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Calpain and calpastatin in porcine retina

Identification and action on microtubule-associated proteins

Nagahisa YOSHIMURA,* Isamu TSUKAHARA* and Takashi MURACHI†‡
**Department of Ophthalmology and †Department of Clinical Science, Kyoto University Faculty of Medicine, Sakyo-ku, Kyoto 606, Japan*

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Two forms of Ca^{2+}-dependent cysteine proteinase (calpain, EC 3.4.22.17) and their specific endogenous inhibitor (calpastatin) were partially purified from porcine retina: calpain I (low-Ca^{2+}-requiring form) was half-maximally activated at 8 μM-Ca^{2+}, and calpain II (high-Ca^{2+}-requiring form) at 250 μM-Ca^{2+}. Both calpain I and calpain II were inhibited by calpastatin. Calpain I from porcine retina was shown to be composed of 83000- and 29000-M_r subunits, and calpain II of 80000- and 29000-M_r subunits, by the use of monospecific antibodies. Calpains I and II were both found to hydrolyse microtubule-associated proteins 1 and 2 rapidly.

Calpain (EC 3.4.22.17; Ca^{2+}-dependent cysteine proteinase) is a typical intracellular non-lysosomal proteinase (Murachi *et al.*, 1981*b*). Two forms of calpain are now known to exist which differ in their Ca^{2+} requirements, calpain I requiring low, and calpain II, high, Ca^{2+} (Mellgren, 1980; Murachi *et al.*, 1981*b*). Although the physiological functions of these two enzymes are still obscure, a number of irreversible phenomena have been attributed to the action of calpain [see recent reviews by Murachi (1983) and Kay 1983)]. Not much is known about calpain and calpastatin in ocular tissues, except that a recent report from this laboratory has shown the presence of calpain II and calpastatin in bovine lens (Yoshida *et al.*, 1984). In the present paper we describe the identification of the two forms of calpain and calpastatin from porcine retina and some enzymological properties of these calpains, which include rapid degradation by either calpain I or calpain II of MAPs from the same tissue.

Abbreviations used: SDS, sodium dodecyl sulphate; IgG, immunoglobulin G; MAP(s), microtubule-associated protein(s); the terms '80kDa' and '30kDa' proteins are used to represent heavy and light subunits of a calpain molecule whose molecular masses have been known to vary from 70 to 85kDa and from 25 to 30kDa respectively, depending on the source of the enzyme.

‡ To whom correspondence and requests for reprints should be sent.

Experimental

DEAE-cellulose (DE 52) was purchased from Whatman, Maidstone, Kent, U.K. Ultrogel AcA 34 was the product of LKB, Bromma, Sweden. Blue Sepharose CL-6B and protein M_r standards were obtained from Pharmacia, Uppsala, Sweden. Nitrocellulose filters were obtained from Schleicher and Schüll, Dassel, Germany, and peroxidase-conjugated goat anti-rabbit IgG from Cappel, Cochranville, PA, U.S.A. An ultrafiltration apparatus and PM-10 membrane were obtained from Amicon, Lexington, MA, U.S.A.

Calpain activity was measured as previously described (Murachi *et al.*, 1981*a*). One unit of calpain activity was defined as the quantity of enzyme that increased the A_{750} by 1.0 after 30min incubation with casein at 30°C. One unit of calpastatin was defined as the quantity of inhibitor that inhibited one unit of calpain II purified from porcine kidney.

Affinity-purified monospecific antibodies against the 80kDa subunit of porcine erythrocyte calpain I and porcine kidney calpain II and a similar antibody against the 30kDa subunit of porcine erythrocyte calpain I were prepared in rabbits as previously described (Yoshimura *et al.*, 1984). After two-step affinity chromatography, anti-(calpain I 80kDa) IgG did not cross-react with calpain II 80kDa subunit and vice versa, whereas anti-(calpain I 30kDa) IgG cross-reacted

equally well with both 30kDa subunits of calpains I and II (Yoshimura *et al.*, 1984).

Polyacrylamide-slab-gel electrophoresis in the presence of 0.1% SDS was performed by the method of Laemmli (1970). Immunoblotting was performed as described by Towbin *et al.* (1979). Antigens were localized by peroxidase staining with *o*-dianisidine as the substrate (Hawkes *et al.*, 1982).

Microtubules were prepared from porcine retina by the method of Shelanski *et al.* (1973), by two cycles of the temperature-dependent polymerization–depolymerization step.

Protein concentration was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard, and that of IgG by measuring absorbance assuming $A_{280}^{1\%} = 15.0$.

Results

Partial purification and properties of calpain and calpastatin from porcine retina

Retinal tissue was homogenized with 3vol. of 20mM-Tris/HCl buffer, pH7.5, containing 1mM-EGTA, 1mM-EDTA, 5mM-2-mercaptoethanol and 0.25mM-sucrose, a Potter–Elvehjem Teflon/glass homogenizer being used. The homogenate was ultracentrifuged at 105000*g* for 90min at 4°C and the supernatant solution was dialysed overnight against 20mM-Tris/HCl buffer, pH7.5, containing 1mM-EGTA, 1mM-EDTA, 5mM-2-mercaptoethanol and 50mM-NaCl (buffer A). The dialysis residue was applied to a column (3cm × 15cm) of DEAE-cellulose pre-equilibrated with the same buffer. After extensive washing with buffer A, the adsorbed protein was eluted with a linear gradient of 50–400mM-NaCl in a total volume of 1 litre. As shown in Fig. 1, when purified calpain II from porcine kidney was added to the assay tubes (0.15 unit each) for measuring the activities of the eluted fractions, a trough in activity at 80mM-NaCl was observed that was found to be due to calpastatin. Just after the calpastatin fractions, a tiny peak of activity was noticed at 100mM-NaCl, which was judged from its Ca^{2+} requirement to be calpain I. At 230mM-NaCl, calpain II was eluted. No Ca^{2+}-dependent proteolytic activity was found in the flow-through fractions. Calpains I and II were further purified on columns (2.5cm × 80cm) of Ultrogel AcA 34 in buffer A. Active fractions were combined and

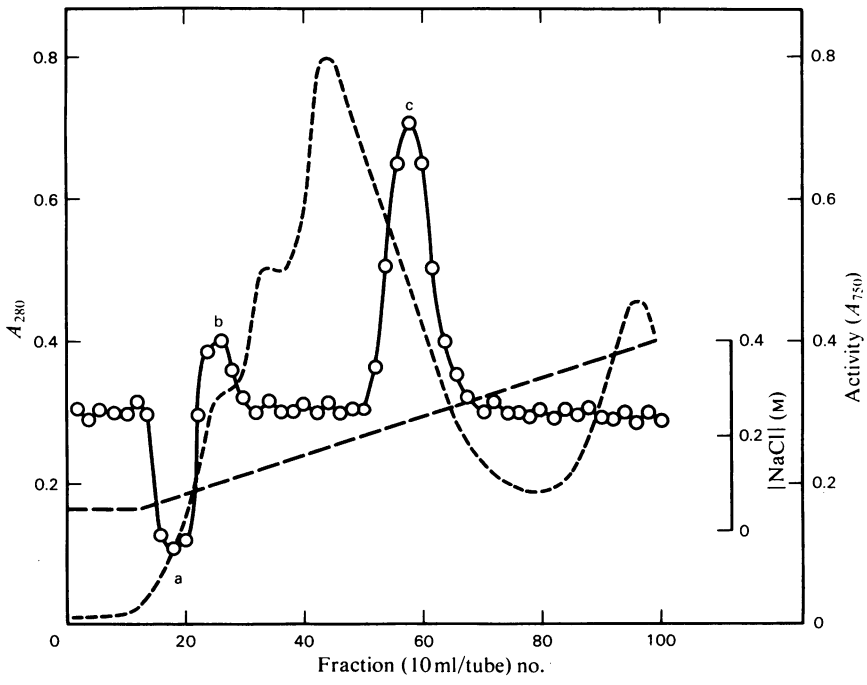

Fig. 1. *DEAE-cellulose chromatography of porcine retina crude extract*
Crude extract from 150 porcine retinae (634mg of protein) was applied to a column (3cm × 15cm) of DEAE-cellulose (DE 52) which was developed with a linear gradient of 50–400mM-NaCl in a total volume of 1 litre. ----, A_{280}; ○, activity; – – – –, [NaCl] gradient. Trough a, calpastatin; peak b, calpain I; peak c, calpain II.

applied to a column (1.0cm × 5.0cm) of Blue Sepharose CL-6B equilibrated with buffer A. Activities were recovered from the column by 1M-urea as previously described for the purification of calpains I and II from rat kidney (Yoshimura *et al.*, 1983). Thus starting with 29.6g wet wt. of retina from 150 porcine eyes, 32 units of calpain I, 167 units of calpain II and 52 units of calpastatin were obtained after Ultrogel AcA 34 chromatography. After Blue Sepharose chromatography, 15 units of partially purified calpain I were obtained with a specific activity of 42units/mg and 98 units of calpain II with specific activity of 174 units/mg. Homogeneously purified calpain I and calpain II of porcine origin have specific activities of 176 units and 302 units/mg respectively (Kitahara *et al.*, 1984).

When caseinolytic activities of partially purified retinal calpains I and II were plotted as a function of free Ca^{2+} concentrations, smooth parallel logarithmic curves were obtained whose features were almost indistinguishable from those reported for the highly purified calpains of other origins (Yoshimura *et al.*, 1983; Kitahara *et al.*, 1984). Retinal calpain I was half-maximally activated at 8 μM-free Ca^{2+} and fully activated at 20 μM-Ca^{2+}, and calpain II half-maximally at 250 μM-Ca^{2+} and fully at 2mM-Ca^{2+}. Calpastatin partially purified from porcine retina was found to inhibit both calpain I and calpain II of the same origin in a dose-dependent manner. At the same concentration of calpastatin, calpain II was inhibited about twice as strongly as was calpain I. Retinal calpastatin had no effect on the hydrolysis of casein by papain, ficin, trypsin and α-chymotrypsin (results not shown).

Immunoelectrophoretic blotting of calpains from porcine retina by monospecific antibodies

The supernatant fraction (50ml) from porcine retina was concentrated with an Amicon PM 10 membrane to 7ml and applied to a column (3.0cm × 90cm) of Ultrogel AcA 34 equilibrated with buffer A. Fractions containing calpains I and II, which were eluted together, were combined, and a portion that contained 100 μg of protein was subjected to immunoelectrophoretic blotting (Fig. 2). When the blot was made with the affinity-purified anti-(calpain I 80kDa subunit) IgG, only one single band of M_r 83000 appeared (Fig. 2, lane B). Anti-(calpain II 80kDa subunit) IgG also revealed only one band, but it corresponded to M_r 80000 (Fig. 2, lane C). The antibody against the 30kDa subunit of calpain I stained only the M_r 29000 band (Fig. 2, lane D). Under the conditions employed, authentic porcine erythrocyte calpain I is known to give, on SDS/polyacrylamide gel, two bands of M_r 83000 and 29000, and porcine kidney calpain II two bands of M_r 80000 and 29000 (Kitahara *et al.*, 1984).

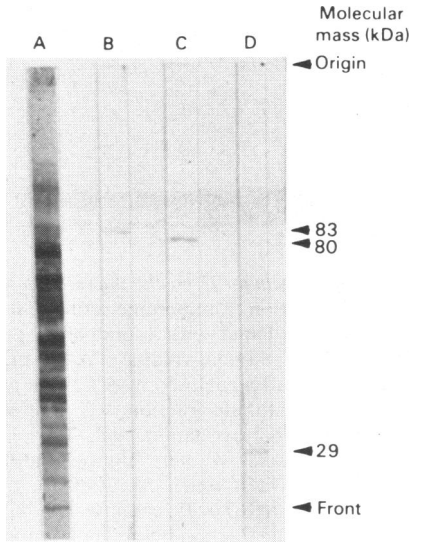

Fig. 2. *Immunoelectrophoretic blotting of calpain fraction from Ultrogel AcA 34 by monospecific antibodies*
Proteins of calpain fraction (100 μg) were subjected to SDS-polyacrylamide gel electrophoresis, and then transferred to a nitrocellulose filter. The filter was cut into strips and stained with Amido Black or immunoblotted with the antibodies. Lane A, Amido Black staining of proteins (about 6 μg); B, immunoblot with anti-(calpain I 80kDa subunit) IgG (2 μg/ml); C, immunoblot with anti-(calpain II 80kDa subunit) IgG (1 μg/ml); D, immunoblot with anti-(calpain I 30kDa subunit) IgG (2 μg/ml).

Effects of calpain I and calpain II on the microtubule fraction

The microtubule fraction prepared from porcine retina was incubated with calpain I and calpain II from the same origin. Calpain I and calpain II were found to act on microtubule proteins in exactly the same fashion. Thus MAP 1 and MAP 2 disappeared rapidly, whereas tubulins were not digested. Fig. 3 shows only the results obtained with calpain II, which were indistinguishable from those obtained with calpain I. Calpastatin (3 units) added to the reaction mixture inhibited the proteolysis of MAP 1 and MAP 2 completely (Fig. 3, lane 7). Leupeptin (acetyl-L-leucyl-L-leucylargininal; 0.2mg/ml) also inhibited the proteolysis of MAP 1 and MAP 2 (results not shown). Exactly the same results were obtained when homogeneously purified calpain II from porcine kidney (Kitahara *et al.*, 1984) was used in place of retinal calpain (results not shown).

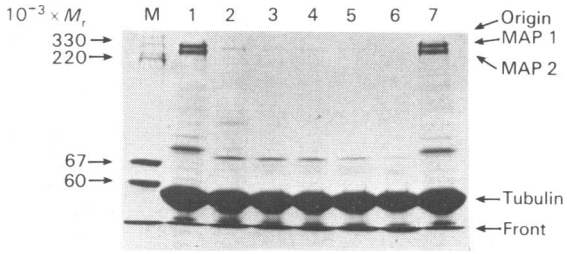

Fig. 3. *Effects of calpain II on the microtubule fraction*
Microtubule fraction from porcine retina (2.0mg of protein) was incubated with 1 unit of of porcine retinal calpain II in a total volume of 0.4ml in 0.1M-imidazole/HCl buffer, pH7.5, at 30°C. The proteolysis of the microtubule fraction was analysed on SDS/7.5% (w/v)-polyacrylamide gel. Marker proteins used for lane M were thyroglobulin (M_r 330000), ferritin (half unit) (M_r 220000), bovine serum albumin (M_r 67000), catalase (M_r 60000). Lane 1, microtubule fraction before incubation with calpain; lane 2, incubation for 1min; lane 3, incubation for 3min; lane 4, incubation for 5min; lane 5, incubation for 10min; lane 6, incubation for 30min; lane 7, incubation for 30min with 3 units of porcine calpastatin. Approx. 90μg of protein was electrophoresed in lanes 1–7.

Discussion

Our knowledge of the enzymological aspects of calpain has expanded recently, but the physiological significance of the enzyme is rather obscure. We have established an efficient method for the purification of calpain I and calpain II, which enabled us to obtain monospecific antibodies that can discriminate between the two forms of calpain (Yoshimura *et al.*, 1983). Applying these methods and materials to the present study on retinal tissues, we have demonstrated that: (1) porcine retina contains two forms of Ca^{2+}-requiring proteinases (calpain I and calpain II) and an endogenous inhibitor that is specific to calpain (calpastatin); (2) calpain I from porcine retina is half-maximally activated at 8μM-Ca^{2+}, whereas calpain II is half-maximally activated at 250μM-Ca^{2+}; (3) both calpain I and calpain II from porcine retina are inhibited by a calpastatin preparation from the same tissue; (4) the immunoblotting analysis shows that calpain I from porcine retina is composed of 83000- and 29000-M_r subunits, and calpain II of 80000- and 29000-M_r subunits; (5) MAP 1 and 2 from porcine retina are rapidly hydrolysed by either calpain I or calpain II.

The chemical, catalytic and immunological properties of porcine retinal calpains I and II, itemized as (2), (3), and (4) above, are consistent with those of calpains that have been highly purified from erythrocytes and kidney of the same species of animal (Sasaki *et al.*, 1983; Kitahara *et al.*, 1984). Their chromatographic behaviours are also in agreement with those reported for calpains from erythrocytes and kidney.

We have examined the quantitative distributions of calpain I, calpain II and calpastatin in various organs of rats, and we have found that rat brain, a typical neural organ, contained 0.12 unit of calpain I, 8.35 units of calpain II and 3.30 units of calpastatin/5g wet wt. of brain (Murachi *et al.*, 1981*a*). Porcine retina has rather high contents of these components. The yields (in units) after Ultrogel AcA 34 chromatography can be converted to 5.41 for calpain I, 28.2 for calpain II and 8.78 for calpastatin/5 g wet wt. of retina. The content of calpain I in porcine retina is more than 40 times higher than that in rat brain. That the content of calpastatin is less than calpain II in porcine retina is in agreement with the situation for rat brain.

Ca^{2+}-dependent proteolysis of MAP 1 and MAP 2 was reported to occur in a system *in vitro* that involved crude enzyme preparations (Sandoval & Weber, 1978; Klein *et al.*, 1981), but it was not clarified which type of calpain I or II was responsible for such proteolysis. Our present data indicate that both calpain I and calpain II can almost equally well digest MAP 1 and MAP 2 at such Ca^{2+} concentrations as are known to cause the full activation of calpain I and calpain II (Fig. 3). It was claimed that when MAPs were Ca^{2+}-dependently proteolysed, microtubules lose their ability to polymerize *in vitro*, and exogenous addition of MAPs restored that ability (Sandoval & Weber, 1978; Klein *et al.*, 1981). One may therefore speculate that, in the retina, calpain and calpastatin regulate the polymerization and depolymerization of the microtubules as triggered by varying Ca^{2+} ion concentrations *in vivo*.

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