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The calpain–calpastatin system in vascular smooth muscle

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Vascular smooth muscle contains large amounts of the Ca^{2+}-dependent protease calpain II. In this study, we compared bovine aortic muscle (muscle phenotype) to cultured bovine aortic cells of smooth muscle origin (modulated phenotype) with respect to major constituents of the calpain–calpastatin system. Bovine aortic muscle contained only calpain II by activity measurements, Western blot of tissue extracts and Northern blot of poly(A)$^+$ RNA. On the other hand, using the same methodologies, both calpains I and II as well as the 110 kDa inhibitor protein, calpastatin, were identified in cultured bovine aortic cells of smooth muscle origin. We conclude that the phenotypic state of smooth muscle cells is associated with differential expression of major components of the calpain–calpastatin system. Moreover, bovine aortic muscle is the only tissue identified to date that contains calpain II exclusively.

Calpain; Calpastatin; Vascular smooth muscle

1. INTRODUCTION

The calpains are Ca^{2+}-dependent thiol proteases that are widely distributed in mammalian tissues [1]. Two major enzymes have been identified: calpain I and calpain II. Both are heterodimers which contain a common 30 kDa regulatory subunit and unique catalytic subunits of approximately 80 kDa that are products of distinct genes [2]. Calpain I is highly sensitive to Ca^{2+} and is the only calpain found in human platelets and erythrocytes [1]. Calpain II is found in many tissues, especially liver and muscle, and is relatively insensitive to Ca^{2+}, requiring concentrations in the millimolar range for full activity [2].

Calpastatin is a highly specific inhibitor of both calpains I and II [3]. It, too, is widely distributed in mammalian tissues and cDNA's encoding the human liver, rabbit skeletal and porcine cardiac calpastatins have been sequenced [4,5]. Calpastatin possesses four inhibitory domains (except the erythrocyte calpastatin which has only three) which accounts for the maximal inhibitory stoichiometry of 1 calpastatin molecule per 4 calpain molecules [4,5].

Although the calpain–calpastatin systems has been implicated in a number of cellular processes, increasing evidence suggests a modulatory role in cellular proliferation [6,7]. For example, cell permeant inhibitors of the calpains inhibit mitosis [8]. Calpain II has been shown to bind to metaphase chromosomes [7]. Quite recently, calpain II has been shown to degrade the meiotic cell cycle progression factor and metaphase inhibitor, c-mos, an event which permits progress into anaphase [9].

Vascular smooth muscle contains relatively large amounts of calpain II [10]. Moreover, this muscle can undergo a marked proliferative response as a reaction to arterial injury [11]. As part of a continuing effort to define the function of the calpain–calpastatin system in vascular smooth muscle, we report that calpain II is the exclusive isoform present in the contractile phenotype of bovine aortic smooth muscle, which is also devoid of calpastatin. In addition, our results also show that cultured bovine aortic cells of smooth muscle origin (modulated phenotype) contain calpain I and II as well as calpastatin.

2. EXPERIMENTAL

2.1. *Materials*

Most chemical reagents were purchased from the Sigma Chemical Company. Supplies for electrophoresis and Western blotting were purchased from Bio-Rad, Schleicher and Schuell and New England Nuclear. Reagents and other materials for molecular biology were obtained from BRL, United States Biochemical, Pharmacia, Promega and Stratagene.

2.2. *Methods*

2.2.1. *Protein purification and assays.* Calpain II was purified from bovine aortic smooth muscle as described previously [12]. Calpain I was purified from bovine erythrocytes as described by Ando et al. [13]. Calpain activity was measured as trichloroacetic acid-soluble peptides liberated from [α-^{3}H]casein under linear reaction conditions [12]. Calpastatin activity was measured as % inhibition of TCA-soluble peptides released from [α-^{3}H]casein by 1.5 μg of calpein II under standard assay conditions. Fresh bovine aortic muscle or cultured cells were

Abbreviations: MOPS, 4-morpholinepropanesulfonic acid; EGTA, [ethylene bis (oxyethylenenitrilo)] tetraacetic acid; EDTA, ethylenediamine tetraacetic acid.

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extracted in 5 volumes of 20 mM MOPS, pH 7.0, 10 mM EGTA, 5 mM EDTA, 1 mM dithiothreitol and 0.5% Triton X-100, sonicated and sedimented (15 000 × *g* for 20 min). The supernatants were applied to 50 (muscle) or 20 (cells) ml columns of DEAE-Sephacel equilibrated in 20 mM MOPS, pH 7.0, 1 mM EGTA, 1 mM EDTA and 1 mM dithiothreitol. Fractions of 5 ml or 2 ml, respectively, were collected by elution with a NaCl gradient (0–0.5 M) in column equilibration buffer. In addition to activity measurements, the calpains and calpastatin were identified by Western blots of 30 μl fractions applied to 7.5% Porzio gels [14]. Antisera to calpains I and II were produced in rabbits [15]. Antiserum to calpastatin was a gift from Dr Darrel Goll (University of Arizona).

2.2.2. *Molecular and cellular biology methods.* Bovine aortic smooth muscle cells were generated by the explantation method [16] and grown to confluence in Dulbecco's Modified Eagles Medium. The smooth muscle origin of these cells was confirmed by immunofluorescence using the anti-α actin antibody, HHF-21 [17]. A cDNA encoding 1.5 kb of calpain I was cloned and sequenced from a rabbit brain λgt11 library [18]. cDNA's for the calpain II catalytic subunit and calpastatin were prepared by the polymerase chain reaction (PCR). For calpain II, poly(A)$^+$ RNA was isolated from fresh bovine aorta, and cDNA synthesis and amplification were performed as described by others [19]. PCR probes were synthesized as: (+) strand to nucleotides 1–24 and (−) strand to nucleotides 991–1017 in reference. Calpastatin cDNA was prepared from rabbit liver poly (A)$^+$ RNA. PCR probes for the liver were: (+) strand to nucleotides 100–123 and (−) strand to nucleotides 1123–1149 in reference [21]. Poly(A)$^+$ RNA was isolated from bovine aorta or cultured cells (1 × 10^7 cells) for Northern analysis using cDNA's labeled by nick translation [22].

3. RESULTS

Elution profiles of extracts of bovine aortic smooth muscle or cultured bovine aortic cells are shown in Fig. 1. By activity measurements, only a single peak of calpain activity was identified in bovine aortic muscle eluted from the DEAE-Sephacel column at an NaCl concentration of about 0.3 M. No calpastatin or calpain I activity was measured. In addition to calpain II activity, the extract of cultured bovine aortic cells yielded calpastatin activity and a small peak of calpain I. As in other reported purification schemes, overlap of calpastatin and calpain I activities was evident.

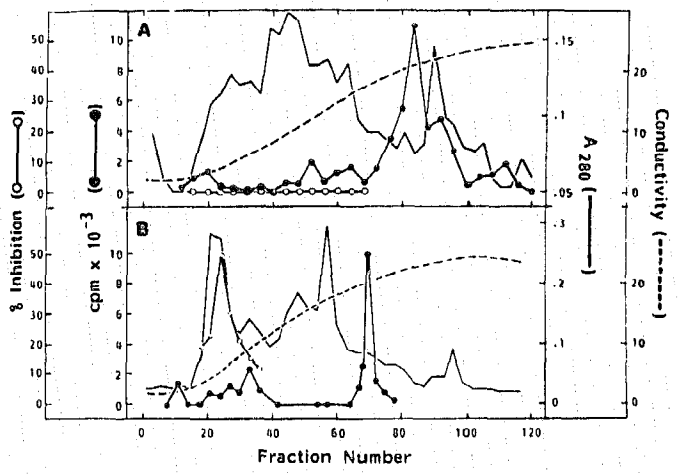

Fig. 1. DEAE-Sephacel chromatography of extracts of bovine aortic muscle (A) or cultured bovine aortic cells (B). Extracts were prepared and assayed for activity as described in section 2. Approximately 100 fractions of either 5 (A) or 2 (B) ml were collected for each run.

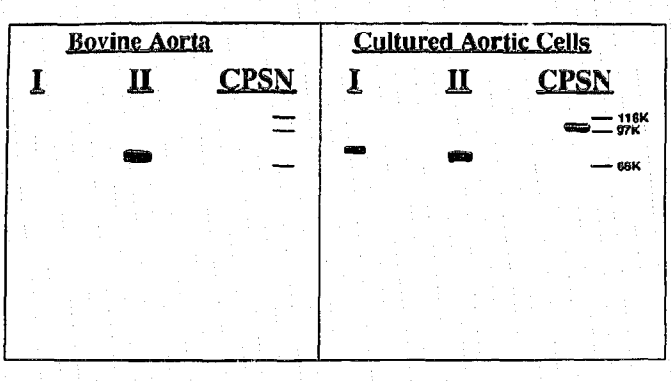

Fig. 2. Western blots of fractions from the DEAE-Sephacel columns. Approximately 30 μl of every third fraction was subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose. The sheets were then incubated with antisera to the catalytic subunits of calpain I (1:5000), calpain II (1:5000) or calpastatin (1:2500) according to a protocol previously published [26]. The lanes of the autoradiogram shown in Fig. 2 correspond to actual or theoretical peaks. For bovine aorta, these are: calpain I, fraction 46; calpain II, fraction 84; calpastatin, fraction 29. For cultured bovine aortic cells, these are: calpain I, fraction 38; calpain II, fraction 69; calpastatin, fraction 24.

Verification of results was obtained by Western blotting of fractions from the DEAE-Sephacel column using antisera specific for calpain catalytic subunits and for calpastatin. Those fractions corresponding to the peak identified by antibody reactivity are shown in Fig. 2. For both bovine aortic muscle and cultured cells, the peak fraction of calpain II identified by Western blot corresponded exactly to the peak fraction identified by activity measurements. Calpain I could not be identified by Western blot in any fraction of vascular muscle. On the other hand, calpain I was detected in the column eluate of cultured bovine aortic cells. The peak identified by activity measurements was clearly distorted due

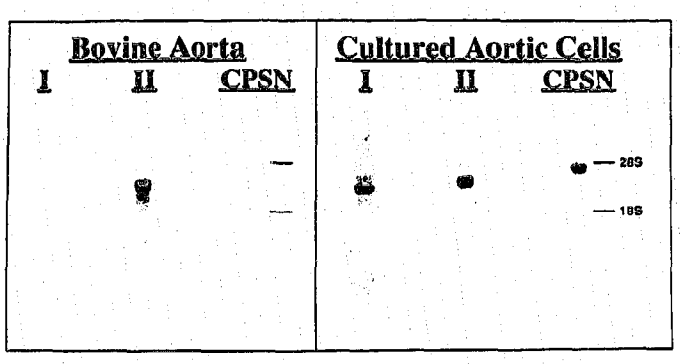

Fig. 3.Northern blots of poly(A)$^+$ RNA from bovine aortic muscle or cultured bovine aortic cells. Approximately 2 μg of poly(A)$^+$ RNA prepared from aortic muscle or cells as described under the section, Experimental, was subjected to agarose gel electrophoresis in formaldehyde buffer and blotted onto nitrocellulose [22]. cDNA species, corresponding to the catalytic subunits of calpains I and II and calpastatin and radiolabeled by nick translation, were hybridized to the mRNA as described by others [22].

to overlap with calpastatin (and vice versa) since separation of these proteins by chromatography on DEAE-Sephacel is incomplete. Confirming the activity measurements, there was no immunological evidence for calpastatin in bovine vascular muscle.

Northern blot analysis of poly(A)$^+$ RNA prepared from either bovine aortic muscle or cultured cells is shown in Fig. 3. Although no attempt was made to quantitate the signal for each of the calpain–calpastatin species present, under identical conditions, only calpain II mRNA was present in muscle while mRNA species for calpains I, II and calpastatin were present in the cultured cells. These results were confirmatory of both activity measurements and Western blots.

4. DISCUSSION

Our study demonstrates that contractile (muscle) and cultured (modulated) phenotypes of bovine aortic smooth muscle express different components of the calpain– calpastatin system. Recent studies from our laboratory have shown that cell permeant tripeptide aldehyde inhibitors of calpains I and II are potent antimitogens [23]. These results are, in general, complementary to the findings of Schollmeyer who showed that calpain II facilitated metaphase to anaphase transition by binding to chromosomes [7]. However, many complex issues remain including the physiological modulation of Ca^{2+} sensitivity of calpain II, the mode of activation of either calpain I or II and an explanation of how calpastatin inhibition of the calpains is circumvented. Moreover, with the exception of c-mos inactivation by calpain II in meiotic cells [9], specific substrates that are targets for activated calpain during cell growth and proliferation remain to be identified.

The situation in vascular muscle is further complicated by segregation of the expression of the calpain–calpastatin system. Vascular calpain II can degrade myosin in vitro but the Ca^{2+} concentrations required for activation of the enzyme are quite high (e.g. > 150 μM) [24]. Certain cytoskeletal elements such as dense bodies, membrane plaques, filamin and intermediate filaments have been shown to be sensitive to calpain proteolysis in chemically-skinned smooth muscle [25]. Thus, it is possible that calpain II could be involved in contractile or cytoskeletal protein turnover or in major changes that occur in vascular myocytes in response to growth factor stimulation.

It is further interesting that calpastatin expression can be segregated from that of calpain II as demonstrated by this study. This does not conclusively establish that calpastatin is unessential for regulation of calpain II. On the other hand, no one has identified a tissue that expresses calpain I that is devoid of calpastatin. Thus, it is possible that the major function of calpastatin is to regulate proteolytic activity of calpain I.

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