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Possible mechanism for the decrease of mitochondrial aspartate aminotransferase activity in ischemic and hypoxic rat retinas

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Abstract

Glutamate is believed to be an excitatory amino acid neurotransmitter in the retina. Enzymes for glutamate metabolism, such as glutamate dehydrogenase, ornithine aminotransferase, glutaminase, and aspartate aminotransferase (AAT), exist mainly in the mitochondria. The abnormal increase of intracellular calcium ions in ischemic retinal cells may cause an influx of calcium ions into the mitochondria, subsequently affecting various mitochondrial enzyme activities through the activity of mitochondrial calpain. As AAT has the highest level of activity among enzymes involved in glutamate metabolism, we investigated the change of AAT activity in ischemic and hypoxic rat retinas and the protection against such activity by calpain inhibitors. We used normal RCS (rdy^+/rdy^+) rats. For the in vivo studies, we clamped the optic nerve of anesthetized rats to induce ischemia. In the in vitro studies, the eye cups were incubated with Locke's solution saturated with 95% N_2/5% CO_2. The activity of cytosolic AAT (cAAT) was about 20% of total activity, whereas mitochondrial AAT (mAAT) was about 75% in rat retina. Ninety minutes of ischemia or hypoxia caused a 20% decrease in mAAT activity, whereas cAAT activity remained unchanged. To examine the contribution of intracellular calcium ions to the degradation of mAAT, we used Ca^{2+}-free Locke's solution containing 1 mM EGTA, ryanodine (Ca^{2+} channel blocker), and thapsigargin (Ca^{2+}-ATPase inhibitor). In the present study, thapsigargin in Ca^{2+}-free Locke's solution, but not ryanodine in this solution, was found to prevent AAT degradation. AAT degradation was also prevented by calpain inhibitors (Ca^{2+}-dependent protease inhibitor) such as calpeptin at 1 nM, 10 nM, 0.1 μM, 1 μM and 10 μM, and by calpain inhibitor peptide, but not by other protease inhibitors (10 μM leupeptin, pepstatin, chymostatin). Additionally, we determined the subcellular localization of calpain activity and examined the change of calpain activity in ischemic rat retinas. Our results suggest that decreased activity of mAAT in ischemic and hypoxic rat retinas might be evoked by the degradation by calpain-catalyzed proteolysis in mitochondria.

Keywords: Ischemia; Aspartate aminotransferase; Mitochondrion; Calcium ion; Calpain

1. Introduction

Considerable evidence indicates that glutamate plays an excitatory neurotransmitter role for glutamate in the mammalian central nervous system [1–3], stimulating glutamate receptors to increase the permeability of the cell membrane. The released glutamate from presynaptic nerve terminals is taken up by Müller cells and converted to glutamine by glutamine synthetase. Glutamine is released by Müller cells and is taken up by neurons for deamination to glutamate by phosphate-activated glutaminase [4]. This process

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is generally known as the glutamine cycle. Because glutamate does not readily cross the blood-brain barrier, it must be synthesized within the central nervous system. It is widely assumed that neurons synthesize glutamate themselves, using aspartate or precursors derived from the Krebs cycle [5]. Aspartate aminotransferase (AAT) catalyzes the interconversion of glutamate and oxaloacetate with aspartate and α-ketoglutarate.

Ischemia reportedly produces a massive efflux of glutamate. Glutamate couples with NMDA glutamate receptors. It is generally agreed that calcium conductance linked to the NMDA receptor ionophore is responsible for both the plasticity and pathology triggered by the receptor [6–8]. Most previous studies have shown that intracellular calcium ions play a fundamental role in regulating numerous enzyme activities and mediating the effects of hormones and growth factors that control a wide variety of cellular processes, such as metabolism, cell differentiation, and secretion. The loss of Ca^{2+} homeostasis due to excess Ca^{2+} influx through voltage-dependent channels and NMDA receptor-operated channels and the decrease of Ca^{2+} pumping resulting from ATP depletion and due to the release of Ca^{2+} from intracellular stores are believed to result in ischemic damage. Loss of Ca^{2+} homeostasis may activate Ca^{2+}-dependent enzymes such as proteases, phospholipases [9], and endonucleases, leading to mitochondrial damage and cell death resulting from energy failure.

In the present study, we investigated the change of AAT activity in ischemic and hypoxic rat retinas. AAT has the highest activity among the enzymes for glutamate metabolism [10–17]. The distribution of AAT activity in retinal layers has been reported predominantly in the outer plexiform layer and photoreceptor inner segments [18,19]. The outer plexiform layer contains photoreceptor terminals and bipolar and horizontal cell processes. AAT might play an important role as a neurotransmitter such as that of glutamate in the outer plexiform layer [20,21], although other functions, such as retinal energy metabolism, are possible. The correlation between the distributions of AAT and malate dehydrogenase (MDH), an enzyme of aerobic metabolism [18,19,22], is consistent with AAT involvement in retinal energy metabolism. We also examined the contribution of calcium ions to the change of mitochondrial AAT (mAAT), using EGTA, ryanodine (Ca^{2+} channel blocker) and thapsigargin (Ca^{2+}-ATPase inhibitor). In addition, we assayed Ca^{2+}-dependent protease (calpain) activities of each retinal fraction under conditions of ischemia and examined the protective effects against decreased mAAT activity using Ca^{2+}-dependent protease inhibitors (calpain inhibitor peptide) and other protease inhibitors.

2. Materials and methods

2.1. Preparation of samples

Normal RCS (rdy^+/rdy^+) rats [23] weighing 200–250 g were used. After administering intramuscular anesthesia, we clamped the left optic nerve with an aneurysm clip (Mizuho Co. Ltd., Tokyo, Japan) to stop the blood flow in retinal vessels. The right eyes were used as sham-operated controls. After 30, 60 or 90 min of clamping, both eyes were enucleated and the retinas were separated. Interruption of blood flow was confirmed ophthalmoscopically, and ammonia concentrations were measured. In the retina, it is thought that ammonia is mainly converted to glutamine by glutamine synthetase (GS) in retinal Müller cells. GS, however, does not work efficiently during ischemia, since this enzyme requires ATP for glutamine formation. We therefore also measured the ammonia concentration of retinas as an indicator of ischemia.

The following steps were carried out at 4°C. The rat retina was homogenized with 500 μl of 0.25 M sucrose using a glass-Teflon homogenizer. The homogenate was dialyzed with distilled water for 60 min to remove endogenous disturbing substances.

To examine the distribution of AAT in the retina, we obtained a nuclear fraction, a mitochondrial fraction, a lysosomal fraction, and a microsomal fraction by differential centrifugation at $600\times g$ for 5 min, $4500\times g$ for 10 min, $20\,000\times g$ for 20 min, and $100\,000\times g$ for 60 min, respectively. These were washed three times by suspending in fresh 0.25 M sucrose and resedimenting under the same conditions. The supernatant obtained was used as a soluble fraction. Each pellet was resuspended in 500 μl of 0.25 M sucrose. The AAT activity was found mainly

in mitochondrial and soluble fractions. The supernatant contained mainly cytosolic AAT (cAAT).

To separate the soluble mAAT, the pellet of the mAAT fraction was disrupted by four freeze-thaw cycles. The sample was centrifuged at $100\,000 \times g$ for 60 min. The supernatant obtained was used as the soluble mitochondrial fraction, and the pellet obtained was used as the membrane-bound mitochondrial fraction. mAAT activity was found in the membrane-bound mitochondrial fraction.

For in vitro experiments, normal RCS rat eyes were enucleated following intramuscular anesthesia. We removed the cornea, lens, and vitreous, and we obtained the eye cups. The eye cups were incubated at 37°C in Locke's solution (Na^+ 157 mM, Ca^{2+} 2.3 mM, Cl^- 164.2 mM, HCO_3^- 3.6 mM, HEPES 5 mM, pH 7.2) and saturated with 95% O_2/5% CO_2 for 30, 60, or 90 min as control samples. We used glucose-free Locke's solution saturated with 95% N_2/5% CO_2 for 30, 60, or 90 min as hypoxic samples. To obtain samples for measuring mAAT and cAAT activities, we used the same methods as described above.

2.2. Effects of EGTA, ryanodine, and thapsigargin on mAAT activity in hypoxic rat retinas

To examine the protective effects of EGTA, ryanodine (Ca^{2+} channel blocker), and thapsigargin (Ca^{2+}-ATPase inhibitor) on AAT activity, eye cups were incubated at 37°C for 90 min in Ca^{2+}-free Locke's solution with 1 mM EGTA in Locke's solution with 1 nM, 100 nM, or 10 μM ryanodine and in Locke's solution with 250 nM or 500 nM thapsigargin.

2.3. Effects of protease inhibitors on mAAT activity of hypoxic rat retinas

To examine the protective effects of Ca^{2+}-dependent protease inhibitors (calpain inhibitor peptide, calpeptin) and other protease inhibitors (pepstatin, leupeptin, chymostatin) on AAT activity, the eye cups under hypoxic condition were incubated in Locke's solution with 1 nM, 10 nM, 0.1 μM, 1 μM, or 10 μM calpain inhibitor peptide (RBI, Natick, MA, USA) and calpeptin (TOCRIS, Bristol, UK), and 10 μM pepstatin, leupeptin, or chymostatin (Peptide Institute, Inc., Osaka, Japan).

2.4. Assay of aspartate aminotransferase activity

Activity of AAT was assayed according to the modified method of Godfrey et al. [24,25]. After 60 min of dialysis, 20 μl (34 μg protein) of samples was added to a 30 μl reaction mixture containing 60 mM imidazole buffer, pH 7.4, 4 mM α-ketoglutarate, 0.7 g/l bovine serum albumin, 1.4 ml/l Triton X-100, and 1.4 mg/l MDH. Each tube was sonicated for 1 min on the ice, and the reaction was started by adding 10 μl of substrate solution containing of 160 mM L-aspartate and 1.44 mM NADH. Samples were incubated at 37°C for 2 min, then the reaction was stopped by adding 40 μl of 0.7 M HCl. After 20 min required to destroy the remaining NADH in the acid solution, 1 ml indicator solution containing 95 mM Tris-HCl buffer, pH 8.5, 860 mM ethanol, 2 mM mercaptoethanol and 11.6 mg/l alcohol dehydrogenase were added to each sample. The NAD^+ was converted to NADH in 30 min. The intensity of fluorescence of each sample was measured with a fluorophotometer (excitation: 340 nm; emission: 460 nm).

Protein concentration was measured by the method of Lowry et al. [26].

2.5. Assay of ammonia concentration

Ten microliters of 7% trichloroacetic acid (TCA) was immediately added to 60 μl of the homogenized solution of the fresh retina. After standing at 4°C for 15 min, the samples were centrifuged at 10 000 rpm for 3 min at 4°C. Distilled water (70 μl) was added to 30 μl of the supernatant. The ammonia concentration was measured by adding 2.9 ml of *o*-phthaldialdehyde/mercaptoethanol reagent. The mixtures were kept in the dark at room temperature for 45 min and then intensities of fluorescence were measured with a fluorophotometer (excitation: 410 nm; emission: 470 nm) [27]. The optical density was converted to ammonia concentration using the NH_4Cl standard curve.

2.6. Antibody preparation

AAT antibody was prepared by the modified method of Altschuler et al. [28]. Commercially available AAT (Boehringer Mannheim) was electropho-

resed on sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) [29]. Of these, a component of about 45 000 Da is the major band and has been identified as the monomeric subunit of AAT [30]. The 45 000 Da component was separated from the other bands and collected by electrophoresis. Re-electrophoresis of this band showed a single major component at 45 000 Da. Antibodies were raised against the prepared isolated 45 000 Da component, using two New Zealand white rabbits. The prepared isolated 45 000 Da component was emulsified in Freund's adjuvant and injected subcutaneously into the rabbits' necks. The antisera were collected and characterized. The collected IgG solution was purified by an affinity column (HiTrap NHS-activated, Amersham Pharmacia Biotech, Bucks, UK). We obtained the two bands by immunoblotting of crude rat retina homogenate (including mitochondrial and soluble fractions) using rabbit antisera against AAT (the 45 000 Da subunit). We also used rabbit antisera against rat liver mAAT (generous gift from Dr. Horio), which have been produced and characterized elsewhere [31,32]. By the immunoblotting of rat retina homogenate (only mitochondrial fraction), one band was obtained with the rabbit antisera against rat liver mAAT.

2.7. Electrophoresis of AAT and immunoblotting

Gel electrophoresis in the presence of SDS was performed according to the method of Laemmli [29] on a 10% polyacrylamide gel. The crude rat retinal homogenates, mitochondrial fraction, and cytosolic fraction (10 μl sample/lane, 170 μg of protein/lane) were applied on the gel. Electrophoresis was carried out for 1 h at 100 V. The ischemic samples (90 min clamping) were analyzed in the same way. Western blotting was performed according to Towbin et al. [33]. After electrophoretic transfer of proteins to nitrocellulose membranes (Cellulose Nitrate, Advantec, Toyo Roshi Kaisha Ltd.), the membranes were treated with 3% gelatin and 5% skim milk in PBS for 1 h at 37°C. We used 1:2000 diluted rabbit antisera against AAT (the 45 000 Da subunit) and 1:1000 diluted rabbit antisera against rat liver mAAT as the first antibody and peroxidase-conjugated goat anti-rat IgG antibody as the second antibody. The antibodies were diluted with phosphate-buffered saline (0.14 M NaCl and 10 mM phosphate buffer, pH 7.4) containing 1% bovine serum albumin and 0.05% Tween 20.

Each incubation time was 1 h at 37°C. Washing was performed after each step with phosphate-buffered saline containing 0.05% Tween 20.

2.8. Assay of calpain activity

Calpain activity was assayed using Cell Probe LY Calpain Enzyme Substrate as substrate (Coulter, Florida, USA). One unit calpain (Sigma Co., St. Louis, MO, USA) produces an A_{280} of 0.5 in 30 min at pH 7.5 at 30°C, measured as TCA-soluble products using *N*,*N*-dimethylated casein as substrate. Cell Probe LY Calpain Enzyme Substrate is cleaved by calpain to form a fluorescent product, rhodamine 110, which can be measured by fluorescence quantification techniques. The intensity of fluorescence of each sample was measured with a fluorophotometer (excitation: 499 nm; emission: 521 nm). To examine the initial rate of relative fluorescence intensity changes as functions of calpain concentration and incubation time, 50 μl of Cell Probe LY Calpain Enzyme Substrate was incubated with various amounts (0.2–0.5 U) of calpain for various incubation times (0, 5, 10, 15, 20 and 30 min). We examined the Ca^{2+} requirement of calpain for the hydrolysis of Cell Probe LY Calpain Enzyme Substrate by assaying calpain hydrolytic activity at various Ca^{2+} concentrations using the Ca^{2+}-EGTA buffer system. Samples used were a crude retinal homogenate, a nuclear fraction, a mitochondrial fraction, a lysosomal fraction, a microsomal fraction and a soluble fraction. These samples were obtained by dialysis, differential centrifugation, and by three times washing, using the same methods as described above. Fifty microliters of each sample (17 μg of protein) was incubated with Cell Probe LY Calpain Enzyme Substrate at 37°C. After 15 min of incubation, 1 ml of ice-cold PBS was added to stop the reaction, and then the samples were kept on ice for 20 min. The resulting fluorescence from the hydrolysis of the Cell Probe LY Calpain Enzyme Substrate provides a measure of the enzyme activity within a sample. Calpain activity was defined as the difference in proteolytic activity between assays in the presence of $CaCl_2$ or EGTA. To control the free calcium, each sample fraction was resuspended in 500 μl of 0.25 M sucrose

containing 50 mM Tris-HCl, pH 7.5, 1 mM EGTA or varying amounts of $CaCl_2$.

To confirm the contamination of the soluble fraction to a mitochondrial fraction, Western blot analysis using a soluble fraction as a marker was performed. Each sample, containing approximately 170 μg of protein/lane, was electrophoresed on 10% SDS polyacrylamide gels and transferred to a nitrocellulose membrane. Western blot analysis was performed using the rabbit antisera against AAT (45 000 Da subunit).

2.9. Immunoprecipitation of calpain activity by monoclonal antibody to m-calpain

The monoclonal antibody to *m*-calpain (Alexis Co., Switzerland) (0.1–10 μg) and the monoclonal antibody to glial fibrillary acidic protein (GFAP) (Boehringer Mannheim, Germany) (0.1–10 μg) were incubated with 5 μl (8.5 μg) sample of sonicated and dialyzed mitochondrial fractions in a total volume of 60 μl of 0.25 M sucrose containing 50 mM Tris-HCl, pH 7.5, 5 mM $CaCl_2$. After 2 h at 4°C, excess goat anti-mouse IgG (Southern Biotechnology Associates Inc., USA) was added (40 μl) and incubated for 18 h at 4°C. The samples were centrifuged at 12 000 rpm for 10 min at 4°C. Fifty microliters of the supernatant was used for assay of calpain activity as described above.

3. Results

AAT activity was found to be present in mitochondrial and cytosolic fractions by differential centrifugation. Specific activity of crude retinal extract was 780 ± 8.0 (mean ± S.D., $n = 20$) nmol/min/mg protein. The mAAT and cAAT activities were 585 ± 6.5 and 170 ± 7.5 ($n = 20$) nmol/min/mg crude retinal protein, respectively. mAAT was 75% of total retinal AAT activity. Centrifugation at $100\,000 \times g$ for 60 min after four freeze-thaw cycles resulted in mAAT activity only in the membrane-bound mitochondrial fraction.

mAAT and cAAT activities in ischemic rat retinas are shown in Fig. 1. Ninety minutes of ischemia caused a 20% decrease in mAAT activity, whereas cAAT activity remained unchanged. Ischemia for

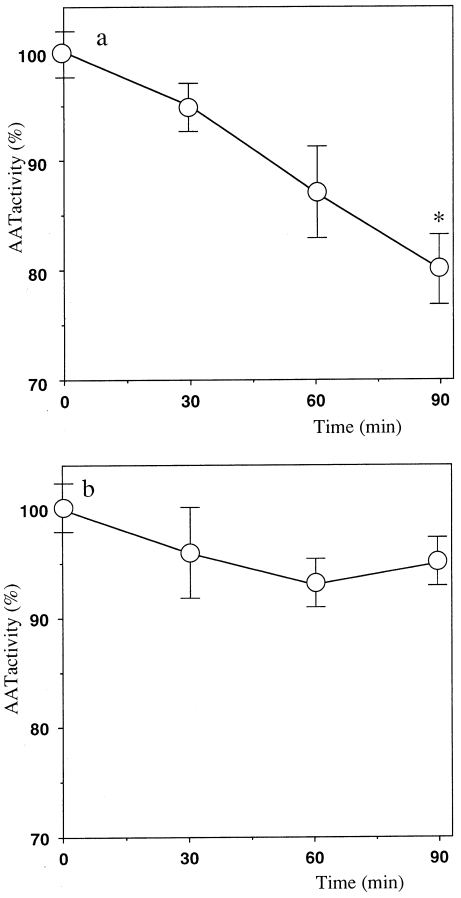

Fig. 1. The activity of AAT under ischemic conditions (in vivo). The optic nerve was clamped to stop the blood flow in retinal vessels for different time periods. After 30, 60, or 90 min of clamping, rat retinas were separated for immediate analysis. Details are described in Section 2. (a) Change of mAAT activity under ischemic conditions (○). As a control, the right eyes were sham-operated and enucleated after 30, 60, or 90 min. (b) Change of cAAT activity under ischemic conditions (○). As a control, the right eyes were sham-operated. Each datum point shows mean ± S.D. ($n = 4$). Ninety minutes of ischemia decreased mAAT activity significantly to 80% (*t*-test, $*P < 0.01$), whereas cAAT activity remained unchanged.

30, 60, or 90 min of ischemia produced elevated ammonia concentrations in rat retinas (30 min of ischemia: control 6.56 ± 0.31; ischemia, 6.78 ± 0.33 nmol/mg crude retinal protein; 60 min of ischemia, control, 6.46 ± 0.51; ischemia, 9.01 ± 0.55 nmol/mg crude retinal protein; 90 min of ischemia, control, 6.63 ± 0.23; ischemia, 11.4 ± 0.43 nmol/mg crude retinal protein). A similar decrease in AAT activity was observed in vitro after 90 min of hypoxia. As shown in Fig. 2, 90 min of hypoxia caused a 20% decrease

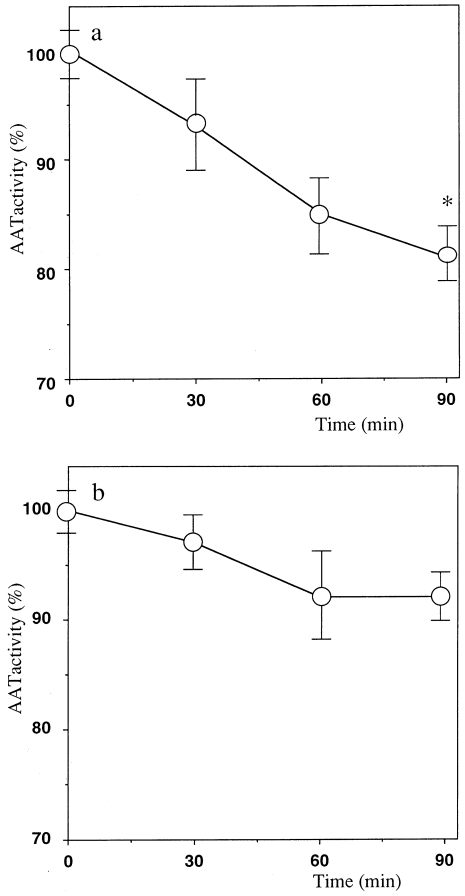

Fig. 2. The activity of AAT under hypoxic conditions (in vitro). The eye cup was incubated in Locke's solution saturated with 95% N_2/5% CO_2 for 30, 60, or 90 min. (a) Change of mAAT activity under hypoxic conditions (○). As a control, the eye cup was incubated in Locke's solution saturated with 95% O_2/5% CO_2 for 30, 60, or 90 min. (b) Change of cAAT activity under hypoxic conditions (○). As a control, the eye cup was incubated in Locke's solution saturated with 95% O_2/5% CO_2 for 30, 60, or 90 min. Each datum point shows mean ± S.D. ($n=4$). Ninety minutes of hypoxia decreased membrane-bound mAAT significantly to 80% (t-test, $^*P<0.01$), whereas cAAT activity remained unchanged.

in mAAT activity. The protective effects of EGTA, ryanodine, and thapsigargin on retinal mAAT were examined. The percentage of protection against decreased mAAT activity in hypoxic rat retinas is shown in Fig. 3. Decreased mAAT activity was 90% effectively prevented by the addition of 1 mM EGTA and 250 or 500 nM thapsigargin, but not by the addition of ryanodine (1 nM to 10 μM).

The protective effects of several protease inhibitors (pepstatin, leupeptin, chymostatin, calpain inhibitor peptide, calpeptin) on retinal mAAT were examined. The percentage of protection against decreased mAAT activity in hypoxic rat retinas is shown in Fig. 4. Decreased mAAT activity was approximately 85% prevented by the addition of calpain inhibitor peptide and calpeptin. The concentrations of calpain inhibitor peptide and calpeptin for 50% protection (EC_{50}) of mAAT activity were about 200 nM and 150 nM, respectively. Each EC_{50} was higher than each IC_{50} (100 nM, 30–50 nM) [34–38].

mAAT and cAAT were stained by immunoblots using the rabbit antisera against AAT (the 45 000 Da subunit). The upper band was cAAT and the lower band was mAAT (mAAT had a molecular weight of 44 500 and cAAT had a molecular weight of 46 000). The mAAT band was also detected by rabbit antisera against rat liver mAAT. The density of the mAAT band under hypoxic condition was lower than that of the control sample mAAT, but that of the cAAT band did not change (Fig. 5).

To confirm the presence of calpain in the retinal mitochondria, we measured the calpain activities in the subcellular fractions. Hydrolysis of the Cell

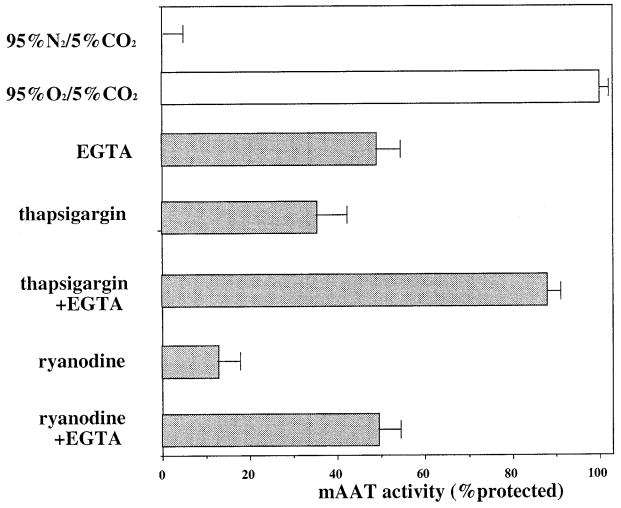

Fig. 3. The effects of EGTA, ryanodine, and thapsigargin on retinal mAAT activity under hypoxic conditions in vitro. The concentrations of reagent used were 1 mM EGTA, 1 nM, 100 nM and 10 μM ryanodine, and 500 nM thapsigargin. The reagents were added to Locke's solution saturated with 95% N_2/5% CO_2. One hundred percent protection represented no degradation of the enzyme. Each datum point shows mean ± S.D. ($n=4$). Treatment with 1 mM EGTA and 500 nM thapsigargin protected against enzyme activity, but ryanodine (1 nM to 100 μM) did not.

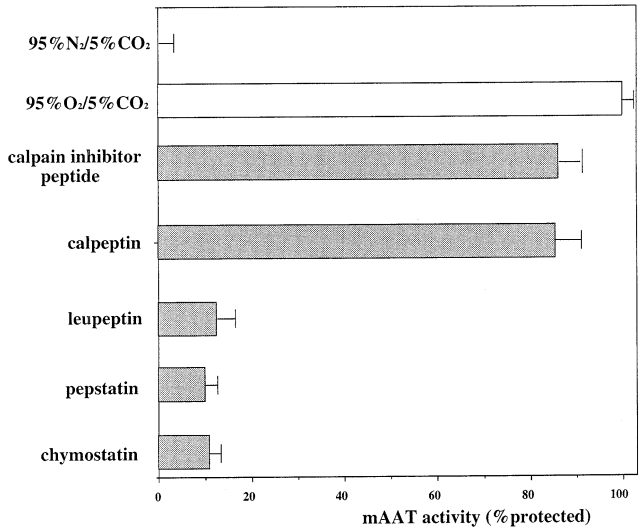

Fig. 4. The effects of protease inhibitors on retinal mAAT activity under hypoxic conditions in vitro. The concentrations of reagents used were 10 μM pepstatin; 10 μM leupeptin; 10 μM chymostatin; 1 nM, 10 nM, 0.1 μM, 1 μM, and 10 μM calpain inhibitor peptide and calpeptin.The reagents were added to Locke's solution saturated with 95% N_2/5% CO_2. One hundred percent protection represented no degradation of the enzyme. Each datum point shows mean ± S.D. ($n=4$). Treatment with calcium-activated neutral protease inhibitors (calpain inhibitor peptide and calpeptin) specifically protected against enzyme activity.

Probe LY Calpain Enzyme Substrate was found to be linear with calpain concentration (0.2–0.5 U) (Fig. 6). After 20 min incubation, the relative fluorescence reached a plateau (Fig. 7). Five millimolar or more calcium ion was required for maximum reaction of calpain (Fig. 8). Total retinal calpain activity was 145 ± 7.5 (mean ± S.D., $n=20$) U/h/mg protein. The amounts of calpain activity found in the soluble fraction (60%), the mitochondrial fraction (30%) and the microsomal fraction (10%) are shown in Fig. 9. To confirm the contamination of cytosolic cAAT to a mitochondrial fraction, Western blot analysis of AATs using a soluble fraction and mitochondrial fraction was performed. We used rabbit antisera

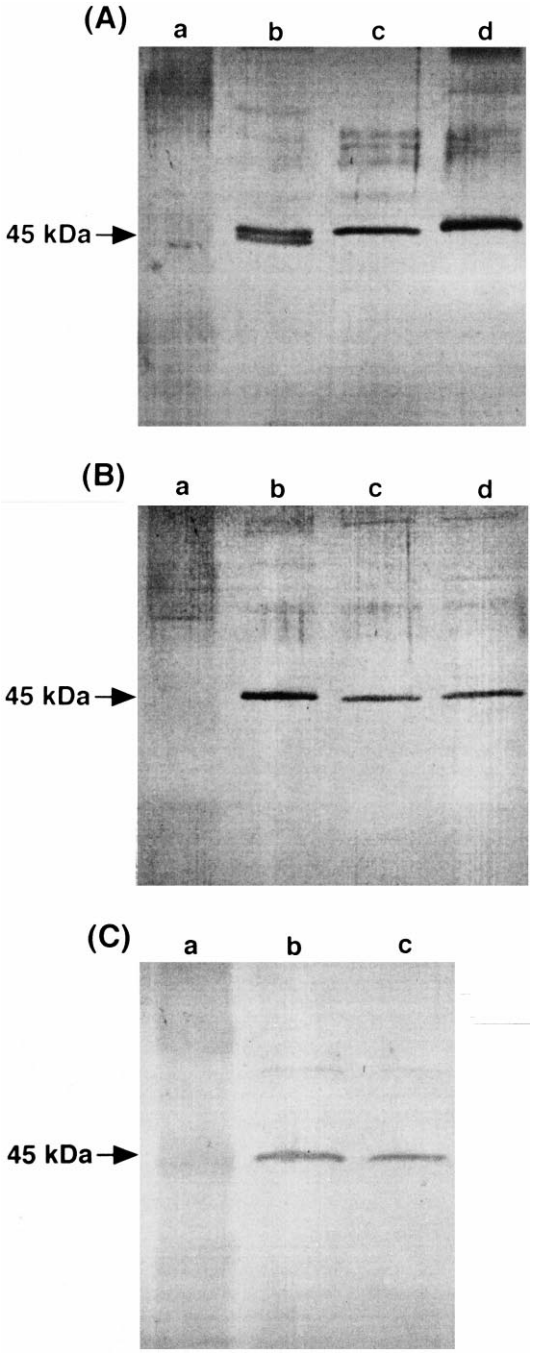

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Fig. 5. Western blot analysis of AAT in rat retina. Crude rat retinal homogenate, the soluble fraction and the mitochondrial fraction, each containing approximately 170 μg of protein/lane, were electrophoresed on 10% SDS polyacrylamide gels and transferred to a nitrocellulose membrane. Western blot analysis was performed using anti-AAT antisera. (A) Immunostaining using the rabbit antisera against AAT (45 000 Da subunit). Lane a, molecular weight standards; lane b, crude retinal homogenate; lane c, soluble fraction; lane d, commercial AAT. (B) Immunostaining using the rabbit antisera against AAT (45 000 Da subunit). Lane a, molecular weight standards; lane b, soluble fraction (control); lane c, soluble fraction (90 min. hypoxia). (C) Immunostaining using the rabbit antisera against rat liver mAAT. Lane a, molecular weight standerds; lane b, mitochondrial fraction (control); lane c, mitochondrial fraction (90 min hypoxia).

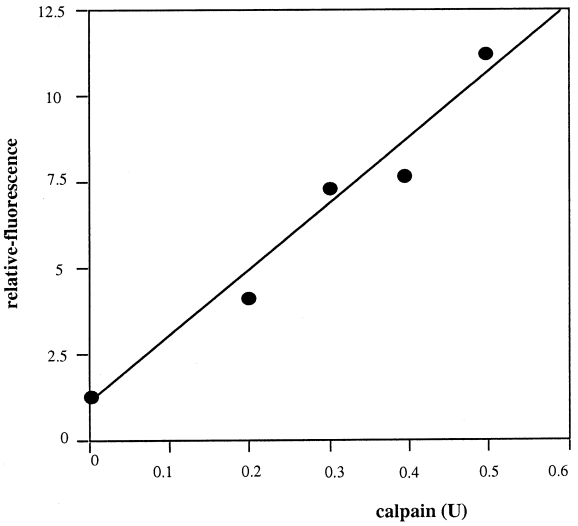

Fig. 6. Determination of the initial rate of relative fluorescence intensity change. Fifty microliters of Cell Probe LY Calpain Enzyme Substrate was incubated with various amounts (0.2–0.5 U) of calpain in the standard kinetic assay, and the initial rate of fluorescence intensity change was determined and plotted as a function of calpain concentration. One unit calpain (Sigma, St. Louis, MO, USA) produces an A_{280} of 0.5 in 30 min at pH 7.5 at 30°C, measured as TCA-soluble products using *N*,*N*-dimethylated casein as substrate.

against AAT (the 45 000 Da subunit), which recognize both mAAT and cAAT. We could obtain only one band of mAAT from a mitochondrial fraction, and the possibility of cytosolic contamination to mitochondrial fractions was eliminated (Fig. 10).

Immunoprecipitation of calpain activity in a mitochondrial fraction was carried out by the monoclonal antibody to *m*-calpain. The monoclonal antibody to GFAP was used as a control. Fig. 11 shows the results of the precipitation. Approximately 80% of the calpain activity was precipitated by the monoclonal antibody to *m*-calpain. The result indicates that our Ca^{2+}-dependent activity is mainly attributed to *m*-calpain activity.

After 90 min of clamping, mitochondrial calpain activity showed a 2.5-fold increase, but cytosolic calpain activity decreased to 60% compared with each activity (Fig. 12).

4. Discussion

It is widely accepted that glutamate acts as a neurotransmitter in the central nervous system [1–3].

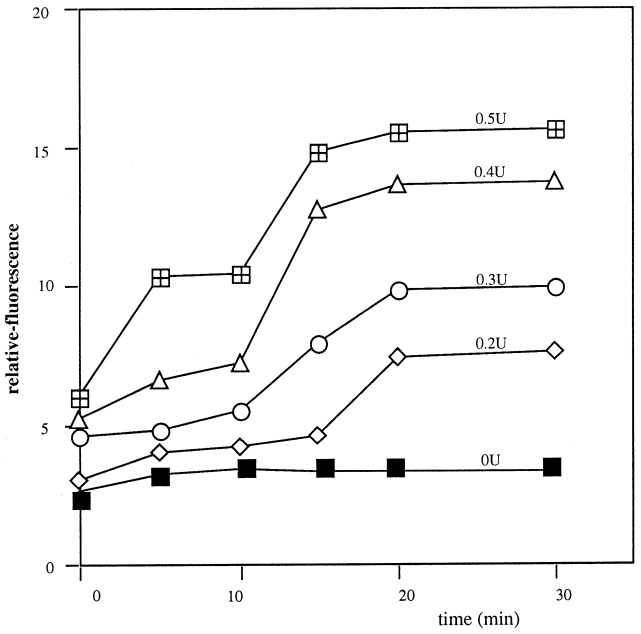

Fig. 7. Initial rate of relative fluorescence intensity change as a function of calpain concentration and incubation time. Fifty microliters of Cell Probe LY Calpain Enzyme Substrate was incubated with various amounts (0.2–0.5 U) of calpain. After incubation for 0, 5, 10, 15, 20 and 30 min, the reaction was stopped by adding 1 ml ice-cold PBS for 20 min. Then the fluorescence intensity was measured and normalized to the values obtained with identically treated samples, without calpain. Datum points represent mean ± S.D. for four experiments.

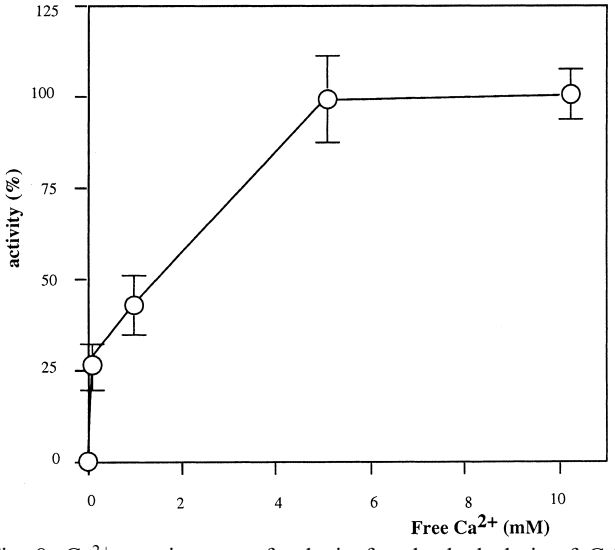

Fig. 8. Ca^{2+} requirement of calpain for the hydrolysis of Cell Probe LY Calpain Enzyme Substrate. Assays of calpain hydrolytic activity were performed at various Ca^{2+} concentrations using the Ca^{2+}-EGTA buffer system.

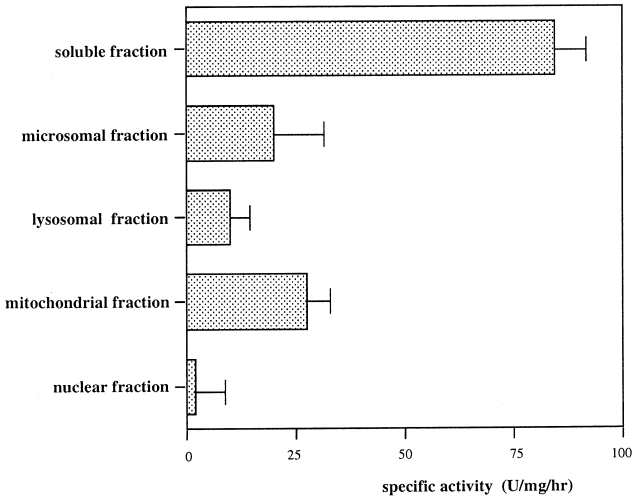

Fig. 9. Subcellular distribution of calpain activity in rat retina. Details are described in Section 2. To examine the distribution of calpain in the retina, we obtained the nuclear fraction, the mitochondrial fraction, the lysosomal fraction, and the microsomal fraction by differential centrifugation. Total retinal calpain activity was 145 ± 7.5 (mean ± S.D., $n = 20$) U/h/mg crude retinal protein. The amount of calpain activity found in the soluble fraction was 60%, that in the mitochondrial fraction was 30%, and that in the microsomal fraction was 10%.

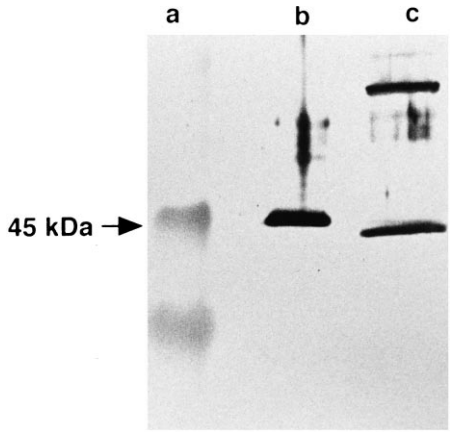

Fig. 10. Western blot analysis of AAT in rat retina. To confirm the contamination of cytosolic cAAT to mitochondrial fraction, Western blot analysis was performed. We used rabbit antisera against AAT (the 45 000 Da subunit), which recognize both mAAT and cAAT. Lane a, molecular weight standards; lane b, soluble fraction; lane c, mitochondrial fraction. We could obtain only one band of mAAT from the mitochondrial fraction, and the possibility of cytosolic contamination to mitochondrial fractions was eliminated.

AAT has the highest activity among the enzymes for glutamate metabolism in the rat retina. The present study showed that mAAT activity decreased under ischemic or hypoxic conditions. Such a decrease of mAAT activity would mean serious injury for retinal functions of metabolism and neurotransmission. Elucidation of the mechanism of this decrease might be helpful for understanding the regulation of AAT in the retina and its contribution to alleviation of ischemic injury.

In the present study, the mAAT degradation under hypoxic conditions was decreased by about 90% in the presence of the endoplasmic reticulum Ca^{2+}-ATPase inhibitor, thapsigargin in Ca^{2+}-free Locke's solution with EGTA, but not by ryanodine. These results indicate that decreased mAAT activity may be Ca^{2+}-dependent. It is widely believed that intracellular Ca^{2+} plays a fundamental role in the regulation of enzyme activation. Under ischemic conditions, the intracellular calcium level is elevated through voltage-dependent and NMDA receptor-operated channels. A decrease of Ca^{2+} pumping results from ATP depletion and the release of Ca^{2+} from intracellular stores [6–8]. Most previous studies have shown ele-

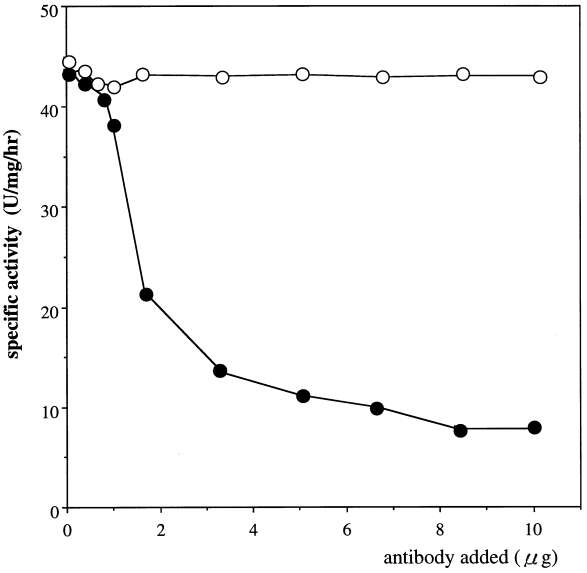

Fig. 11. Immunoprecipitation of calpain activity by monoclonal antibody to *m*-calpain. The monoclonal antibodies (0.1–10 μg) to *m*-calpain or GFAP were incubated with 5 μl (8.5 μg) of sonicated and dialyzed mitochondrial fractions. When a precipitation had formed, these samples were centrifuged at 12 000 rpm for 10 min at 4°C. Fifty microliters of the supernatant obtained was used for assay of calpain activity as described in Section 2. Approximately 80% of the calpain activity was precipitated by the monoclonal antibody to *m*-calpain (●). The monoclonal antibody to GFAP was used as a control (○).

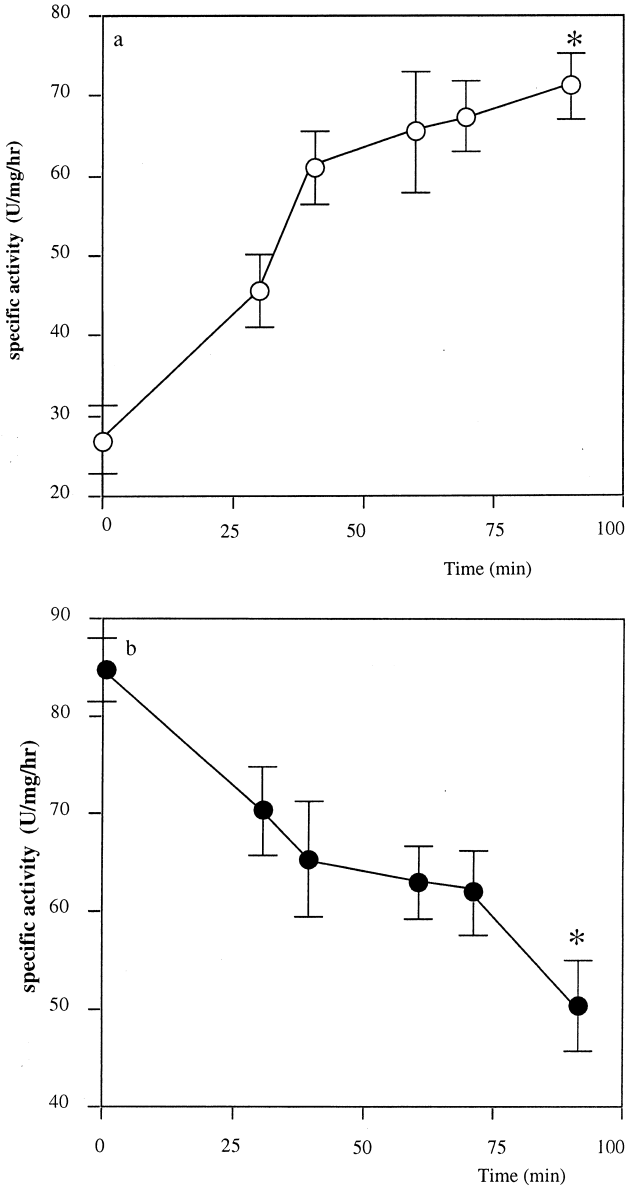

Fig. 12. Activity of calpain in hypoxic rat retina. After 90 min of clamping, rat retinas were separated for immediate analysis. Details are described in Section 2. (a) Change of mitochondrial calpain activities under hypoxic conditions (○). (b) Change of cytosolic calpain activities under ischemic conditions (●). Ninety minutes of ischemia mitochondrial calpain activity resulted in a 2.5-fold increase (t-test, $*P < 0.05$), whereas cytosolic calpain activity decreased significantly to 60% (t-test, $*P < 0.05$).

vated levels of intracellular Ca^{2+} under ischemic conditions. This loss of Ca^{2+} homeostasis then triggers cellular events that lead to cell damage and death. The intracellular calcium stores from which calcium is released contain three major components: pumps which sequester calcium, binding proteins (such as calsequestrin and calreticulin) which store calcium, and specific inositol (1,4,5)-triphosphate receptor (IP_3R) or ryanodine receptor (RyR) channels which release calcium back into the cytosol. Ryanodine releases Ca^{2+} through the RyR, which is negatively regulated by calcium-calmodulin (Ca-CaM). RyR also responds to an increase in the intracellular concentration of Ca^{2+}, thereby mediating Ca^{2+}-induced Ca^{2+} release (CICR) [39]. These receptors are normally located on modified portions of the endoplasmic reticulum. The distribution of such IP_3-sensitive or ryanodine-sensitive calcium stores varies considerably among cells. Some cells have either ryanodine-sensitive stores or IP_3-sensitive stores, whereas others contain both [40,41]. Little is known as yet about the exact localization and mechanism of Ca^{2+} stores in retinal cells [42,43]. In this study, under hypoxic conditions, it was suggested that the release of Ca^{2+} may occur from thapsigargin-sensitive stores, because the elevation of intracellular Ca^{2+} concentration was protected by the depletion of Ca^{2+} from Ca^{2+} stores using thapsigargin (Ca^{2+}-ATPase inhibitor), but not by ryanodine. It was also suggested that RyR might be negatively regulated by intracellular Ca^{2+} concentration (due to CICR) and by Ca-CaM under hypoxic conditions.

The present study showed that 90 min of ischemia or hypoxia caused a 20% decrease in mAAT activity. Reportedly the distribution of AAT activity in retinal layers is found predominantly in the outer plexiform layer and photoreceptor inner segments [18,19]. Most previous studies showed that retinal ischemia thinned the inner retinal layers and reduced the number of cells in those layers, but not in the photoreceptor nuclear layer. However, it should be noted that the photoreceptor segments were shorter and more disorganized than normal [44]. Therefore, the photoreceptor cells might be sublethally affected by the cytotoxic processes and this effect could be due either to the photoreceptors themselves or to their interacting Müller cells or retinal pigment cells. This suggests that a 20% decrease in mAAT activity by a 90 min ischemic insult might be induced by the damage of specific cells. This decrease was inhibited by calpain inhibitor but not by leupeptin and other protease inhibitors. Calpain, a calcium-activated neutral protease ubiquitously present in animal cells, has been

implicated in a large variety of cellular processes [45]. Reportedly, the nature of calpain-catalyzed proteolysis is not digestive, but proceeds in a limited manner, resulting in alteration of the biochemical and structural parameters of the substrate proteins [46–48]. The physiological behavior of calpain in cells, however, has remained unclear. Histochemical studies have indicated that calpain is present in all neurons and can be detected in the inner and outer segments of the photoreceptor cells, the outer and inner parts of the Müller cells corresponding to the outer and inner limiting membranes, and the outer and inner plexiform layers in the rabbit retina [49]. In this study we determined the amount of calpain activity in the soluble fraction, the mitochondrial fraction and the microsomal fraction and found that 90 min of ischemia resulted in a 2.5-fold increase in mitochondrial calpain activity. It is therefore suggested that under the hypoxic condition in this study, the activation of calpain is likely to occur because of the elevated level of intracellular Ca^{2+}, and that the decrease of mAAT activity may have been evoked by the degradation of mAAT due to the activation of calpain-catalyzed proteolysis in mitochondria.

We did not, however, find an obvious decrease in cAAT activity under hypoxic conditions. Unchanged cAAT activity can be explained by one of the following hypotheses: first, that cAAT is not recognized by calpain as a substrate; second, that cAAT does not exist near activated calpain; or third, that under ischemic conditions Ca^{2+} concentration is insufficient for calpain activation in cytosol.

Great differences in sequence (peptides) have been found between mAAT and cAAT [50,51]. Substrate specificity [52] and the preferred sequence of cleavage by calpain [53] have not been elucidated. A large variety of cellular proteins have been reported to be subject to cleavage by calpain [48]. We cannot eliminate our first hypothesis that calpain might not recognize cAAT as a substrate.

It has been reported that neutrophils and erythrocytes that bind calpain to membranes appear to play important roles in the expression of proteolytic activity at physiological Ca^{2+} concentration [54–57]. Calpain results in degradation of membrane-bound substrates or intracellular protein substrates. In the presence of a pathologically high Ca^{2+} concentration calpain might bind to the membrane and prefer membrane-bound protein to proteolysis. In this study, mAAT activity was found in the membrane-bound mitochondrial fraction. Most likely, cAAT does not exist near activated calpain, contrary to our second hypothesis.

Ca^{2+} accumulation in the mitochondria occurs during ischemia [58–61]. In this study, however, Ca^{2+} concentration in cytosol did not change significantly. It is thought that mitochondria play a part in intracellular calcium regulation in pathological states. If this is so, unchanged cAAT can also be explained by the third hypothesis, namely, that the level of Ca^{2+} is insufficient for calpain activation in cytosol under intracellular Ca^{2+} regulation.

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