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The Calpain-Calpastatin System and the Calcium Paradox in the Isolated Perfused Pigeon Heart

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Key Words

Calpain • Calcium paradox •Calpastatin •Pigeon heart • Avian heart •Protection

Abstract

To examine whether the calpain-calpastatin system is activated during the calcium paradox in the isolated perfused pigeon heart, we separated the protease from its inhibitor calpastatin and studied its kinetic properties. The protease exhibits kinetic properties similar to those of mammalian m-calpains. Ca^{2+} requirements for half and maximum activities are 220 μM and 2 mM, respectively. In the absence of Ca^{2+} the protease is strongly activated by Mn^{2+} or Sr^{2+}. In the presence of Ca^{2+}, Mn^{2+} and Sr^{2+} exhibit a synergistic effect; Mg^{2+} and Ba^{2+} have no effect, whereas Co^{2+}, Ni^{2+} and Cd^{2+} completely inhibit its activation. Furthermore, we measured the activity of calpain and calpastatin under either conditions inducing a calcium paradox, or protecting the heart against this phenomenon. Although the calpain/calpastatin ratio is lowered during Ca^{2+} depletion, during Ca^{2+} repletion it is markedly inverted. Calpain activation during reperfusion is inhibited by the presence of 200 μM Mn^{2+} or Ba^{2+}, in the Ca^{2+}-free medium. Gel filtration of calpastatin, isolated from either untreated hearts or during Ca^{2+} depletion, produces two main peaks of ~150 and 40 kDa of molecular mass, respectively, whereas calpastatin isolated during the 2nd min of reperfusion appears to be shifted to the 150 kDa form. All the above data suggest that this system may be involved in the induction of the calcium paradox in pigeon heart.

Introduction

Calpain (Ca^{2+} activated neutral protease), the cysteine protease that absolutely require calcium for catalytic activity, and its endogenous inhibitor calpastatin, have been implicated in many physiological and pathological conditions of increased protein degradation associated with increased intracellular Ca^{2+} concentration [1-5]. Altered calpain activities have also been measured in myocardial injury, due to hereditary cardiomyopathy [6] or ischaemia and reperfusion [7]. Upon autoproteolytic activation, the protease can selectively cleave a subset of cellular proteins including membrane

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receptors, cytoskeletal proteins, and transciption factors. Calpain is therefore well suited for executing messages conveyed by shifting intracellular calcium concentrations [4-5].

On the other hand, the Ca^{2+} paradox, namely the unexpected heart necrosis during Ca^{2+} repletion, following a short period of Ca^{2+} deprivation [8], is caused by a massive Ca^{2+} influx [9-10]. The Ca^{2+} paradox has been experimentally induced in mammalian [8], amphibian [11] and avian [12] myocardium and although the exact mechanisms are uncertain, some major points have been noted: First, a Ca^{2+} depletion results in an increase of membrane permeability for Ca^{2+} [13-15]. Second, the Ca^{2+} influx during reperfusion leads to the irreversible myocardial contraction (formation of contraction bands), which in turn, is succeeded by sarcolemmal ruptures, Ca^{2+} overload and eventually, cell death [16-20].

Calpain activation, resulting from the massive Ca^{2+} influx, could be accounted for at least some of these observations. First, Ca^{2+} overload has been correlated with Z lines dissolution and/or myofibrillar disruption [21-22] and calpain is localised to the Z lines of myofibrils [23]. Second, contraction bands have also been observed in post-ischaemic reperfusion of myocardium [24] and these conditions are known to alter calpain activity [7]. The common basis of these two phenomena (the calcium and the oxygen paradox) has already been underlined [25-26].

In our previous studies we had described the characterisation of the calcium paradox [12], as well as the protective effects of various divalent cations such as manganese, barium, nickel, and cobalt against this phenomenon in the isolated perfused pigeon heart (unpublished data). The results of those studies clearly showed that despite the fundamental structural and functional differences between mammalian and avian heart, the characteristics of this phenomenon induced upon Ca^{2+} repletion following a 40 min Ca^{2+} depletion, at normal body temperature (42 °C), are quite similar.

In the present study we examined the regulation of the calpain-calpastatin system under conditions inducing a calcium paradox as well as under conditions protecting the pigeon heart against this phenomenon and provide evidence for a potential role of this system in the calcium paradox.

Material and Methods

Animals

Isolated hearts of the pigeon *Columba livia* were used. Domestic animals were obtained from a commercial dealer and kept in the laboratory with free access to water and food. All animals received humane care in accordance to the Guidelines for the Care and Use of Laboratory Animals published by the Greek government (160/1991) based on EC regulations (86/609).

Chemicals

The following materials were purchased from the sources indicated: DEAE-cellulose DE-52 (Serva, Heidelberg, Germany); Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden); casein, Hammarsten grade (Merck, Darmstadt, Germany); E-64, leupeptin, antipain, N-ethylmaleimide (NEM), phenyl methyl sulfoxide (PMSF) (Sigma Chemical Co, St. Louis, U.S.A.). All other chemicals used were of analytical grade and purchased from Sigma Chemical Co.

Perfusion procedure

Pigeons (*Columba livia*) weighing 300-350 g were anaesthetised with 20-25 mg sodium pentobarbital and received heparin (400 IU) intravenously. The hearts were excised and mounted onto the aortic cannula of a conventional Langendorff perfusion system. All perfusions were carried out at a constant perfusion pressure of 90 cm H_2O and a flow of 15-18 ml/min. The normal perfusion medium was a Krebs-Henseleit's (KH) bicarbonate buffer which consisted of (in mM): 118 NaCl, 2.96 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 2 $CaCl_2$, 25 $NaHCO_3$, 10 glucose and 1 sodium pyruvate. The pH of the oxygenated KH buffer was adjusted at 42°C to 7.35-7.40. All KH buffers were equilibrated with 95% O_2-5% CO_2. In the calcium-free medium calcium was omitted and EGTA was added at a final concentration of 10 μM to ensure removal of any contaminant calcium. In different sets of experiments 200 μM of $BaCl_2$, or $MnCl_2$, along with 10 μM EGTA were added in the calcium free KH buffer. In all experiments an equilibration period of 15 min was allowed during which the hearts were perfused with the normal KH buffer. This was followed by a 40 min period of calcium depletion in the presence or absence of added divalent cations and finally by the reperfusion with standard KH buffer for increasing time intervals. At the end of perfusion, the hearts were rapidly frozen at -170 °C and the ventricles were removed and stored at -80 °C.

Partial purification of calpain

All purification procedures were carried out at 2-4 °C. The frozen ventricles (4-5 g of weight) were ground to powder under liquid nitrogen and homogenized in 5 volumes of Tris-EGTA (TE) buffer, which contained (in mM): 20 Tris-HCl, pH 7.4, 1 EGTA, 5 NaN_3 and 10 2-mercaptoethanol. The homogenate was centrifuged at 11,000 x g for 30 min and the supernatant was precipitated by a 30% ammonium sulfate saturation (16.4 g/100 ml), stirred for 30 min and centrifuged at 11,000 x g for 20 min. The supernatant was then brought to a 70% ammonium sulfate saturation (24.9 g/100 ml), stirred for 60 min and centrifuged at 11,000 x g for 20 min. The precipitate was suspended in TE buffer

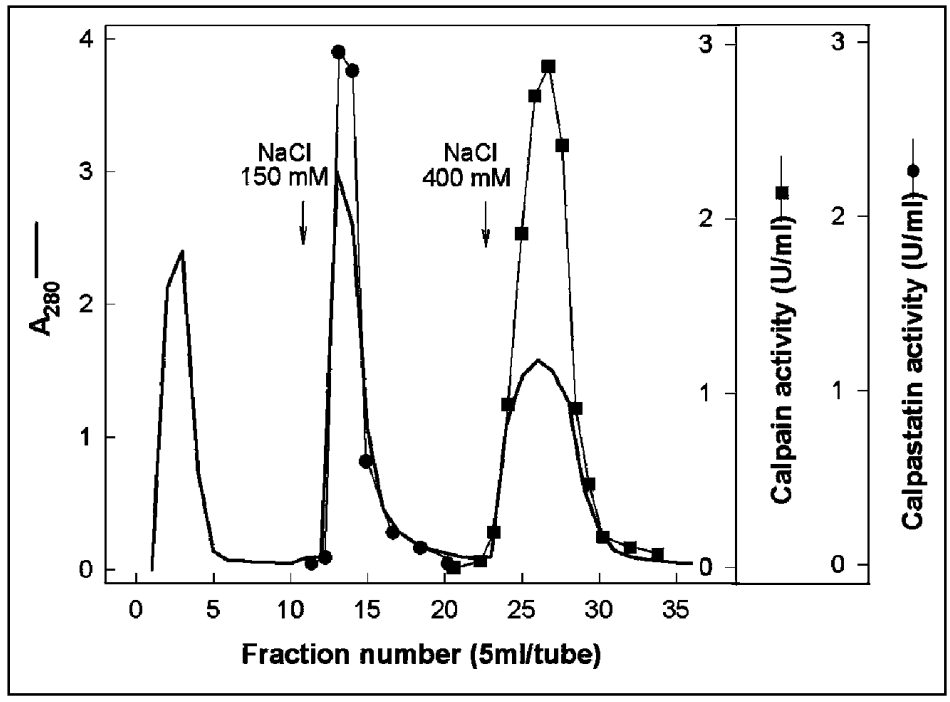


Fig. 1. Representative elution profile of calpain and calpastatin from the DEAE cellulose DE-52 column as determined by monitoring for protein and calpastatin and calpain activities. The arrows indicate the application time of the different, NaCl containing, TE buffer.

and salt excess was removed by overnight dialysis against an excess volume of the same buffer. The suspension was then loaded onto a DEAE cellulose DE 52 column (20 ml packed volume, equilibrated with TE buffer). After being washed with 60 ml of TE buffer, the column was eluted with 80 ml of TE buffer, which contained 150 mM NaCl followed by 80 ml of TE buffer containing 400 mM NaCl. The eluted fractions (5 ml) were monitored for protein (A_{280}) and calpain and calpastatin activities. The calpain active fractions were pooled, dialysed overnight in TE buffer and concentrated by ultrafiltration using an Amicon membrane (PM30). This final enzyme preparation was stored at -20 °C in 50% (v/v) glycerol for at most 3 weeks, without any loss of calpain activity during this period. Fractions of the final preparation were dialysed in 20 mM imidazole-HCl buffer, pH 7.4, 50 µM EGTA, 10 mM 2-mercaptoethanol and 10% (v/v) glycerol, a few hours before the assay of calpain activity was performed.

Subcellular fractionation

Homogenates (1 g of tissue), prepared as described above, were centrifuged at 11,000 x g for 30 min and the supernatants were then centrifuged at 100,000 x g for 60 min. The particulate fractions were suspended in TE buffer. The suspension of the 11,000 and 100,000 x g pellets and the 100,000 x g supernatants were subjected to DEAE cellulose columns (2 ml of packed volume, equilibrated with TE buffer). Fractions (1 ml) were collected as described above and monitored for calpain and calpastatin activities.

Protein determination

Protein was determined by the method of Lowry et al. [27], using bovine serum albumin as the standard.

Assay of calpain and calpastatin activity

Calpain activity was measured as a release of peptides from alkali denatured casein, as described by Ishiura et al. [28], with slight modifications [29]. The standard assay mixture (0.5 ml) contained 50 mM imidazole-HCl buffer, pH 7.4, 10 mM 2-mercaptoethanol, 5 mg/ml alkali denatured casein, 5 mM $CaCl_2$ and 20-100 µl of each sample. Standard assay mixtures without the sample or with 5 mM EGTA instead of $CaCl_2$ were used as blanks. The mixtures were incubated at 30 °C for 60 min and the reactions were terminated by the addition of 0.5 ml of 10% (w/v) trichloroacetic acid (TCA) solution. The samples were allowed to stand on ice for 30 min and then centrifuged at 11,000 x g for 8 min. The proteolytic products were measured at 750 nm by the method of Ross and Schatz [30]. For this, 0.2 ml of the supernatant was mixed with the reagents and diluted to a final volume of 3.25 ml. One unit of calpain activity was defined as an increase in A_{750} of 1.0 per ml of sample per hour. The determination of calpastatin activity was performed with a fixed amount of calpain and one unit of the inhibitor was defined as a decrease in A_{750} of 1.0 under standard assay conditions.

Gel filtration

Calpastatin molecular weight was assessed by gel filtration, performed as described by Whitaker [31], with slight modifications. Samples (2 ml final volume containing 0.5 M sucrose) from the calpastatin active fractions, eluted from the DEAE cellulose column, were applied to a Sephadex G-200 column (0.70 x 32.5 cm) and the eluted (with TE buffer, at 4 °C and ionic strength of 0.200) fractions (2 ml) were monitored for calpastatin activity. The void volume was determined by using Blue Dextran 2000, mixed with 0.5 M sucrose and calpastatin

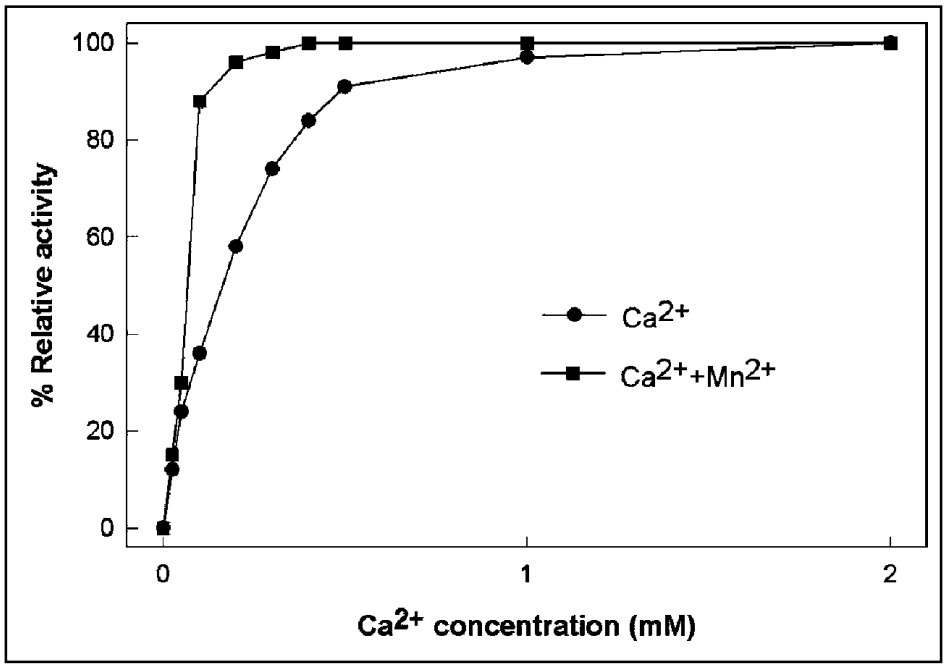


Fig. 2. Effect of Ca^{2+} concentration on calpain activity and synergistic effect of Mn^{2+}. The activity measured at 2 mM Ca^{2+} was considered as 100 %. SEM are less than 0.02% of mean values.

	% Relative calpain activity	
Cation	5mM	5mM + 1mM Ca^{2+}
Ca^{2+}	100	100
—	—	89±0.15
Sr^{2+}	84±0.35	97±0.42
Mn^{2+}	77±0.27	95±0.18
Ba^{2+}	30±0.12	90±0.22
Mg^{2+}	20±0.14	89±0.18
Co^{2+}	22±0.17	22±0.11
Ni^{2+}	19±0.11	17±0.12
Cd^{2+}	25±0.12	25±0.13

Table 1. Activation, inhibition of calpain activity, as well as synergistic effect of various divalent cations. The activity measured at 5 mM Ca^{2+} was considered as 100 %. Values are the mean±SEM of 5 determinations

molecular weight was estimated by using gel filtration protein markers: apoferritin (443 kDa), alcohol dehydrogenase (150 kDa) and carbonic anhydrase (29 kDa), also mixed with 0.5 M sucrose.

Data analysis

The results are presented as mean±SE of 4-5 independent experiments. The statistical significance was determined by using the Student's unpaired t-test at $P<0.05$ level of confidence.

Results

Chromatographic separation of calpain from calpastatin

A) Separation of calpain from calpastatin. Calpain activity, measured in myocardium extracts without the DEAE cellulose chromatographic procedure, was not detectable, due to the presence of calpastatin. The determination of calpain and calpastatin activities in the 11,000 x g supernatant eluted from the DEAE cellulose column, showed that calpastatin is eluted by 0-150 mM NaCl, in TE buffer, and completely separated from calpain, which is eluted by 150-400 mM NaCl, in the same buffer (Fig. 1).

B) Fractional localisation of calpain. The activity found at the 11,000 x g supernatant was the same with the activity measured at the 100,000 x g supernatant following the same chromatographic procedure, while there was no, detectable with the employed procedure, calpain or calpastatin activity at the 11,000 and 100,000 x g particulate fractions (data not shown).

Properties of partially purified calpain

A) Effect of various divalent cations. Incubation of calpain with increased Ca^{2+} concentrations showed that maximum and half-maximum calpain activities are attained at 2 mM and 220 μM Ca^{2+} respectively (Fig. 2). No activity is detected at 50 μM Ca^{2+} indicating that the

Inhibitor	IC_{50}
Leupeptin	0.438 ± 0.03 μM
E-64	1.630 ± 0.13 μM
Antipain	3.156 ± 0.23 μM
NEM	404.94 ± 49.2 μM
PMSF	5.391 ± 0.481 mM

Table 2. Required concentration of the inhibitors for 50% inhibition of calpain activity. Values are the mean ±SEM of four determinations.

Sample	Calpain (U/g)	Calpastatin (U/g)
Control	3.42 ± 0.19	4.77 ± 0.16
Ca^{2+} depletion		
20 min	3.96 ± 0.14	5.45 ± 0.20*
40 min	4.65 ± 0.28*	8.90 ± 0.47**
Mn^{2+} (1)	4.16 ± 0.56	8.59 ± 0.38**
Ba^{2+} (1)	4.57 ± 0.38	8.56 ± 0.52**
Ca^{2+} repletion		
30 sec	6.73 ± 0.29**	4.53 ± 0.21
1 min	7.38 ± 0.45**	4.63 ± 0.11
2 min	7.89 ± 0.27**	7.38 ± 0.51**
Mn^{2+} (1)	4.68 ± 0.56	5.79 ± 0.30*
Ba^{2+} (1)	5.77 ± 0.67*	7.81 ± 0.21**
3 min.	3.56 ± 0.28	4.18 ± 0.16

Table 3. Effect of Ca^{2+} depletion and reperfusion on calpain and calpastatin total activities measured in the DEAE cellulose fractions. Values are mean± SEM of four different determinations. *, P<0.05; **, P<0.001 versus control. (1) 200 μM of each cation were added during Ca^{2+} depletion.

enzyme is a millimolar isoform (m-calpain). As shown in Table 1, at 5 mM, only Mn^{2+} and Sr^{2+}, activate calpain, substituting for Ca^{2+}, which activates calpain to a maximum extent. Furthermore, in the presence of 1 mM Ca^{2+}, Mn^{2+} and Sr^{2+}, show a synergistic effect (Table 1). Among the other cations that do not activate calpain, Mg^{2+} and Ba^{2+} do not inhibit calpain activation by Ca^{2+} whereas Co^{2+}, Ni^{2+} and Cd^{2+} completely inhibit calpain activation, showing an effect of inhibition by this group of cations.

In the presence of increased Ca^{2+} concentrations (Fig. 2), Mn^{2+} (400 μM) causes a decrease in Ca^{2+} requirement, from 220 μM to 160 μM and from 2.0 to 0.5 mM (for half-maximum and maximum activity, respectively). Neither cation showed any synergistic effect at 200 or 300 μM (data not shown).

B) Effect of various protease inhibitors. In order to study the effect of various protease inhibitors on calpain activity, standard assay conditions were used. Inhibition grade was expressed as a % decrease in calpain activity, considering the activity measured in the absence of the inhibitors as 100%. Thiol-protease inhibitors, such as E-64, leupeptin and antipain, strongly inhibit calpain activity (Table 2), at a concentration lower than 10 μM. The alkylating reagent, N-ethylmaleimide (NEM), also inhibits calpain activity but at a much higher concentration (1 mM). PMSF even at 3 mM, has no significant inhibitory effect (Table 2), indicating that this enzyme is a member of the thiol-protease group.

C) Effect of the Ca^{2+} paradox.. The effect of the Ca^{2+} paradox on calpain activity was tested by perfusion of the hearts for 40 min with a Ca^{2+}-free medium, in the presence or absence of 200 μM Mn^{2+} or Ba^{2+}, and subsequent reperfusion with the Ca^{2+} containing KH buffer. The hearts were frozen in specific intervals and calpain activity was measured.

In Table 3 calpain and calpastatin total activities measured at the DEAE cellulose fraction, eluted by 400 and 150 mM NaCl, respectively are shown. During Ca^{2+} depletion both enzyme and its endogenous inhibitor (to a larger extent) activities linearly increase. The presence of Mn^{2+} or Ba^{2+} during this period has no effect on the observed calpain and calpastatin activation.

During calcium repletion followed a 40 min calcium depletion however, calpain activity is continuously raised to a maximum attained at the 2nd min of reperfusion, while calpastatin activity is lowered at the first 30 sec of reperfusion to be raised to a maximum also attained at the 2nd min (Table 3). Calpain activation during reperfusion is inhibited by the presence of 200 μM Mn^{2+}

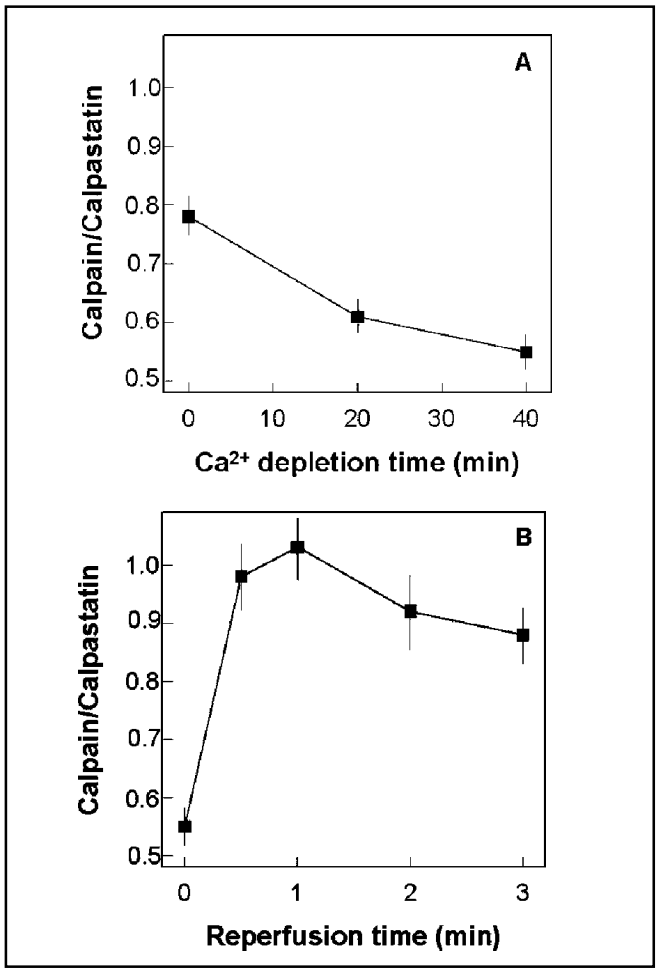


Fig. 3. The alteration of calpain/calpastatin ratio during Ca^{2+} depletion (A) and reperfusion (B). After a relative decrease during the first period the ratio is inverted at the second period with calpain exceeding calpastatin.

or Ba^{2+}, in the Ca^{2+}-free medium, while calpastatin activation is inhibited only by Mn^{2+}.

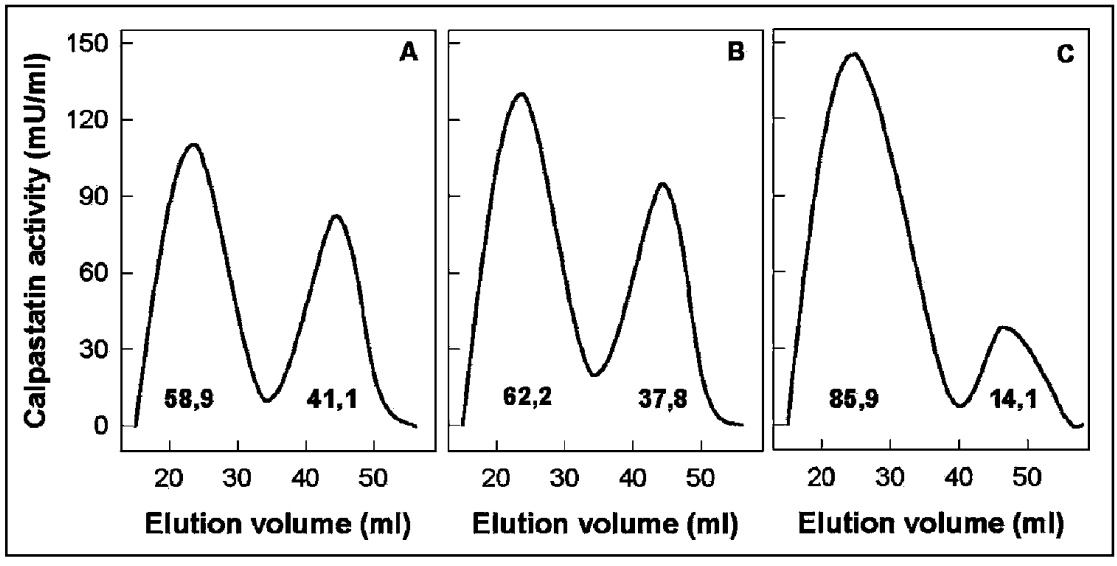
The presence of Mn^{2+} or Ba^{2+}, at 200 μM, during Ca^{2+} depletion, powerfully protects the heart (recovery of mechanical and maintenance of the electrical activity of the heart) against the induction of the Ca^{2+} paradox (Gaitanaki et al., unpublished data).

Although calpain/calpastatin ratio is lowered during Ca^{2+} depletion, during reperfusion it is markedly inverted, with the maximum value attained at the first min of reperfusion (Fig. 3).

D) Calpastatin molecular mass. In order to estimate whether the alteration of calpastatin activity is mainly due to diffusional loss and/or intracellular translocation, or it is the result of affected equilibrium between two different calpastatin forms, we assessed calpastatin molecular mass during Ca^{2+} depletion and reperfusion.

Gel filtration of calpastatin, isolated from untreated hearts, produces two main peaks of calpastatin activity (Fig. 4A) at 150 and 40 kDa approximate molecular mass, respectively. The recovery of calpastatin activity was in every case over 98%. Calpastatin isolated at the end of Ca^{2+} depletion shows an almost identical elution pattern (Fig. 4B), while the ratio of the two different molecular mass forms, of calpastatin isolated at the 2nd min of reperfusion, appears to be shifted to the 150 kDa form (Fig. 4C).

Fig. 4. Elution pattern on gel filtration, of calpastatin isolated from control (A), Ca^{2+} depleted (B), and reperfused (C) hearts. The numbers under the peaks indicate the (%) ratio of each calpastatin form.



Discussion

The alteration of calpain and calpastatin activities, demonstrated by this study, during Ca^{2+} depletion and reperfusion, indicates that this Ca^{2+} activated neutral protease system might be implicated in the Ca^{2+} paradox.

The millimolar form of calpain, isolated from the pigeon myocardium by ion exchange chromatography (Fig. 1), is activated by high Mn^{2+} and Ba^{2+} concentration (Table 1), similarly to m-calpain from other sources [29, 32], but differently to others [28, 33]. The synergistic effect of Mn^{2+} and Ca^{2+} (Fig. 2, Table 1) is also in agreement with other reports [34-35].

Measurements of calpain and calpastatin activities at the end of the Ca^{2+} depletion and during the Ca^{2+} repletion and comparison with those under physiological conditions (Table 3), shows that moderately the enzyme and mostly the inhibitor are activated after Ca^{2+} depletion. This activation is probably indirect, due to a decreased intracellular Ca^{2+} concentration, since the presence of Ca^{2+} is considered to be necessary for enzyme and inhibitor interaction [36-37]. Furthermore, a functionally active fraction of the endogenous inhibitor, known to be associated with membranes [38-39], could be released by structural changes of sarcolemmal phospholipids and carbohydrates, which are observed during Ca^{2+} depletion [40-41]. In particular, the plasma membrane phospholipid turnover has been shown to be stimulated in mammalian myocardium during ischaemia or reperfusion [42]. It seems therefore that an elevated phosphoinositol level may also contribute to the activation of the calpain-calpastatin system in our experimental model.

Calpain activity continuously increases (Table 3) during the first 2 minutes of reperfusion, probably due to a massive Ca^{2+} influx. Mn^{2+} and Ba^{2+}, at a concentration (200 μM) that powerfully protects the heart against the induction of a Ca^{2+} paradox and having no direct effect on calpain activity, appear to inhibit calpain activation during reperfusion, reducing, each with a different possible mechanism, the Ca^{2+} influx. Although the reduction of calpastatin activity at the first 30 sec of reperfusion (Table 3) probably indicates diffusional loss or association with membranes, the inhibitor is also activated at the 2nd min of reperfusion, without, however preventing the inversion of calpain/calpastatin ratio (Fig. 3). Mn^{2+} appears to inhibit calpastatin activation during reperfusion, while Ba^{2+} does not (Table 3); the protective action of each cation, against a Ca^{2+} paradox, is also considered to be resulting of a different mechanism [43-44]. Finally, the loss of calpain and calpastatin activities at the third min of reperfusion (Table 3) possibly coincides with irreversible damages of cardiac cells, following the massive Ca^{2+} influx.

Although calpastatin appears to migrate anomalously on gel filtration [45-46], two main peaks of inhibitory activity are found (Fig. 4). The assessed molecular masses of 150 and 40 kDa, are quite comparable to the two most commonly reported values: 120 and 70 kDa [5]. It is uncertain whether multiple forms of calpastatin exist *in vivo* or they are the product of differential susceptibility to proteolysis during purification procedure. Nevertheless, the altered ratio of the two forms, at the 2nd min of reperfusion, could also be explained by a possible association with membranes of the smaller calpastatin molecules at the first 30 sec of reperfusion.

In conclusion, the calpain system could possibly provide a basis for the elucidation of the Ca^{2+} paradox mechanism, either by degrading myofibrillar or cytoskeletal proteins, indispensable for conservation of contractile capability and sarcolemmal integrity or by activating Ca^{2+} dependent enzymes that initiate an irreversible, self destructive pathway. However, further studies on this protease and inhibitory protein system, with cell permeable, calpain specific inhibitors are necessary for the clarification of this system's participation in the Ca^{2+} paradox induction.

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