A CALCIUM-DEPENDENT PROTEASE SELECTIVELY DEGRADING THE 160 000 M_r COMPONENT OF NEUROFILAMENTS IS ASSOCIATED WITH THE CYTOSKELETAL PREPARATION OF THE SPINAL CORD AND HAS AN ENDOGENOUS INHIBITORY FACTOR

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1. Introduction

A class of calcium-activated proteases has been found in a wide variety of tissues (reviews [1,2]). In vertebrate [3–7] and invertebrate [8,9] nerves, similar type of proteolytic activity has been detected in the soluble compartment, acting preferentially on the neurofilament proteins. As neurofilaments are the major structural elements of the axoplasm, significant roles for this enzyme in pathological [3–5] and developmental [6] aspects have been suggested.

Here we report the presence of a calcium-activated protease associated with the cytoskeletal preparation of the mammalian spinal cord. This enzyme has 10-times higher calcium sensitivity compared to those reported in the nerve, and shows a very narrow substrate specificity directed towards 1 of the 3 major polypeptides of the mammalian neurofilaments, the 160 000 M_r polypeptide. Furthermore, a soluble inhibitory factor for this enzyme was found in the same tissue, suggesting a possible involvement of this enzyme–inhibitor system in the regulation of neurofilament organisation.

2. Materials and methods

2.1. *Cytoskeletal preparation from the spinal cord*

Neurofilament-enriched cytoskeletal material was prepared from rat spinal cords as in [10] with a slight modification: Spinal cords from adult rats were homogenised in TKM buffer (50 mM Tris (pH 7.6), 25 mM KCl, 10 mM $MgCl_2$) containing 1% Triton X-100, 5 mM EGTA and 5 mM β-mercapthoethanol in addition. The homogenate was stirred at room temperature for 1 h, which was effective in increasing the yield of neurofilaments. The homogenate was then layered on TKM buffer containing 1 M sucrose, and centrifuged at 78 000 × *g* for 2 h. The neurofilament-enriched pellet from three animals was usually suspended in 10 ml TKM buffer. It was either assayed immediately for the proteolytic activity or stored at −80°C.

2.2. *Protease assay*

Proteolytic activity was assessed by gel electrophoresis on SDS slab gels as breakdown of polypeptides. Standard incubation mixture contained (final vol., 1 ml); 0.5 ml 20 mM Tris (pH 7.6) containing 5 mM β-mercaptoethanol, 0.1 ml 50 mM $CaCl_2$ (final conc. 5 mM), 0.2 ml 0.1 M cysteine, and 0.2 ml cytoskeletal preparation suspended in TKM buffer. After incubation at 37°C for 30 min, the reaction was terminated by the addition of 0.2 ml of ice-cold 70% trichloroacetic acid. The trichloroacetic acid-insoluble material was collected by centrifugation, washed successively with ethanol, ethanol–ether (1:1) and ether, dried, and finally dissolved in SDS sample buffer containing 2% SDS, 10% glycerol, 5 mM β-mercaptoethanol and 80 mM Tris (pH 6.8). Aliquots were subjected to SDS gel electrophoresis as in [11], and stained with Coomassie brilliant blue R-250.

2.3. *Preparation of the inhibitory factor*

Fresh spinal cords were homogenised in 20 mM Tris (pH 7.6) containing 5 mM β-mercaptoethanol and 5 mM EGTA, and centrifuged at 100 000 × *g* for

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1 h. The supernatant was applied onto a column of DEAE-cellulose (Whatman DE-52), washed extensively with the same buffer and eluted with a linear [NaCl] gradient of 0–0.5 M in the same buffer. Inhibitory activity was assayed by including 0.5 ml of the gradient fraction in the standard incubation mixture in place of the buffer and raising $CaCl_2$ to 10 mM.

3. Results

3.1. *Proteolytic activity associated with the cytoskeletal preparation of the spinal cord*

The Triton-insoluble cytoskeletal preparation of the spinal cord is composed mainly of the neurofilament triplet (200, 160 and 68 × 10^3 M_r), glial fibrillary acidic protein (GFA protein, 51 000 M_r [12]), actin (43 000 M_r) (fig.1a). Incubation of this preparation in the presence of mM levels of calcium revealed a proteolytic activity associated with this fraction (fig.1b). The 160 000 M_r polypeptide of the neurofilament triplet was degraded preferentially, followed by a partial loss of the 68 000 M_r component. GFA protein and actin remain intact after 30 min at 37°C.

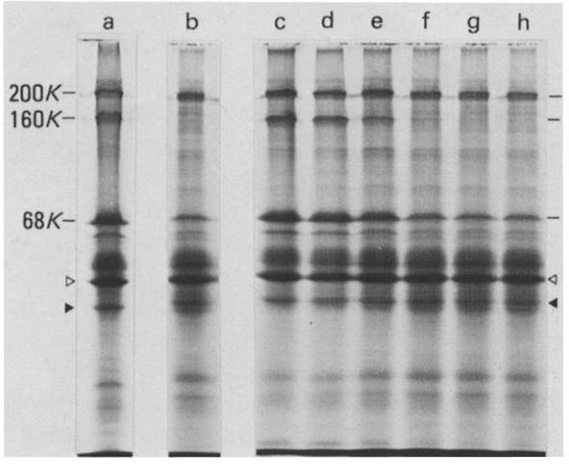

Fig.1. Calcium-dependent breakdown of the 160 000 M_r polypeptide in the cytoskeletal preparation of the spinal cord. Triton-extracted cytoskeletal preparation from the spinal cord was incubated for 30 min at 37°C in the presence of different concentrations of calcium. After incubation, trichloroacetic acid-insoluble material was recovered and analysed by SDS gel electrophoresis on 10% acrylamide gels: (a) cytoskeletal preparation without incubation; (b–h) cytoskeletal preparation incubated with (b) 5 mM $CaCl_2$, (c) 10 mM EGTA, (d) no addition, (e) 0.1 mM $CaCl_2$, (f) 0.5 mM $CaCl_2$, (g) 1 mM $CaCl_2$, (h) 5 mM $CaCl_2$. 200 K, 160 K and 68 K are the 3 major polypeptides of the neurofilaments (K = × 10^{-3} M_r): (◁) GFA protein; (◀) actin.

The enzyme activity was already detectable at 0.1 mM Ca^{2+} (fig.1e), reaching full activity at 0.2 mM Ca^{2+} (fig.1c–h). It was strictly dependent on Ca^{2+}, as Mg^{2+} and Mn^{2+} could not replace Ca^{2+}.

Breakdown of the 160 000 M_r component was complete within 5 min (fig.2). The other two components of the neurofilaments were more resistant

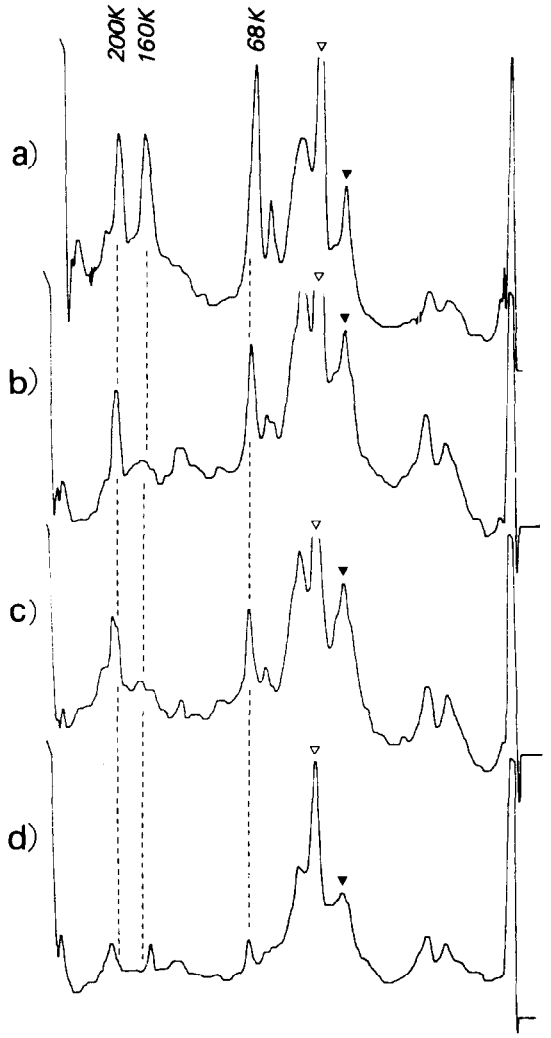

Fig.2. Densitometric tracings of the Coomassie-stained gel showing progressive breakdown of the neurofilament proteins during incubation with 5 mM $CaCl_2$. Cytoskeletal preparation was incubated at 37°C in the standard medium containing 5 mM $CaCl_2$ for time periods. Trichloroacetic acid-insoluble material recovered after incubation was electrophoresed on a SDS gel 10% in acrylamide. Incubation times were: (a) 0; (b) 5 min; (c) 30 min; (d) 16 h. Three major polypeptides of the neurofilaments are marked with 200 K, 160 K and 68 K (×10^{-3} M_r): (◁) GFA protein; (◀) actin.

to this protease and were degraded only upon much longer incubations (16 h; fig.2d). The GFA protein was remarkably stable even after prolonged incubation, showing that the enzyme had a very narrow substrate specificity.

The pH optimum for the proteolytic breakdown was between pH 7–8. The activity was effectively inhibited by 1 mM leupeptin, an oligopeptide inhibitor of some proteases such as papain, cathepsin B, trypsin and the squid neurofilament protease [9].

The proteolytic activity could be separated from the cytoskeletal proteins by further extraction with 0.6 M KCl.

3.2. *Soluble inhibitory factor of the cytoskeleton-associated protease*

Incubation of the whole spinal cord homogenate under similar conditions in the presence of 5 mM Ca^{2+} did not lead to clear and reproducible breakdown of the proteins as with the cytoskeletal preparation. This suggested a possible occurrence of an inhibitory factor which might have been dissociated from the protease in the course of Triton extraction.

Soluble supernatant of the spinal cord homogenate was applied onto a column of DEAE-cellulose, and the fractions eluted with a linear salt gradient were tested for their effects on the cytoskeleton-associated protease activity. Material eluted over 25–110 mM NaCl completely inhibited the breakdown of the 160 000 M_r polypeptide (fig.3). This inhibitory factor was non-dialysable and heat-stable.

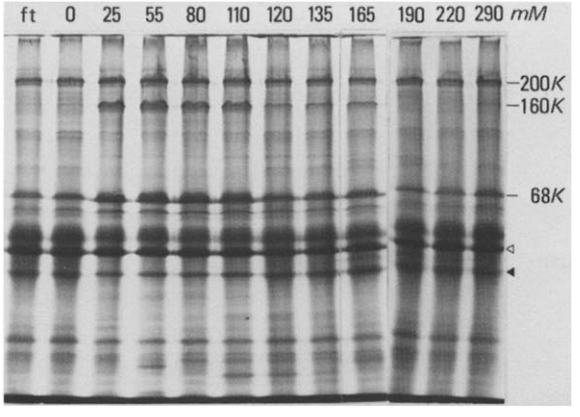

Fig.3. Detection of a soluble inhibitory factor for the cytoskeleton-associated protease. Soluble fraction of the spinal cord homogenate was applied onto a column of DEAE-cellulose and eluted with a linear [NaCl] gradient. Fractions were tested for their effects on the protease by including them in the assay mixture. After incubation at 37°C for 30 min, trichloroacetic acid-insoluble material was recovered and analysed by SDS gel electrophoresis. Number on top of each column denotes [NaCl] in mM of that fraction: (ft) flow-through fraction; (◁) GFA protein; (◀) actin. The 3 neurofilament components are marked by 200 K, 160 K and 68 K ($\times 10^{-3}$ M_r).

4. Discussion

Although the proteases dependent on mM levels of calcium have been reported in various tissues [1,2], the high calcium concentration required was a major problem in understanding the physiological significance of this type of enzymes. However, another type of calcium-activated protease with 10-times higher calcium sensitivity was found in cardiac muscle [13]. Further studies revealed that the two types of proteases with different calcium requirements coexist in a wide variety of tissues at varying ratios [1]. Generic names have been proposed for these two enzymes; 'calpain I' for the enzyme which is fully activated with 0.1 mM calcium; and 'calpain II' for the other which requires 4–5 mM calcium [1]. From the properties of the proteolytic activity described here, the cytoskeleton-associated enzyme seems to be classified as calpain I.

In the soluble compartment of the invertebrate and vertebrate nerves, calpain-type enzyme activities have been reported which selectively degrade the neurofilaments [3–9]. However, in all cases the calcium level required for activity was >1 mM. Thus those enzymes are probably of the calpain II-type. Substrate specificities reported for the mammalian enzymes were broader as well, compared to this enzyme described here. The protease in [3–5] degrades all 3 polypeptides of the neurofilament triplet, and that in the brain acts on actomyosin and tubulin as well as the neurofilaments [7]. The protease reported here is specific towards the 160 000 M_r component of the neurofilament triplet, being much less active towards the other 2 components and virtually inactive towards the coexisting actin and the glial filament protein. The enzyme seems also to be more potent than those reported, as much less time was required to detect the activity. This may be a consequence of direct structural association between the enzyme and the substrate.

This is the first report concerning the endogenous inhibitor of the neurofilament degrading enzyme. In

other tissues, endogenous high M_r inhibitors of calpains, 'calpastatins', have been found [1]. Experiments are in progress to purify the inhibitory factor from the spinal cord for further characterisation and comparison of its properties with the other 'calpastatins'.

From immunocytochemical studies using antibodies against each of the triplet proteins, it was concluded that the 68 000 M_r component makes up the core structure of the neurofilament, while 160 000 and 200 000 M_r components are located peripherally [14]. Presence of an enzyme specific towards one of the outer components suggests interesting possibilities that such a selective proteolysis may be involved in the control of filament–filament interaction.

In [15], a calcium-activated protease specific for vimentin, another type of intermediate-sized silament protein, was associated with the cytoskeleton of Ehrlich ascites tumor cells. This enzyme was inactive towards other types of intermediate-sized filament proteins such as neurofilament proteins and GFA protein. With this observation, controlled proteolysis by tissue specific proteases may be an important general mechanism for the regulation of intermediate-sized filament organisation in the cell.

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