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Characterization of proteoglycan degradation by calpain

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Degradation of cartilage proteoglycans was investigated under neutral conditions (pH 7.5) by using pig kidney calpain II (EC 3.4.22.17; Ca^{2+}-dependent cysteine proteinase). Aggregate and monomer degradation reached a maximum in 5 min at 30 °C when the substrate/enzyme ratio was less than 1000:1. The mode of degradation was limited proteolysis of the core protein; the size of the products was larger than that of papain-digested products and comparable with that of trypsin-digested products. The hyaluronic acid-binding region was lost from the major glycosaminoglycan-bearing region after incubation with calpain II. Calpains thus may affect the form of proteoglycans in connective tissue. Ca^{2+}-dependent proteoglycan degradation was unique in that proteoglycans adsorb large amounts of Ca^{2+} ions rapidly before activation of calpain II: 1 mg of pig cartilage proteoglycan monomer adsorbed 1.3–1.6 μequiv. of Ca^{2+} ions before activation of calpain II, which corresponds to half the sum of anion groups in glycosaminoglycan side chains. This adsorption of Ca^{2+} was lost after solvolysis of proteoglycan monomer with methanol/50 mM-HCl, which was used to desulphate glycosaminoglycans. Therefore cartilage proteoglycans are not merely the substrates of proteolysis, but they may regulate the activation of Ca^{2+}-dependent enzymes including calpains through tight chelation of Ca^{2+} ions between glycosaminoglycan side chains.

INTRODUCTION

Degradation of proteoglycans is thought to have important roles in turnover of extracellular matrix in morphogenesis and organogenesis (Caplan *et al.*, 1983), calcification of growth cartilage (Backwalter, 1983), age-related degenerative changes in articular cartilage (Thonar & Kuettner, 1987) and initiation and progression of inflammatory reactions such as arthritic changes. In proteoglycan degradation *in vivo*, proteinases that can degrade core proteins of proteoglycans in the physiological (neutral) pH range are thought to play a major role (Werb, 1989). Proteoglycan-degrading proteinases are suggested to be mainly metalloproteinases, or Ca^{2+}-dependent proteinases (Ehrlich *et al.*, 1982).

Calpains are unique among cysteine proteinases in that they are activated by Ca^{2+} ions and their optimum pH is 7.0–7.5 (Murachi, 1983). Two types of calpain are known: calpain I and II, designated as such by the order of their appearance in DEAE-cellulose fractions. They differ in the Ca^{2+} concentration needed for activation; calpain I is activated by micromolar concentrations of Ca^{2+}, whereas calpain II is activated at over 100 μM-Ca^{2+}.

Because calpains have been classified as intracellular proteinases located mostly in the cytosolic fraction (Murachi, 1983), no studies on extracellular matrix proteins as substrates for calpains have been reported. Even reports on non-matrix extracellular proteins and calpains are limited. Proteins of blood coagulation systems, including fibrinogen (Kunicki *et al.*, 1984), kininogens (Schmaier *et al.*, 1986) and von Willebrand factor (Kunicki *et al.*, 1985), have been shown to be substrates for Ca^{2+}-dependent hydrolysis in platelet lysates *in vitro*. However, the manner of their degradation is sometimes different from that seen *in vivo* (Berkowits *et al.*, 1988). In previous studies, we have demonstrated the presence of extracellular calpain II in normal rat growth cartilage by immunohistochemical methods (Shimizu *et al.*, 1991). The presence of extracellular calpain II has also been demonstrated in skeletal muscle, lung and aorta by Adachi *et al.* (1990). We also found extracellular activity of calpains and calpastatin (the specific endogenous inhibitor of calpains) in synovial fluid of patients with osteoarthritis (Suzuki *et al.*, 1990). These results support our hypothesis that calpains function as proteoglycan-degrading enzymes *in vivo*. In this investigation, we characterized the potency of calpains as proteoglycan-degrading proteinases of proteoglycan monomer and aggregate. Proteoglycans also have a physicochemical feature that is unique among matrix proteins in that they adsorb many bivalent cations, including Ca^{2+}, mainly through interactions with glycosaminoglycan side chains. The effect of this feature on enzyme activation was also evaluated.

MATERIALS AND METHODS

Materials

Chondroitinase ABC (proteinase-free), sodium hyaluronate (800–900 kDa) purified from cockscomb and type C chondroitin sulphate (40–80 kDa) purified from shark cartilage were obtained from Seikagaku-Kogyo, Tokyo, Japan. Proteoglycan aggregate extracted from Swarm rat chondrosarcoma under associative conditions was obtained from Miles Scientific, Elkhart, IN, U.S.A. Leupeptin was the product of Peptide Institute, Osaka, Japan. Agarose and twice-crystallized papain were from Sigma. EGTA was obtained from Dojin, Kumamoto, Japan. Crystallized trypsin and EDTA were from Wako, Osaka, Japan. Casein (Hammarsten grade) and monoiodoacetic acid were obtained from E. Merck, Darmstadt, Germany. Other chemicals used were of reagent grade.

Purification of cartilage proteoglycan monomer and aggregate

Proteoglycans were extracted fresh from young pig articular cartilage and isolated by the method of Oegema *et al.* (1975). The solvent for dissociative extraction consisted of 0.05 M-sodium acetate, 4 M-guanidinium chloride and a proteinase-inhibitor mixture (pH 6.0). The proteinase-inhibitor mixture consisted of 0.1 M-6-aminohexanoic acid, 0.01 M-EDTA, 0.005 M-benzamidine/HCl and 0.001 M-phenylmethanesulphonyl fluoride (Rosenberg, 1975). Proteoglycan monomer was purified as the

Abbreviation used: K_a, concentration of Ca^{2+} ions required for 50% activation of calpains.

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A1D1 fraction. Proteoglycan aggregate was collected as the A1 fraction, or further purified from the A1 fraction by Cs_2SO_4 rate-zonal centrifugation by the method of Kimata *et al.* (1982), when it was necessary to exclude non-aggregating proteoglycans. After purification, proteoglycans were extensively dialysed against 0.05 M-sodium acetate, pH 6.0, then twice against deionized and distilled water and finally against imidazole buffer (110 mM-imidazole/1 mM-EGTA, pH 7.5). Samples of proteoglycan monomer in distilled water were freeze-dried for solvolysis (described below). The proportion of aggregate in pig articular cartilage A1 fraction was 67%. The proportions of uronic acid to total proteoglycans used were 27% (A1D1 fraction) and 24% (A1 fraction).

Purification of calpains and calpastatin

Calpain II and calpastatin were purified from pig kidney and human erythrocytes respectively, as previously described (Takano & Murachi, 1982; Kitahara *et al.*, 1984). The specific activity of calpain II was determined with casein as substrate after a 30 min incubation at 30 °C and pH 7.5 in the presence of 5 mM-cysteine (Yoshimura *et al.*, 1983), and was 290 units/mg, where 1 unit of activity is defined as the amount of enzyme that increased the absorbance at 750 nm by 1.0. Assays of calpastatin activity were carried out by using a known amount of calpain I and measuring the inhibition of caseinase activity (Murakami *et al.*, 1981).

Degradation of proteoglycan monomer (fraction A1D1) or aggregate by calpain II

Degradation of proteoglycans by calpain II was carried out at 30 °C for 30 min in most of the experiments. The reaction mixture contained various amounts of calpains and proteoglycans in 5 mM-2-mercaptoethanol/1 mM-EGTA/110 mM-imidazole buffer, pH 7.5 (buffer A). The reaction mixture was prepared immediately before use. Degradation was initiated by addition of $CaCl_2$ solution to a final concentration of 7 mM (resulting in 6 mM free Ca^{2+} over EGTA) in most of the experiments. Digestion was stopped by adding monoiodoacetic acid solution adjusted to pH 7.5 to a final concentration of 20 mM or EDTA to a final concentration of 10 mM. The effect of 5 mM-2-mercaptoethanol as a reducing agent over proteoglycan substrate causing partial disaggregation of proteoglycan aggregate or disturbing reassociation of proteoglycan monomers with hyaluronic acid was not observed if the incubation time was less than 5 h at 37 °C. This was established by using Sepharose 2B gel-filtration-chromatographic analysis and agarose/PAGE.

Agarose/PAGE

Proteoglycans and their degradation products (less than 2 mg/ml) were subjected to electrophoresis by the method of Heinegård *et al.* (1985) in slab gels made of 0.6% (w/v) agarose and 1.2% polyacrylamide in a horizontal system for submerged-gel electrophoresis (Thornton *et al.*, 1986). Gels were stained with 0.2% (w/v) Toluidine Blue in 3% (v/v) acetic acid.

Degradation of proteoglycan monomers determined by colorimetry

Degradation of proteoglycan monomers was determined by the method of Sapolsky *et al.* (1975). The modified incubation mixture in buffer A consisted of 0.05 ml of 1.5% proteoglycan monomer (750 μg), 0.1 ml of calpain solution, 0.03 ml of inhibitors (or buffer only) and 0.02 ml of $CaCl_2$ solutions (70 mM in most experiments) that produced 6 mM free Ca^{2+} over EGTA. Degradation with crystallized trypsin was performed in Tris/HCl buffer (50 mM, pH 7.5) containing 1 mM-$CaCl_2$ (Roughley & Barrett, 1977). The incubation was begun by adding enzyme solution in 1 mM-HCl. Proteoglycan-monomer-degrading activity was defined as the increase in trichloroacetic acid-soluble (fragmented) products from the precipitate that was formed after degradation by the addition of 5% cetylpyridinium phosphate. The activity was monitored by colorimetric estimation of uronic acid at 530 nm and defined as the increase in absorption units after incubation (for 30 min in most experiments) at 30 °C. Values reported are the means of two or three experiments.

Degradation of proteoglycan monomers by chondroitinase ABC

Proteoglycan monomers (600 μg) dialysed into 0.1 M-Tris/HCl, pH 8.0, containing 0.03 M-sodium acetate was incubated with 5×10^{-1} to 5×10^{-7} unit of chondroitinase ABC (proteinase free) at 37 °C for 40 min (Oike *et al.*, 1980). The reaction was stopped by the addition of 5 mM-$ZnCl_2$ and products were exhaustively centrifuged through Centricon 10 membranes. The amount of uronic acid in the filtrate was determined by colorimetry at 530 nm (Bitter & Muir, 1962). The degradation products of proteoglycan monomer produced by calpain I or II were similarly ultrafiltered and determined by colorimetry for detection of fragmentation of glycosaminoglycan side chains, as reported by Sapolsky *et al.* (1975).

Determination of Ca^{2+} concentration

Distilled water was further purified with a Milli-Q water purification system (Millipore Corp. Bedford, MA, U.S.A.). Ca^{2+}/EGTA buffers were prepared in 110 mM-imidazole/HCl (pH 7.3) / 5 mM - 2 - mercaptoethanol / 1 mM - EGTA / various amounts of $CaCl_2$. For calculation of free Ca^{2+} concentrations, an association constant of 5.05×10^6 M^{-1} (Harafuji & Ogawa, 1980) was used.

Solvolysis of proteoglycan monomer

Methanolysis of proteoglycan monomer was performed in order to desulphate the glycosaminoglycan side chains. Freeze-dried proteoglycan monomer was mixed with methanol containing 0.05 M-HCl at 25 °C for 4 h (Yamashita *et al.*, 1983). The supernatant was discarded and evaporated to dryness, and HCl was removed from the residue by rinsing and evaporation six times with cold methanol. The products were dissolved in cold imidazole buffer and the pH of the solutions was 7.0–7.5.

Rate-zonal sedimentation on Cs_2SO_4 density gradients

Rate-zonal centrifugation on preformed Cs_2SO_4 gradients was performed under associative conditions by the method of Kimata *et al.* (1982). For purification of proteoglycan aggregate from A1 fractions, 27 ml linear gradients of CS_2SO_4, 0.15–0.50 M in 0.1 M-sodium acetate/0.1 M-Tris/HCl, pH 7.2, were prepared in polyethylene tubes on cushions of 3 ml of 2 M-Cs_2SO_4 in the same solvent. The gradients were preformed before centrifugation and kept at 4 °C for 12 h. After layering 3 ml of sample in the same solvent, the gradients were centrifuged in a Beckman SW 27 swinging rotor at 25000 rev./min for 6 h at 4 ± 2 °C. The tubes were then fractionated into 1.5 or 2.4 ml portions. An analytical study of the proteoglycan aggregate or its degradation products was carried out in a Hitachi RPS 50-2 swinging rotor, where the volume of each component was proportionally scaled. The amount of proteoglycan or degraded products in each fraction was determined by colorimetric estimation of uronic acid content.

Reassociation with hyaluronic acid

The presence of a hyaluronic acid-binding region in proteoglycans or in the major glycosaminoglycan-bearing fragments was verified by rate-zonal centrifugation and by agarose/PAGE. Proteoglycan monomer (2.5 mg) was first degraded with calpain II or incubated with buffer A containing

5 mM-2-mercaptoethanol as control. After incubation and inactivation, 1% (w/w) hyaluronic acid was mixed with the degraded products or with the control (Kimata *et al.*, 1982). These mixtures were then dialysed against an associative solution containing 0.5 M-sodium acetate/2 mM-EGTA, pH 7.0, for 1 day at 4 °C. Thereafter the samples were analysed by rate-zonal centrifugation. Samples for electrophoresis were also incubated with 20% (w/w) exogenous hyaluronic acid at room temperature for 4 h before being analysed for the presence of hyaluronic acid-binding regions in the glycosaminoglycan-bearing fragments.

Comparison of products of limit (maximum) degradation by trypsin, papain and calpain

Limit (maximum) degradation of proteoglycan monomer and aggregate (A1) by trypsin, papain and calpain II was performed. Crystallized trypsin was dissolved in 1 mM-HCl at 1 mg/ml. The digestion mixture containing 20 μg of enzyme/ml of proteoglycan solution (2 mg/ml) in 50 mM-Tris/HCl, pH 7.5, containing 1 mM-$CaCl_2$ was incubated at 37 °C for 24 h (Roughley & Barrett, 1977). Digestion of proteoglycans by papain was also carried out at 37 °C for 24 h. The reaction mixture contained 10 μg of enzyme/mg of proteoglycan in 5 mM-dithiothreitol/5 mM-EDTA/0.1 M-sodium acetate buffer, pH 5.0. The digestion was stopped by adding monoiodoacetic acid at a final concentration of 20 mM (Nguyen *et al.*, 1989). Limit degradation by calpain II was performed at 37 °C for 24 h in buffer A, but 5 mM-dithiothreitol was used instead of 5 mM-2-mercaptoethanol. The ratio of substrate/enzyme was adjusted to 100:1 for comparison. The degradation products were subjected to electrophoresis on 0.6%-agarose/1.2%-polyacrylamide gels. The products were also subjected to electrophoresis on 4–20% gradient vertical slab gels of SDS/polyacrylamide.

RESULTS

Degradation of proteoglycan monomer by calpain II

Degradation of cartilage proteoglycan monomer was investigated by agarose/PAGE (Fig. 1) and by colorimetric estimation of the degradation products. Degradation of proteoglycan monomer did not occur without addition of Ca^{2+} (Fig. 1, lane 2), and the degradation was totally inhibited by calpastatin (lane 3). The decrease in the size of the proteoglycan monomer was produced in a dose-dependent manner (lanes 4–8). With large amounts of calpain II, limit-degradation products were obtained as a single band (lane 4). The dose-dependence of proteoglycan monomer degradation was determined by colorimetry. There was a linear relationship between uronic acid release and log [enzyme concentration (units)]. This linearity held where the value of uronic acid release was between 0.03 and 0.45 absorption units.

To confirm that the action of calpain II on proteoglycan monomer was that of a Ca^{2+}-dependent cysteine proteinase, quantitative inhibition studies were performed and degradation of the glycosaminoglycan side chain was examined by colorimetry. Inhibition of 0.3 unit of calpain II in three experiments was 100% by 40 μg of leupeptin/ml, 98% by 20 mM-monoiodoacetic acid and 95% by 1.2 units of calpastatin. The production of small glycosaminoglycans that could pass through Centricon-10 ultrafiltration membranes was investigated by colorimetry to rule out the possibility of glycosaminoglycan-chain degradation by calpains. This system could measure the action of as little as 1×10^{-3} unit of chondroitinase ABC. No production of small glycosaminoglycans by 1 and 2 units of calpain I and II could be detected.

The time course of proteoglycan monomer degradation by calpain II is shown in Figs. 2(*a*) and 2(*b*). Proteoglycan monomer degradation stopped within 5 min at 30 °C when the substrate/enzyme ratio was 1000:1 (Fig. 2*a*). The result of a colorimetric assay using the same conditions for the enzyme reaction is shown in Fig. 2(*b*). Time courses of proteoglycan monomer degradation by trypsin with the same substrate/enzyme ratio or with a similar molecular ratio (1:5000, w/w) are shown for comparison in Fig. 2(*b*). Comparable results were ob-

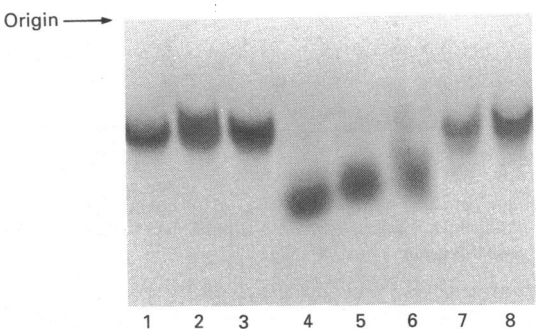

Fig. 1. Inhibition studies (lanes 1–3) and dose-dependence (lanes 4–8) of proteoglycan monomer degradation by calpain II

Proteoglycan monomer (400 μg) was incubated without calpain II (lane 1) or with 1 unit (3.5 μg) of calpain II (lanes 2 and 3), without Ca^{2+} (lane 2) or in the presence of 2 units of calpastatin (lane 3). Digestion mixtures containing 10, 1, 0.1, 0.01 and 0.001 μg of calpain II (lanes 4–8 respectively)/mg of proteoglycan monomer were subjected to electrophoresis on 0.6%-agarose/1.2%-polyacrylamide gel.

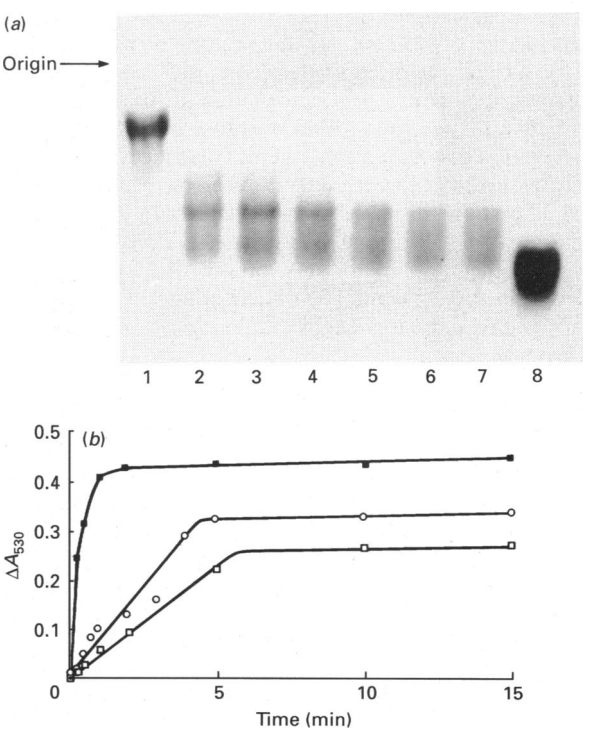

Fig. 2. Time course of proteoglycan monomer degradation by calpain II

(*a*) Substrate/enzyme ratio was 1000:1. Proteoglycan monomer preparation (lane 1) and 15 and 30 s (lanes 2 and 3) and 1, 2, 5 and 60 min (lanes 4–7) digests were analysed by agarose/PAGE. Chondroitin sulphate (40–80 kDa, lane 8) was used as a marker. (*b*) Colorimetric measurement of time-course of proteoglycan monomer degradation. Proteoglycan monomer (750 μg) was incubated with calpain II (1:1000, w/w; 0.2 unit, ○) or trypsin [1:1000, w/w (■) or 1:5000, w/w (□)]. Increase in absorption as a function of incubation time at 30 °C is shown.

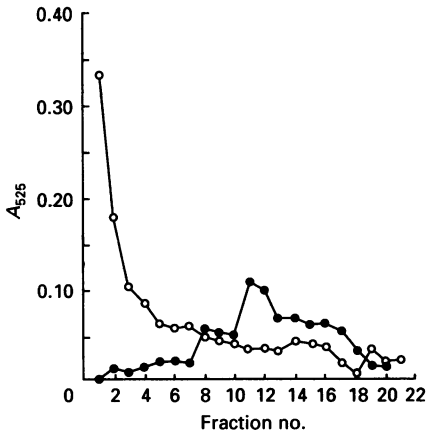

Fig. 3. Loss of aggregating capacity of proteoglycan monomer with hyaluronic acid caused by calpain II; analysis by rate-zonal centrifugation

Intact proteoglycan monomer (incubated without calpain II) plus 1% hyaluronic acid (○) and degraded proteoglycan monomer (substrate/enzyme ratio 2500:1) plus 1% hyaluronic acid (●) were submitted to Cs_2SO_4 rate-zonal centrifugation and fractionated from the bottom. Values on the *y*-axis represent the results of colorimetric estimation of the amount of uronic acid in each fraction.

tained for calpain digestion and trypsin digestion of proteoglycan monomer.

Proteoglycan monomer reassociated with hyaluronic acid was sharply separated by rate-zonal-centrifugation analysis, and loss of the hyaluronic acid-binding region from the major glycosaminoglycan-bearing region was detected at a substrate/enzyme ratio of 2500:1, as illustrated in Fig. 3. Loss of the hyaluronic acid-binding region did not occur when proteoglycan monomer was incubated in the presence of calpain II without Ca^{2+} ion, or with an overdose of calpastatin (results not shown). These inhibition studies confirmed that loss of the hyaluronic acid-binding region was due to calpain II degradation. When digestion mixtures were incubated with exogenous hyaluronic acid before analysis by agarose/PAGE, there was no change in the mobility of any of the degradation products observed in Figs. 1 and 2(*a*), indicating that these glycosaminoglycan-containing fragments do not possess a hyaluronic acid-binding region.

Ca^{2+}-dependence of proteoglycan monomer degradation by calpain II

Quantification of Ca^{2+}-dependence is illustrated in Fig. 4. Activation of calpain II occurred with a significantly higher concentration of Ca^{2+} ($K_a = 3.2 \pm 0.26$ mM, $n = 4$) than was required for casein degradation, which occurred at $K_a = 200$ μM under the same conditions. When proteoglycan monomer was subjected to solvolysis with methanol/50 mM-HCl to desulphate glycosaminoglycan, the Ca^{2+}-dependence curve shifted markedly to the left, and increased without a lag period from zero. The K_a of calpain II for proteoglycan monomer after solvolysis was 0.12 mM. The amount of Ca^{2+} not activating calpain II was calculated as the difference between the curves without and with solvolysis or the difference between K_a values in the cases of proteoglycan monomer and casein degradation (2.50 and 3.08 mM) multiplied by the volume of the solution (200 μl). These Ca^{2+} ions were adsorbed on proteoglycan in the solution (750 μg) before activation of calpain II. The ratio of adsorbed Ca^{2+}/proteoglycan monomer became 1.3–1.6 μequiv./mg of proteoglycan monomer, which corresponds to 1.3×10^3–1.6×10^3 Ca^{2+} ions/molecule of proteoglycan monomer. (This assumed

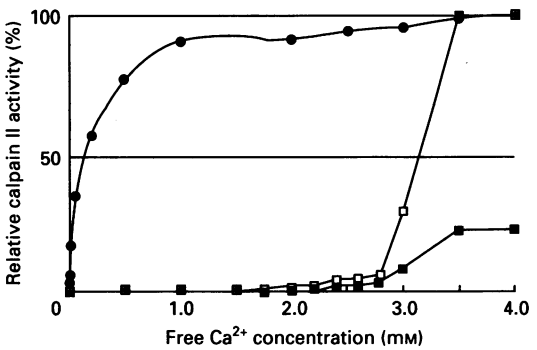

Fig. 4. Ca^{2+}-dependence of proteoglycan monomer degradation by calpain II

Proteoglycan monomer preparation (750 μg) was incubated with 0.5 unit (□) or 0.04 unit (■) of calpain II. Proteoglycan monomer after solvolysis (●) was incubated with 0.5 unit of calpain II. Values are expressed as percentage of maximum activity.

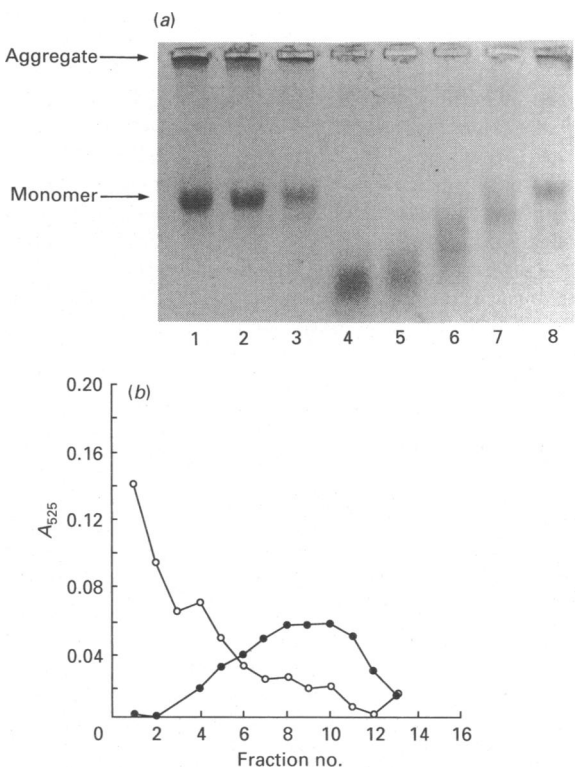

Fig. 5. Inhibition studies and dose-dependence of aggregate degradation by calpain II

Lanes 1–3 show the results of inhibition studies: fraction A1 incubated without calpain II (lane 1) or in the presence of 1 unit of calpain II without addition of Ca^{2+} (lane 2) or with 2 units of calpastatin (lane 3). Digestion mixtures containing 10, 2, 1, 0.2 or 0.1 μg of calpain II (lanes 4–8 respectively)/mg of proteoglycan A1 fraction were subjected to electrophoresis on 0.6%-agarose/1.2%-polyacrylamide slab gel. (*b*) Aggregate degradation by calpain II analysed by rate-zonal centrifugation. Proteoglycan aggregate purified from A1 fraction incubated without calpain II (○) or with calpain II (●; substrate/enzyme ratio 1:600) was subjected to Cs_2SO_4 rate-zonal centrifugation.

the average molecular mass of proteoglycan monomer to be 2×10^6 Da as determined by Sepharose 2B gel-filtration chromatography.) The value corresponds to 0.47–0.58 mol of Ca^{2+} ion/mol of uronate, and this coincides with half of the average of two anion groups (the ester sulphate and the uronic

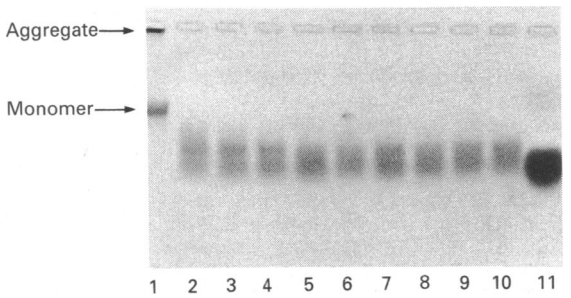

Fig. 6. Time course of fraction A1 degradation

Substrate/enzyme ratio was 500:1. A1 preparation (lane 1) and 15 and 30 s (lanes 2 and 3) and 1, 2, 5, 10 and 30 min (lanes 4–8), 1 and 24 h (lanes 9 and 10) digests were subjected to agarose/PAGE. Chondroitin sulphate (40–80 kDa, lane 11) was used as a marker.

acid carboxy groups)/chondroitin sulphate unit. The presence of 140 mM-NaCl in the incubation buffer did not significantly influence the K_a value (2.95 ± 0.36 mM, $n = 3$) for proteoglycan monomer degradation. The effect of separate chondroitin sulphate chains on Ca^{2+}-dependence was examined for comparison by using casein as substrate. No changes in the Ca^{2+}-dependence of casein degradation were detected with chondroitin sulphate at a final concentration of 1 mg/ml or less (results not shown).

Degradation of proteoglycan aggregate by calpain II

Degradation of A1 fractions was investigated by agarose-gel electrophoresis (Figs. 5*a* and 6) and Cs_2SO_4 rate-zonal centrifugation (Fig. 5*b*). The specificity of fraction A1 degradation by calpain II was confirmed by the Ca^{2+}-dependence of the reaction (Fig. 5*a*, lane 2) and by inhibition with calpastatin (Fig. 5*a*, lane 3). The decrease in the size of the proteoglycan aggregate was produced in a dose-dependent manner (Fig. 5*a*). The size of the products of calpain II limit degradation was larger than that of papain-degraded products and comparable with that of trypsin-degraded products. The same results were obtained when calpain I was used and when the amount of calpains was increased (results not shown).

Degradation of proteoglycan aggregate (purified further from fraction A1) by calpain II is shown in Fig. 5(*b*).

The time course of degradation of fraction A1 by calpain II is shown in Fig. 6. Aggregate degradation reached a maximum within 5 min. The same result was obtained when a smaller (one-tenth) amount of calpain II was used, and when Swarm rat chondrosarcoma proteoglycan aggregate was used as the substrate (results not shown).

DISCUSSION

In previous studies, we identified extracellular activities of calpains in joint spaces from osteoarthritic synovial fluid (Suzuki *et al.*, 1990). The zonal appearance of calpain II coincided with a decrease in matrix proteoglycans in rat growth cartilage (Shimizu *et al.*, 1991). In the present investigation, the mode of proteoglycan degradation by calpain II was verified to be proteolytic and Ca^{2+}-dependent. Both cysteine proteinase inhibitors (monoiodoacetic acid, leupeptin) and calpastatin inhibited proteoglycan degradation totally. Degradation of articular cartilage proteoglycan monomer (fraction A1D1) and aggregate was produced by calpain II. The size of the products decreased in a dose-dependent manner and limit-degradation products were larger than papain-digested products and comparable with trypsin-digested products. No degradation of glycosaminoglycan repeats and linkage regions of side chains was produced by calpains because of the limited manner of degradation. No small products that could pass through a Centricon 10 membrane were detected. When the major glycosaminoglycan-bearing region was fragmented, it did not possess a hyaluronic acid-binding region. Thus the features of proteoglycan degradation by calpains were confirmed by the present studies *in vitro*.

The time course of proteoglycan degradation produced by calpain II was similar to calpain degradation of cytoskeletal proteins, talin (Beckerle *et al.*, 1987) and MAP-1 (microtubule-associated protein-1)-related 350 kDa protein (Sato *et al.*, 1986).

The degradation of large cartilage proteoglycans by proteinases is commonly classified by the size of limit-degradation products. Elastase and papain cleave the chondroitin sulphate-attaching region into fragments containing one or two glycosaminoglycan chains. Trypsin, cathepsins D, G and metalloproteinases are reported to liberate rather large peptide fragments, containing approximately five to twelve chondroitin sulphate chains. Calpains also caused limited proteolysis of core protein at the glycosaminoglycan-attachment region into large fragments, but the significance of this degradation is not known.

Activation of calpains is strictly regulated by the free Ca^{2+} ion concentration (Murachi, 1983). When proteoglycan monomer was used as the substrate for calpain II, curves of Ca^{2+}-dependence shifted significantly to the right compared with those for casein hydrolysis ($K_a = 3.2$ mM). The Ca^{2+} activation requirement for proteoglycan monomer was above the Ca^{2+} concentration found in extracellular fluids *in vivo*. In the case of casein degradation, the K_a value was 200 μM when we used the same calpain II fraction. Similar results for K_a values for synthetic peptides used as substrates were reported by Sasaki *et al.* (1984) and closely examined by Barrett *et al.* (1991). The amount of Ca^{2+} not effective for activation of calpain II was calculated as 1.3–1.6 μequiv./mg of proteoglycan, 1300–1600/proteoglycan subunit molecule, or 0.47–0.58/uronate. This ratio was reproducible, remained stoichiometrically constant when the amount of proteoglycan monomer was varied (results not shown), and corresponded to half the number of anion groups in the glycosaminoglycan side chains. It is probable that Ca^{2+} ions that failed to activate calpain II were rapidly adsorbed on proteoglycans at the glycosaminoglycan side chains. This value is similar to data reported by Dziewiatkowski (1987) and Woodward & Davidson (1968). They measured the amount of Ca^{2+} bound to cartilage proteoglycans from titration curves measured with a Ca^{2+}-specific electrode. They derived a value of 1.11–1.27 μequiv. of Ca^{2+}/mg of proteoglycan. These data indicate that proteoglycans can regulate the concentration of free Ca^{2+} ions.

The unique Ca^{2+}-dependence of proteoglycan monomer degradation was lost after pretreatment of proteoglycan monomer by the rather simple procedure of methanolysis. This confirmed that Ca^{2+} ions had been adsorbed on intact proteoglycan monomer, and strongly suggests that these Ca^{2+} ions are attached to glycosaminoglycan side chains at ester sulphate groups or carboxy groups. The methylation of carboxy groups as a side reaction of methanolysis can also be postulated.

The adsorption of Ca^{2+} on free glycosaminoglycan was reported by several authors using equilibrium dialysis, and the association constant of separate chondroitin sulphate on Ca^{2+} binding was determined by Scatchard-plot analysis (Hunter *et al.*, 1988). In our studies, the adsorption of Ca^{2+} ions on separate chondroitin sulphate chains was not observed, which was demonstrated by the finding of no changes in the activation of calpain II with casein as substrate. This shows that adsorption of Ca^{2+} occurs between glycosaminoglycan side chains in proteoglycan monomer, and that the affinities (association

constants) of the free and bound form of glycosaminoglycan side chains for Ca^{2+} could be compared by effects on enzyme activation. Any discrepancies would reflect the rapid formation and stability of the Ca^{2+}–proteoglycan monomer complex. Chondroitin sulphate chains can act as chelators of Ca^{2+} when they are attached to core protein, with the formation of chelate rings. From these results, the conformation of the Ca^{2+}–proteoglycan monomer complex might be postulated as helices of egg-box-junction type at chondroitin sulphate chains. The loss of Ca^{2+} adsorption on proteoglycan after solvolysis may be caused by desulphation of glycosaminoglycan side chains, but may also be due to smaller changes. Indeed, insufficient desulphation or methylation of glycosaminoglycan side chains might be enough to cause destructive changes in the basic repeat structure which maintains the stable conformation of the Ca^{2+}–proteoglycan complex.

It may be concluded that cartilage proteoglycan can act functionally as a chelator of Ca^{2+}. This causes an inhibitory effect on activation of metal-dependent enzymes such as calpains, even at much lower concentrations than found *in vivo*. The effect is lost in the case of free chrondroitin sulphate chains (a fragmented form of proteoglycan monomer). This may partially explain why metal-dependent enzymes are synchronously activated in closed avascular circumstances when proteoglycans are in dynamic conditions. An example can be found during calcification in the hypertrophic zone of growth cartilage.

Among neutral proteinases, calpains share common characteristics with stromelysin. They are both Ca^{2+}-dependent, and both appear in the hypertrophic zone of growth cartilage and in synovial joints, which are the main sites of degradation of cartilage-type proteoglycan *in vivo*. Both can degrade proteoglycan aggregate into large fragments through limited proteolysis of core protein. Stromelysin requires an extrinsic activation process for proteoglycan degradation. However, some types of arthritis occur rapidly. Fragmentation products of proteoglycan molecules are reported to appear in synovial fluid 30 min after a single injection of interleukin-1 into a synovial joint (Pettipher *et al.*, 1989). The entry of neutrophils is denied by the same authors. Direct participation of calpains in proteoglycan degradation may be postulated.

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