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Ca^{2+}-activated proteinase in the rat

Quantification by immunoassay in the uterus during pregnancy and involution, and in other tissues

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1. Rat uteri were taken at various stages of pregnancy and involution *post partum*, and several other tissues were taken from pregnant and non-pregnant animals. Portions of each tissue were homogenized in the presence of proteinase inhibitors, and the amounts of the high-Ca^{2+}-requiring Ca^{2+}-activated proteinase in the supernatants were measured by a two-site immunoradiometric assay using ^{125}I-immunoglobulin G. 2. The proteinase was shown, by protein blotting, to be immunologically identical in all tissues. 3. The amounts in the various tissues, expressed in units of proteinase activity/g wet wt., were: lung, 95; kidney and small intestine, 42; liver, 20; brain, heart and skeletal muscle, 13. 4. Uterine wet weight increased at the end of pregnancy by about 8-fold, but the amounts of proteinase per uterus increased by about 22-fold; alternatively, expressed in units of proteinase activity/g wet wt., the mean uterine values were: non-pregnant, 28.6; term-pregnant, 77.0. 5. As the wet weight of the uterus fell rapidly during involution, the amounts of proteinase activity remained relatively high. 6. The data suggest that the Ca^{2+}-activated proteinase may have some role in tissue resorption during uterine involution, but the high proteinase activity present before parturition must be regulated in ways which are not yet clear.

Although first well characterized in muscle (Dayton *et al.*, 1976) and thought to have a role in muscle degradation initiated by Z-disc destruction (Reddy *et al.*, 1975), Ca^{2+}-activated neutral proteinases have since been demonstrated in many mammalian tissues. The enzyme was originally described as cytoplasmic on the basis of its ready solubility, but has been localized by immunological techniques on the Z-disc (Ishiura *et al.*, 1980), or on the plasma membrane (Barth & Elce, 1981), or in both of these places (Dayton & Schollmeyer, 1981). These conflicting results, which affect arguments about the physiological role of the enzyme, doubtless reflect the various antibody preparations, and probably require the use of monoclonal antibodies for their unambiguous resolution.

The wide tissue distribution of the enzyme indicates that it has roles other than muscle degradation (Waxman, 1981), and the muscle role was itself disputed on the basis of inhibitor experiments which supported an extracellular localization (Gerard & Schneider, 1980), and on the basis of the effects of prostaglandins on muscle protein turnover (Rodemann *et al.*, 1982).

The evidence from several laboratories has now made clear that two Ca^{2+}-activated proteinases exist: these are a high-Ca^{2+}-requiring form, or calpain II, active at about 0.5mM-Ca^{2+} (which was the first form isolated, and whose Ca^{2+} requirement seemed to be inconsistent with an intracellular function), and a low-Ca^{2+}-requiring form, or calpain I, active at about 10μM-Ca^{2+}; the precise Ca^{2+} requirements vary in the many different reports (for review, see Yoshimura *et al.*, 1983). The low-Ca^{2+} form is eluted at lower ionic strength from DEAE-cellulose columns (Croall & DeMartino, 1983), and is normally observed in much smaller amounts, than the high-Ca^{2+} form, although this ratio may depend on the tissue (Murachi, 1983). Both enzymes are reported to have subunits of approx. M_r 80000 and 30000, although it is not certain that the M_r-30000 protein can validly be described as a subunit. The M_r-80000 subunit can be isolated alone and is fully active in the casein assay (Ishiura *et al.*, 1978;

Abbreviations used: PBS (phosphate-buffered saline), 0.15M-NaCl/10mM-sodium phosphate, pH7.4; SDS, sodium dodecyl sulphate; IgG, immunoglobulin G.

Azanza *et al.*, 1979; Barth & Elce, 1981), and in many published gel pictures the M_r-30000 subunit appears to be present in much less than equivalent amounts. Both enzymes become more sensitive to Ca^{2+} as a result of autolysis (Dayton, 1982), which at first suggested that the low-Ca^{2+} form might be a degradation or auto-activation product of the high-Ca^{2+} form (Suzuki *et al.*, 1981; Mellgren *et al.*, 1982). This does not appear to be the case, however, since the M_r-30000 subunits were found to be immunologically identical, but the M_r-80000 catalytic subunits did not cross-react (Wheelock, 1982). The amino acid compositions also differ (Yoshimura *et al.*, 1983).

In pursuit of our efforts to elucidate the physiological role of the proteinase, we have established an immunoassay to quantify it, first in denervated rat muscle (Elce *et al.*, 1983) and now in pregnant and involuting rat uterus. Both of these systems show rapid loss of wet weight without necrosis, and are therefore very useful for studies of protein turnover and its regulation. An immunoassay was necessary to measure the Ca^{2+}-activated proteinase in crude extracts, both because of the small samples available from single rat tissues, and also because of the existence of proteinase inhibitors in the extracts. As shown below, it is likely that our measurements refer only to the high-Ca^{2+} form of the enzyme, owing to the specificity of the antibody.

Methods and materials

The standard Ca^{2+}-activated proteinase was purified from rat carcasses (decapitated, skinned and eviscerated, the remaining total muscle and bone being minced before extraction) by published procedures involving a sequence of column-chromatography steps on DEAE-cellulose, Sepharose 6B, thiopropyl-Sepharose, antipain–Sepharose and DEAE-Sephacel. The product was stored in the presence of 50% (w/v) glycerol at −20°C and showed no change in caseinolytic activity over 3 months. With the unit of caseinolytic activity defined by Barth & Elce (1981), this proteinase had a specific activity of approx. 1000 units/mg.

The protein blotting and immunodetection (Towbin *et al.*, 1979; Gershoni & Palade, 1983) and the solid-phase immunoradiometric assay (Hales & Woodhead, 1980) were as previously described (Elce *et al.*, 1983), but a new antibody preparation was obtained. The antiserum used in the present work, termed '177' for purposes of discussion, was obtained by inoculation of fresh rabbits with an immunoprecipitate prepared by incubating a partially purified rat proteinase (high-Ca^{2+} form), at 4°C overnight in the presence of 5mM-EDTA, with the antiserum 628 used previously (Elce *et al.*, 1983). The immunoprecipitates were washed in PBS, emulsified in PBS and Freund's adjuvants, and injected intramuscularly three times at 3-week intervals. The IgG fraction was isolated from the pooled sera by $(NH_4)_2SO_4$ precipitation and DEAE-Sephadex chromatography. An affinity column was prepared by applying 4mg of purified rat Ca^{2+}-activated proteinase in 5mM-EDTA/0.1M-NaCl/10mM-Tris/HCl, pH8.0, under N_2 and in the absence of 2-mercaptoethanol, to a column of 2g of the pyridyl-2-disulphide form of thiopropyl-Sepharose, followed by washing of the column in 1M-NaCl. The IgG fraction of the antiserum 177 was applied to the column in 5mM-EDTA at pH8.0, followed by further washing of the column with 1M-NaCl, and the specifically bound IgG was then eluted with 0.2M-glycine/0.6M-NaCl, pH2.4. The eluted fractions were adjusted immediately to pH8.0 and tested by immunodiffusion. Fractions containing the specific IgG were stored in the presence of 20% (w/v) glycerol at −20°C, and a portion of the IgG was radioiodinated in the presence of chloramine-T (McConahey & Dixon, 1980).

Non-pregnant and timed-pregnant Sprague–Dawley rats were obtained from Canadian Breeding Farms, St. Constant, Que., Canada. The animals were killed by CO_2 anaesthesia and cervical dislocation. The uteri were excised and slit open for removal of the products of conception, but the endometrium was not scraped off (Afting *et al.*, 1979). Pregnant uteri with very few foetuses or signs of extensive resorption were rejected. Two samples (each about 0.2g) from each uterus and one sample (about 0.2g) from other tissues were homogenized in 2ml of 0.1M-NaCl/5mM-EDTA / 0.1% 2-mercaptoethanol / 1mM-phenylmethanesulphonyl fluoride/2.5% (v/v) ethanol/20mM-Tris/HCl, pH7.5, at 4°C in a Polytron homogenizer for 15s. Portions of the homogenates were removed for determination of non-collagen protein (Kar & Pearson, 1972), and the homogenates were then centrifuged at $15000g_{av.}$ for 20min at 4°C. Portions of the supernatants were taken immediately for immunoassay, and other portions were prepared if required for SDS/polyacrylamide-gel electrophoresis. The remainder of each supernatant was stored at −70°C and reassayed at a later date.

Results

Subunits of the enzyme, and specificity of the 177 antibody

The Ca^{2+}-activated proteinase fully purified from rat carcasses, and used as the standard for the

immunoassay, consisted of a single protein of M_r 78000 (not significantly different from the value of approx. 80000 usually reported), and did not possess an M_r-30000 component detectable by staining of SDS/polyacrylamide gels or immunologically on nitrocellulose blots. The enzyme was eluted from DEAE-Sephacel at about 0.25M-NaCl, and reached half of its maximum activation at 0.25mM-Ca^{2+}; these properties identify the enzyme as the high-Ca^{2+} form (Waxman, 1981; Croall & DeMartino, 1983).

The specificity of the affinity-purified antibody preparation, 177, which was used in the immunoassay, was defined by the protein-blot experiment shown in Fig. 1. This demonstrates that in the total soluble protein extracted from several rat tissues, only two proteins, of M_r 78000 (corresponding to the purified standard proteinase) and of M_r 30000, were able to bind antibody. Equal amounts of proteinase activity from the various tissues, as measured by immunoassay, were applied to each track in the experiment leading to Fig. 1. The immunodetection process on protein blots is not strictly quantitative, but the results in Fig. 1 tend to suggest that the M_r-78000 and M_r-30000 proteins were present in the same ratio in all tissues. The same ratio was observed with extracts of non-pregnant and late-pregnant uterus.

The relationships of the various proteinase subunits were clarified by two further experiments. The specifically bound IgG was eluted from the M_r-78000 zone of a blot as described by Olmsted (1981), and was shown to be able to re-bind to the M_r-78000 protein, but not to the M_r-30000 protein, on a fresh blot. In addition, the high-Ca^{2+} and low-Ca^{2+} forms of rat muscle proteinase were separated as described by Croall & DeMartino (1983) and subjected to the blot procedure and immunodetection with 177 antibody (Fig. 2). This experiment confirmed the result of Wheelock (1982), in that the M_r-30000 subunits of both enzymes and the M_r-78000 subunit of the high-Ca^{2+} form bound antibody, but the M_r-78000 subunit of the low-Ca^{2+} form did not bind antibody. Sasaki *et al.* (1983), using an enzyme-linked immunosorbent assay, found that the M_r-30000 subunits of high-Ca^{2+} and low-Ca^{2+} forms were immunologically very similar; in contrast with our blot results, the M_r-80000 subunits were found also to cross-react, although much less strongly.

Immunoassay

Although the standard Ca^{2+}-activated proteinase did not contain an M_r-30000 protein, whereas

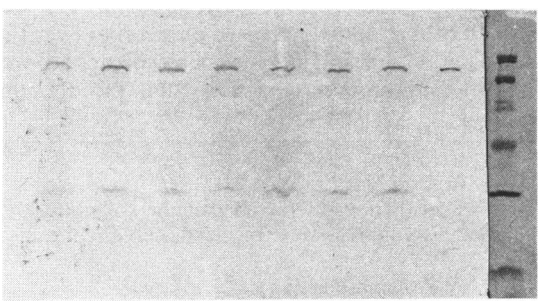

Fig. 1. *Immunodetection of the Ca^{2+}-activated proteinase on nitrocellulose sheets after Western blotting*

The lanes contained 0.1 unit of proteinase activity in the crude tissue supernatant from each of several tissues. From the left, the samples were: liver, lung, kidney, brain, skeletal muscle, small intestine, heart, pure standard proteinase; on the right are M_r standards, stained with Amido Black, having M_r values (from the top) of 92500, 66200 (contamination at approx. 57000), 45000, 31000, and 21000.

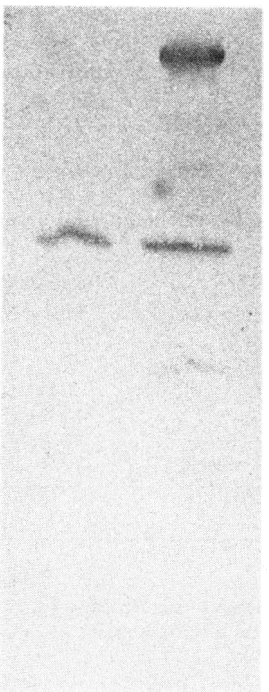

Fig. 2. *High-Ca^{2+} and low-Ca^{2+} forms of the Ca^{2+}-activated proteinase*

The high-Ca^{2+} and low-Ca^{2+} forms of the Ca^{2+}-activated proteinase from rat skeletal muscle were separated on DEAE-Sephacel (Croall & DeMartino, 1983), and obtained in an activity ratio of approx. 4:1. Samples from the peak fraction of each form were subjected to protein blotting and immunodetection with antibody 177. The left-hand lane contained the low-Ca^{2+} form (0.2 unit), and the right-hand lane the high-Ca^{2+} form (0.6 unit).

the crude tissue supernatants did, the plots in both cases of ^{125}I radioactivity bound in the immunoassay wells against log (dilution) were nonetheless parallel. Under the conditions of assay, the maximum binding was 50% of the added radioactivity, and the radioactivity bound in the controls or at extreme dilution of any sample was less than 2% of the added radioactivity. Recovery of pure standard proteinase mixed with a tissue supernatant before immunoassay was 100%; when the standard was added to a tissue sample before homogenization, the recovery was 87%. The smallest amount of Ca^{2+}-activated proteinase distinguishable from zero in this assay was approx. 0.1 unit/ml, which is of the order of 1.25 pmol. In practice, most tissues contained between 10 and 100 units/g wet wt., corresponding to 1–10 units/ml in the extract supernatant. The immunoassay results are reported in terms of the catalytic activity of the pure standard proteinase, although the assay actually measures enzyme protein and not activity.

Tissue distribution

The amounts of proteinase detected in various tissues are shown in Table 1. No differences were observed between pregnant and non-pregnant animals in tissues other than uterus. The data shown are those observed on the first occasion of assay: at the end of the series, most of the extracts were re-assayed together in a single set, after storage for various periods at −70°C, and with few exceptions the re-assay results were consistent with the original values.

Rat blood was collected in the presence of heparin, and the total blood cells were separated by centrifugation. A trace of Ca^{2+}-activated proteinase was detected in the cells, but not in the plasma, both by immunoassay and on a blot, in accordance with the reported presence of the enzyme in platelets (Phillips & Jakabova, 1977; Truglia & Stracher, 1981) and in erythrocytes (Ahkong *et al.*, 1980), but the amounts were not quantified. The low blood value indicates that blood contamination of the tissue samples could not affect the validity of the tissue results.

Pregnant and involuting uterus

The amounts of proteinase detected in the uterus during pregnancy and involution are shown in Table 2, together with the wet-weight data. To permit comparison with the results of similar studies which have been published previously in several different forms, the data in Table 2 are given both as units/uterus and as units/g wet wt. When expressed as units/mg of non-collagen protein, the data exhibited the same pattern as those in units/g wet wt. The data shown are those obtained on the first occasion of assay in two series of pregnant rats, over several months. The re-assays, all performed on a single day, gave the same pattern of the data.

The mean Ca^{2+}-activated proteinase activity per uterus at term was 22 times the mean of the non-pregnant uterine activity, whereas the wet weight of the uterus increased only 8-fold over the same period. During involution, although both wet weight and proteinase activity fell rapidly, the

Table 1. *Ca^{2+}-activated proteinase in rat tissues*
The data are given in units/g wet wt. of tissue, as means ± S.D., for the numbers of animals shown in parentheses.

Tissue	Proteinase activity
Lung	95.3 ± 24.6 (18)
Kidney	41.8 ± 12.3 (18)
Small intestine	41.3 ± 12.0 (4)
Liver	20 (1)
Brain	13.8 ± 2.8 (4)
Heart	13.3 ± 4.2 (4)
Skeletal muscle	12.3 ± 2.9 (4)
Blood cells	Trace (2)

Table 2. *Ca^{2+}-activated proteinase in rat uterus*
The proteinase activities (means ± S.D.) are listed both in units/g wet wt. and in units/uterus. The numbers of animals included in each measurement are shown in parentheses. When only two animals were included, only the mean value is shown.

Day	Wet wt. (g)	Proteinase activity (units/g wet wt.)	Proteinase activity (units/uterus)
Non-pregnant (3)	0.51 ± 0.15	28.6 ± 7.0	13.8 ± 1.35
14 days pregnant (3)	1.17 ± 0.29	39.6 ± 8.3	71.8 ± 20.6
15 days pregnant (4)	2.05 ± 0.52	41.3 ± 1.0	84.2 ± 19.5
17 days pregnant (2)	3.97	41.8	165.8
20 days pregnant (3)	3.89 ± 0.25	77.0 ± 21.3	302.2 ± 98.5
1 day *post partum* (3)	2.74 ± 0.20	116.1 ± 23.1	316.0 ± 48.3
2 days *post partum* (4)	2.00 ± 0.37	84.9 ± 11.4	167.3 ± 21.5
3 days *post partum* (2)	1.28	67.5	86.4
4 days *post partum* (3)	0.62 ± 0.23	67.7 ± 3.1	41.8 ± 14.9
5 days *post partum* (3)	0.46 ± 0.05	65.1 ± 29.1	28.6 ± 6.1

units/g wet wt. column in Table 2 shows that the proteinase activity remained relatively high; even at 5 days *post partum*, when the wet weight had returned to that for non-pregnant animals, the proteinase activity was twice that for the non-pregnant animals.

The amounts of DNA in the uterus were not measured during the present work. From earlier DNA measurements (Afting & Elce, 1978), it may be estimated that the mean Ca^{2+}-activated proteinase activity per mg of DNA increased 11-fold during pregnancy.

Discussion

Specificity of the 177 antibody

As described in the Results section, the affinity-purified 177 antibody was specific for M_r-78000 and -30000 proteins in the total soluble protein extracted from several rat tissues (Fig. 1); IgG eluted from the larger protein on a blot did not rebind to the smaller; and the antibody bound to an M_r-30000 protein, but not to an M_r-80000 protein in the low-Ca^{2+}-requiring form of the enzyme (Fig. 2). Apart from confirming the report by Wheelock (1982), that the two forms of the enzyme are immunologically different, these results indicate that incubation of 628 antiserum with partially purified high-Ca^{2+}-requiring proteinase gave rise to an immunoprecipitate which contained both enzyme subunits. On inoculation into fresh rabbits, this immunoprecipitate therefore gave rise to two separate antibody populations. The thiopropyl-Sepharose–proteinase column used to purify the anti-proteinase IgG, although enriched in M_r-78000 protein, clearly also contained sufficient M_r-30000 protein to retain some of its corresponding IgG.

Given this specificity of the antibody, it is probable that the immunoassay results reflect only the amounts of the high-Ca^{2+} form of the Ca^{2+}-activated proteinase. This assumption would be wrong if considerable variation occurred in the amounts of the low-Ca^{2+} form, or of the M_r-30000 subunit alone, without variation in the high-Ca^{2+} M_r-78000 subunit. This is unlikely, since the two subunits appeared to be present at least approximately in a constant ratio in all tissues. Any remaining uncertainty probably cannot be resolved without monoclonal antibodies.

Quantitative distribution of the proteinase in rat tissues

The Ca^{2+}-activated proteinase, as expected, was immunologically identical in all tissues tested (Fig. 1), suggesting some general function which is not yet clear. The quantitative distribution (Table 1) showed that lung contains much the highest amount of the enzyme, that smooth muscle and kidney have intermediate amounts, and that skeletal muscle, cardiac muscle and brain have low amounts.

There is a rough agreement between the distribution reported in Table 1, and that reported previously, also in rat tissues (Murachi *et al.*, 1981), except that our value for lung is much greater. The values reported by Azanza *et al.* (1981) for some rabbit tissues do not appear to agree with ours. Both of these previous reports depended on enzyme assay after some purification steps, and it is possible that the results were affected by losses on columns and by incomplete removal of proteinase inhibitors.

An explanation of the physiological role of the Ca^{2+}-activated proteinase must take into account the quantitative distribution shown in Table 1, although the effective activity of the enzyme *in vivo* is difficult to estimate, since regulation by thiol-proteinase inhibitors, by compartmentation and membrane binding, and by a possible role of high-Ca^{2+} and low-Ca^{2+} forms, must be considered.

Specific roles and substrates for the proteinase in particular tissues have been argued by many workers, such as oestrogen receptors in uterus (Puca *et al.*, 1977), progesterone receptors in oviduct (Vedeckis *et al.*, 1980), platelet proteins (Fox *et al.*, 1983) and neurofilament proteins (Baudry & Lynch, 1980; Burgoyne & Cumming, 1982; Ishizaki *et al.*, 1983), in addition to the original suggestion of a role in muscle Z-line degradation (Reddy *et al.*, 1975; Dayton *et al.*, 1976). In none of these cases has it yet been unequivocally shown that the proteinase and the proposed substrate have access to each other and to sufficient Ca^{2+} in the native tissue.

There appears, however, to be some interesting correlation between the proteinase amounts reported here, and the relative content of stroma in the various tissues, which is consistent with the earlier suggestion from our laboratory that the proteinase in skeletal and cardiac muscle appeared to be membrane-bound or even extracellular (Barth & Elce, 1981). (The word 'stroma' means the connective-tissue framework, which consists of collagen and elastin fibres, glycosaminoglycans, and the ground substance closely surrounding cells; it includes also several different connective-tissue cell types.) In the rat muscle denervation study (Elce *et al.*, 1983), it was suggested that the rather small increase in Ca^{2+}-activated proteinase activity could also be explained in terms of the increase in membrane content of muscle which follows denervation. Lung, with the highest proteinase activity, possesses also the highest proportion of stroma; brain and skeletal muscle, with the least proteinase activity, also possess the least stroma, among the tissues that we have investigated (R. Kisilevsky, personal communication). We have not yet found published data on the pro-

portion of extracellular connective tissue in various organs, to pursue this correlation further, but immunohistochemical studies in several different tissues are clearly desirable.

Uterine involution

In some earlier, similar, experiments (Afting *et al.*, 1979), activities of an acid proteinase and an acid phosphatase increased by about the same degree as the wet weight, and that of a neutral proteinase did not increase during pregnancy. The increase observed in the Ca^{2+}-activated proteinase activity in the uterus during pregnancy, which was about 2.75 times that of the wet weight, is therefore much greater than the increases in any other hydrolases so far measured, and is matched only by the increase in a soluble proteinase inhibitor (Afting *et al.*, 1979).

During involution, the Ca^{2+}-activated proteinase activity stayed relatively high with respect to the wet weight, a pattern which has been observed for some other uterine hydrolase activities (Woessner, 1965; Etherington, 1973; Afting *et al.*, 1979; Sellers & Woessner, 1980). These high proteinase activities during involution have usually been considered to imply a role in tissue resorption, although a rigorous proof of this assumption is hard to devise.

It is clear that several proteinases must be involved in the extensive loss of intracellular and extracellular protein during the first few days of involution. At present we are pursuing the hypothesis that the Ca^{2+}-activated proteinase is effectively extracellular, and might therefore be involved in metabolism of the extracellular matrix. Both to prove this point, and also to determine the relative contribution to involution of lysosomal and non-lysosomal proteinases, and of proteinases imported into the myometrium with macrophages (Parakkal, 1969), will require further immunohistochemistry.

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