thus, it is possible that calpain regulates the function of endothelial cells by modifing adhesion between cells or between cells and extracellular matrix.
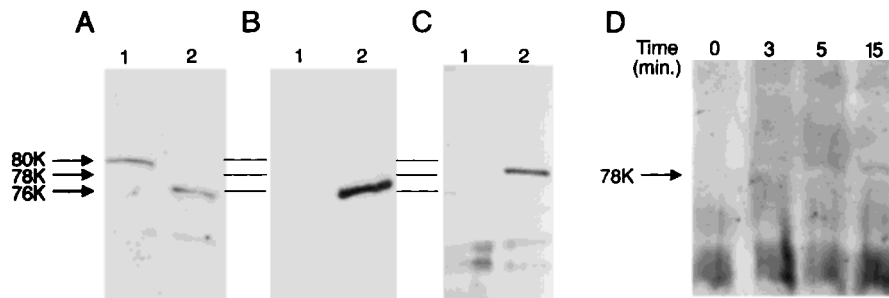
Fig. 7. Specificity of the anti-µ-calpain antibodies and capture of µ-calpain activation in endothelial cells stimulated with calcium ionophore. The autolyzed forms of µ-calpain were prepared by incubating the purified enzyme in the presence of 2 mM CaCl₂ and 25 mM β-mercaptoethanol at 4°C for 5 min. Western blot analysis of pre-autolysis form (**lane 1**) and autolyzed form (**lane 2**) of µ-calpain was performed using different types of anti-µ-calpain antibodies. (**A**) 1A8A2, (**B**) anti-ACTµ, and (**C**) antibody against autolytic intermediate form of µ-calpain. **D:** Western blot analysis of activated µ-calpain in endothe-lial cells was performed with an antibody against the autolytic intermediate form of µ-calpain. Confluent endothelial cells in T-75 flasks (2.0 × 10⁶) were incubated with A23187 (2 µM) in the presence of 2 mM CaCl₂ for the indicated time periods at 37°C. Then, endothelial cells solubilized in an SDS-containing buffer were subjected to SDS-PAGE and electroblotted onto PVDF membranes. The membranes were then immunostained with an antibody against the autolytic intermediate form of µ-calpain as described in Methods.

Talin is also considered to play an important role in linkage of actin filaments to the plasma membrane [Burridge et al., 1988]. Several other actin binding proteins (e.g., filamin and spectrin) are also known to be cleaved by calpain. Therefore, calpain may be involved in cytoskeletal reorganization of endothelial cells in some pathological conditions. Indeed, cytoskeletal reorganization of endothelial cells (e.g., loss of peripheral actin bands, increases in number and thickness of stress fibers as well as centralization of cytoplasmic stress fibers) is induced by a variety of inflammatory mediators such as histamine [Rotrosen and Gallin, 1986], α-thrombin [Garcia et al., 1986; Stasek and Garcia, 1992] and oxidants [Shasby et al., 1985; Phillips and Tsan, 1988], all of which have been shown to increase [Ca²⁺]i. As these bioactive substances induce barrier dysfunction characterized by an increase in albumin permeability and development of intrercellular gaps, the intracellular activation of calpain may be involved in endothelial barrier dysfunction via cytoskeletal reorganization. This possibility is further supported by the evidence that calpain activates both MLCK [Tsujinaka et al., 1988a] and PKC [Kishimoto et al., 1983, 1989] by limited proteolysis, the activation of which is a prerequisite for the increase in endothelial permeability [Geeraerts et al., 1991; Wysolmerski and Lagunoff, 1990; Lynch et al., 1990].

Ischemic injury can cause enhanced capillary permeability, morphological changes in endothelial cells and finally tissue destruction [Mc-Cord, 1985]. Recently, non-lysosomal proteolysis has been implicated in ischemic lethal cell injury. An increase in non-lysosomal proteolysis was identified in hepatocytes during anoxia and the close relationship between cell death and non-lysosomal proteolysis by a calpain-like protease was demonstrated [Bronk and Gores, 1993]. In other tissues such as neurons and myocardia, calpain has also been implicated in induction of ischemic lethal cell injury [Lee et al., 1991; Saido et al., 1993b; Tolnai and Korecky, 1986]. Geeraerts et al. also reported that the prevention of the increase in [Ca²⁺]i and the concomitant proteolysis resulted in prolonged protection of endothelial cell viability from oxidative injury [Geeraerts et al., 1991]. These findings suggest the possible involvement of calpain activation in lethal anoxic changes in endothelial cells.

Although these possibilities are totally speculative at present, our newly developed antibody capable of specifically recognizing the autolytic intermediate form of µ-calpain will provide a novel approach to assess its pathophysiological roles by determinating the physiological targets of the enzyme and the local intracellular sites of µ-calpain activation.

In conclusion, the calpain-calpastatin system was identified in endothelial cells at both protein and mRNA levels, and elevation of [Ca²⁺]i resulted in slow and incomplete autolysis of µ-calpain, accompanied by cytoskeletal proteolysis. These findings suggest the µ-calpain involvement in endothelial cell function mediated

by Ca^{2+} via the limited proteolysis of various proteins, although its pathophysiological roles in endothelial cell function remain to be elucidated.

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