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Degradation of myofibrillar proteins by a calpain-like proteinase in the arm muscle of *Octopus vulgaris*

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Abstract The effects of a calpain-like proteinase (CaDP) isolated from the arm muscle of *Octopus vulgaris* on the myofibrils and myofibrillar proteins isolated from the same tissue were examined. Our studies clearly showed that treatment of intact myofibrils with CaDP in the presence of 5 mM Ca^{2+} results in the degradation of the major myofibrillar proteins myosin, paramyosin, and actin. From the isolated α- and β-paramyosins only β-paramyosin is degraded by CaDP in the presence of 5 mM Ca^{2+} producing three groups of polypeptides of 80, 75, and 60 kDa, respectively. The degradation rate depends on the proteinase to substrate ratio, temperature, and time of proteolysis and is inhibited by the endogenous CaDP inhibitory factor (CIF), as well as by various known cysteine proteinase inhibitors (E-64, leupeptin, and antipain). From the other myofibrillar proteins examined myosin, but not actin, is degraded by CaDP; myosin heavy chain (MHC, 200 kDa) is degraded by CaDP producing four groups of polypeptides of lower molecular masses (155, 125, 115, and 102 kDa, respectively); the degradation rate depends on the incubation time and the proteinase to substrate ratio. Furthermore, CaDP undergoes limited autolysis in the presence of both the exogenous casein and the endogenous β-paramyosin producing two large active fragments of 52 and 50.6 kDa, respectively; CIF reversibly inhibits this CaDP autolysis.

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Key words Marine invertebrate · *Octopus vulgaris* · Paramyosin · Myofibrillar protein · Calpain · Calpastatin · Autolysis · Protein degradation

Abbreviations *CaDP* Ca^{2+}-dependent proteinase · *DTT* dithiothreitol · *EGTA* ethyleneglycol-bis-(β-aminoethyl-ether) tetraacetate · *EDTA* ethylenediamino-tetra-acetate · *E-64* L-trans-epoxysuccinyl-leucylamide-4-guanidino-butane · *IAA* iododacetic acid · *IAM* iodoacetamide · *CIF* CaDP inhibitory factor

Introduction

Calpain [EC 3.4.22.17], a Ca^{2+}-dependent proteinase (CaDP) and its endogenous inhibitor (calpastatin) are widely distributed in vertebrate and invertebrate tissues (Murachi et al. 1981; Pontremoli and Meloni 1986; Suzuki et al. 1988; Croall and DeMartino 1991; Saido et al. 1994; Suzuki et al. 1995; Mykles 1998) and play an important role in various Ca^{2+}-regulated cellular functions in response to Ca^{2+} mobilisation. Initial studies on the calpains isolated from various vertebrate tissues had shown that these proteinases exist in two forms; μ-calpain (or calpain I) and m-calpain (or calpain II), showing low (micromolar) or high (millimolar) Ca^{2+} requirements for activation, respectively (Murachi et al. 1981; Pontremoli and Melloni 1986; Suzuki et al. 1988). Both μ- and m-calpain are heterodimers, each consisting of a large catalytic (~80 kDa) and a small regulatory (~30 kDa) subunit, which are possibly activated by autolysis of their subunits and seem to be present in all vertebrate cell types examined. Recent studies, however, have shown that calpains now constitute a large family with at least six distinct members (Saido et al. 1994; Suzuki et al. 1995). In addition to the ubiquitous μ- and m-calpains, tissue-specific or novel calpains have been identified in the stomach and the skeletal muscle (Sorimachi et al. 1989, 1993, 1994; Saido et al. 1994; Suzuki et al. 1995).

CaDPs from invertebrate tissues are not as well characterised as those from vertebrate tissues. CaDP activities have been identified in molluscs (Stafford and Yphantis 1972; Pant et al. 1979; Oldenburg and Hubbell 1990; Hatzizisis et al. 1996), crustaceans (Nosek and Crosland 1983; Mykles and Skinner 1982a, b, 1983, 1986; Mykles 1990, 1992; Mattson and Mykles 1993; Beyette and Mykles 1997), an annelid (Eagles et al. 1981), and insects (Pinter and Friedrich 1988; Muller and Spatz 1989; Muller and Atlfelder 1991; Pinter et al. 1992; Emori and Saigo 1994; Beyette et al. 1997). cDNAs encoding invertebrate CaDPs have been identified in *Schistosoma mansoni* (Anderson et al. 1991; Karcz et al. 1991; Siddiqui et al. 1993), *Drosophila melanogaster* (Emori and Saigo 1994; Theopold et al. 1995) and *Caenorhabditis elegans* (Wilson et al. 1994).

Comparative studies on the Ca^{2+} requirements of vertebrate and invertebrate calpains have shown that vertebrate calpains show higher sensitivity to Ca^{2+} than invertebrate CaDPs. More specifically, m-calpains from various vertebrate muscles seem to require approximately 0.3–0.7 mM of Ca^{2+} for half maximal activity and 1–1.5 mM of Ca^{2+} for maximal activity (for a review see Croall and DeMartino 1991) . On the contrary, calpain-like proteinases from various invertebrate tissues seem to require 1–1.5 mM of Ca^{2+} for half maximal and 5–10 mM of Ca^{2+} for maximal activity (for a review see Mykles 1998). The lower Ca^{2+} sensitivities of invertebrate CaDPs compared with the vertebrate calpains gives evidence that Ca^{2+} sensitivity of calpains becomes higher depending on the evolutionary step in vertebrates.

Although the studies on the kinetic and molecular characteristics of the calpains in vitro are enormous, very little is known about their real physiological roles in vivo. However, it is generally believed that they may play an important role in different calcium-activated events in cells. In the skeletal muscle of several vertebrates, they appear to play an important role in myofibril protein turnover (Sugita et al. 1980; Ishiura 1981; Kay et al. 1982; Barrett et al. 1991; Sargianos et al. 1996).

Endogenous proteins that have been shown to be substrates of the invertebrate CaDPs are cAMP-dependent protein kinase (Muller and Spatz 1989; Muller and Altfelder 1991), neurofilaments (Eagles et al. 1981), rhodopsin (Oldenburg and Hubbell 1990) and myofibrillar proteins (Stafford and Yphantis 1972; Mykles and Skinner 1982a, 1983; Mykles 1990; Mattson and Mykles 1993).

In our previous paper, we described the purification and characterisation of a calpain-like proteinase from the arm muscle of the marine invertebrate *Octopus vulgaris* (Hatzizisis et al. 1996). In particular, we showed that this proteinase has a native molecular mass of ~520 kDa, possibly consisting of eight similar, if not identical, subunits of ~65 kDa each, and exhibits biochemical properties quite similar to those described for CaDPs isolated from other invertebrate tissues. This proteinase undergoes a Ca^{2+}-dependent autolytic modification in the absence of substrate, producing two major fragments of 50.6 kDa, and 31.5 kDa, respectively. In this paper we describe the possible physiological function of CaDP in myofibrillar degradation in the arm muscle of *O. vulgaris*.

Materials and methods

Animals

Animals (*Octopus vulgaris*) with a weight of 500–1000 g were supplied by a local dealer, having been caught on the shores of the large Emvolon, near Thessaloniki, Greece. They were kept in containers in recirculating fresh seawater and used 24–48 h after arrival.

Chemicals

Unless indicated, all chemicals and reagents were purchased from Serva (Heidelberg, Germany) or Sigma (St. Louis, Mo., USA), in the highest grade available. Casein (Hammarsten grade) was obtained from Merck (Darmstadt, Germany). Proteinase inhibitors E-64, leupeptin, antipain, iodoacetic acid, and iodoacetamide were purchased from Sigma. All other reagents used were of analytical grade.

Preparation of CaDP

CaDP was purified from ~120 g of arm muscle of the marine invertebrate *O. vulgaris*, as previously described (Hatzizisis et al. 1996), and was stored in 20 mM imidazole/HCl buffer, pH 7.2, which contained 1 mM EDTA, 1 mM EGTA and 10 mM 2-mercaptoethanol, and 50% (v/v) glycerol at 4 °C. One unit of CaDP was defined as the amount of the proteinase which catalysed an increase of 1.0 absorbance unit at 750 nm in 30 min at 30 °C, using alkali denatured casein as substrate (Hatzizisis et al. 1996). The specific activity of the purified CaDP was 464 U/mg of protein. Storage of the isolated enzyme under these conditions did not affect its proteolytic activity for at least 6 months.

Preparation of the CaDP inhibitory factor

CaDP inhibitory factor (CIF) was partially purified from the arm muscle of *O. vulgaris* according to the method described by Murachi et al. (1981). The CIF fraction recovered from the first DEAE-Sepharose CL-6B ion exchange chromatography was heated at 95 °C for 30 min and centrifuged at 20,000 g in a Sorvall centrifuge (rotor type SS-34). The supernatant was collected and dialysed overnight against an excess of 50 mM imidazole/HCl buffer, pH 7.2, which contained 10 mM 2-mercaptoethanol. The dialysate was then concentrated by ultrafiltration using an Amicon (Danvers, Mass., USA) membrane (UM 2) and stored at −20 °C in 50% (v/v) glycerol. One unit of CIF is defined as the amount of the inhibitory factor that inhibits one unit of CaDP under standard assay conditions.

Preparation of myofibrils

Myofibrils were isolated from the arm muscle of *O. vulgaris* according to the method described by Szent-Gyorgyi et al. (1971).

Preparation of myosin

Myosin was prepared from the isolated myofibrils according to the method described by Szent-Gyorgyi et al. (1971).

Preparation of paramyosin

Paramyosin was isolated by the combination of two methods: (1) the selective extraction from the thick filaments according to that described by Szent-Gyorgyi et al. (1971), and (2) the ethanol extraction according to that described by Johnson et al. (1959), as modified by Stafford and Yphantis (1972). The method described by Stafford and Yphantis (1972) is identical to that described by Johnson et al. (1959) except that in all buffers used for the preparation of α-paramyosin 10 mM EDTA and 1 mM 2-mercaptoethanol are included, whereas for the preparation of β-paramyosin EDTA and 2-mercaptoethanol are omitted.

Preparation of G-actin

G-actin was prepared according to the method of Spudich and Watt (1971) as modified by Mykles and Skinner (1982a).

Effect of calpain on the isolated myofibrils

Myofibrils (30 μg of protein), were added to the assay mixture which contained 50 mM imidazole/HCl buffer, pH 7.2, 100 mM KCl, 5 mM $CaCl_2$, 10 mM 2-mercaptoethanol, in a total volume of 30 μl. The enzyme-to-substrate ratio was kept at 1:10 or 1:50 (w/w). The reaction was started by the addition of purified CaDP. The incubation was performed for 30 min at 30 °C, and the reaction was terminated by the addition of 0.33 volumes of SDS-PAGE sample buffer which contained 0.33 M Tris/HCl, pH 6.8, 10% (w/v) SDS, 13% (v/v) glycerol, 133 mM DTT, and 0.2 mg/ml bromophenol blue. The samples were boiled for 3 min and subjected to SDS-PAGE. As controls, myofibrils incubated (1) in the presence of 10 mM EDTA instead of $CaCl_2$, (2) in the presence of 5 mM $CaCl_2$, without CaDP, and (3) in the presence of 5 mM $CaCl_2$, with CaDP plus CIF, were included.

Effect of calpain on the isolated myofibrillar proteins

Isolated myofibrillar proteins from the arm muscle of *O. vulgaris* were tested as endogenous substrates of CaDP. For these experiments, a constant amount of each isolated myofibrillar protein (10 μg) was incubated under various conditions with CaDP, such as: varying calpain to substrate ratios, increasing incubation times and/or temperatures, and including various proteinase inhibitors. The reactions were terminated by the addition of 0.33 volumes of sample buffer for SDS-PAGE and the samples were boiled for 3 min and subjected to SDS-PAGE.

SDS-PAGE

Polyacrylamide slab gel electrophoresis in the presence of 0.1% (w/v) SDS was performed according to the method described by Laemmli (1970) with 10% separating and 5% stacking gels. Gels were fixed and stained for 6–8 h in 0.1% (w/v) Coomassie brilliant blue R-250, 50% (v/v) methanol, 10% (v/v) glacial acetic acid solution, and then destained with 50% (v/v) methanol – 10% (v/v) acetic acid solution. The molecular mass markers used were: α_2-macroglobulin (180 kDa), β-galactosidase (116 kDa), fructose-6-phosphate kinase (84 kDa), pyruvate kinase (58 kDa), fumarase (48.5 kDa), lactate dehydrogenase (36.5 kDa) and triose phosphate isomerase (26.6 kDa).

Protein determination

The concentration of protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Results

Effect of CaDP on intact myofibrils isolated from the arm muscle of *O. vulgaris*

Intact myofibrils isolated from the arm muscle of *O. vulgaris* were used as endogenous substrates of CaDP isolated from the same tissue. As can be seen in Fig. 1, major myofibrillar proteins present in intact myofibrils such as myosin, paramyosin and actin were partially degraded by the CaDP, even at a proteinase to substrate ratio of 1:50 (w/w). The electrophoretic pattern obtained exhibited the characteristic bands of myosin heavy chain (MHC, 200 kDa), and light chains (MLCs, 17 kDa, and 15 kDa, respectively), paramyosin (PM, 105 kDa), actin (43 kDa), and tropomyosin (TM, 37 kDa). As controls, intact myofibrils incubated without CaDP (Fig. 1, lane 4), with CaDP in the absence of Ca^{2+} (Fig. 1, lane 5), or intact myofibrils incubated with CaDP and Ca^{2+} in the presence of 1 U of CIF (Fig. 1, lane 6) were included. The results in Fig. 1 (lanes 7 and 8) clearly show that all

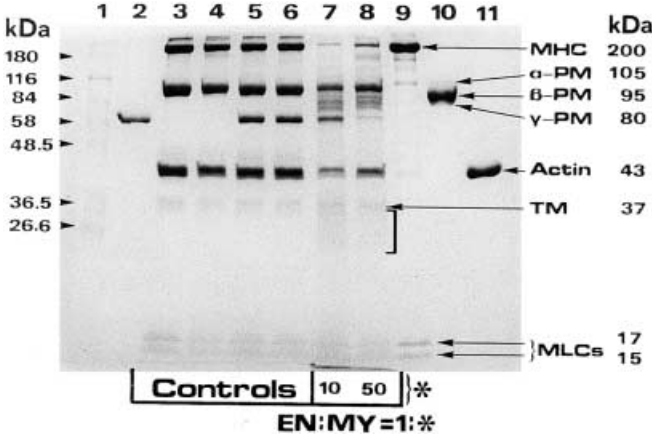

Fig. 1 Degradation of intact myofibrils from the arm muscle of *O. vulgaris* by CaDP isolated from the same tissue of *O. vulgaris*. *Lanes: 1* mixture of protein molecular mass markers; *2* intact CaDP (3 μg); *3* intact myofibrils (30 μg); *4* myofibrils incubated in the assay mixture in the absence of CaDP; *5* myofibrils incubated in the assay mixture with CaDP in the presence of 10 mM EDTA instead of $CaCl_2$; *6* myofibrils incubated in the assay mixture in the presence of 1 U of CIF; *7* myofibrils incubated in the assay mixture with CaDP at a ratio 1:10 (EN:MY); *8* myofibrils incubated in the assay mixture with CaDP at a ratio 1:50 (EN:MY); *9* intact myosin (10 μg) isolated from the arm muscle of *O. vulgaris* (*MHC* heavy chain of myosin, *MLCs* light chains of myosin); *10* intact paramyosin (10 μg) isolated from the arm muscle of *O. vulgaris* (α-, β- and γ-PM: α-, β- and γ-paramyosin, respectively); *11* actin (10 μg) isolated from the arm muscle of *O. vulgaris*. The numbers (in kDa) *to the right* correspond to the molecular masses of the isolated myofibrillar proteins. Brackets in *lane 8* represent various polypeptides of 25–35 kDa of molecular mass. *TM* tropomyosin, *EN* CaDP, *MY* myofibrils

major myofibrillar proteins such as myosin, paramyosin, and actin, were partially degraded by CaDP in the presence of Ca^{2+} and that this degradation was completely inhibited by CIF even at a proteinase to substrate ratio of 1:10 (w/w).

Effect of CaDP on the isolated myofibrillar proteins

Degradation of paramyosin (PM) by CaDP

In our preliminary experiments we isolated the paramyosin (PM) forms α and β from the arm muscle of *O. vulgaris* according to the methods described in the Materials and methods section. Both, α- and β-paramyosins were examined for their susceptibility to degradation by CaDP. The CaDP to PM ratios tested were 1:10 and 1:50. As can be seen in Fig. 2, β-PM was extensively degraded by CaDP, even at a ratio of 1:50 (Fig. 2, lane 2), producing large proteolytic fragments of lower molecular masses, whereas α-PM was only slightly degraded by CaDP at a ratio of 1:10 (Fig. 2, lane 6). We therefore decided to examine the profile of β-PM degradation by CaDP.

β-PM was isolated to apparent homogeneity and the mass of the intact subunit was estimated to be 95 kDa, although small α-PM (105 kDa) as well as γ-PM (80 kDa) impurities were also evident after the SDS-PAGE. However, as we showed above (Fig. 2, lanes 5 and 6), isolated α-PM was basically not degraded by CaDP at proteinase to substrate ratios of more than 1:10. We therefore examined the degradation of β-PM in relation to the CaDP to β-PM ratio. The electrophoretic pattern obtained (Fig. 3) clearly showed that β-PM was degraded by CaDP. The degree of proteolysis was dependent on the CaDP to β-PM ratio (w/w). Apparent degradation occurred even at a ratio of 1:300 (Fig. 3, lane 12). The β-PM band almost disappeared at a ratio of 1:10, and three major groups of polypeptides with molecular masses of 80, 75, and 60 kDa, respectively, were produced. Furthermore, CIF completely inhibited the β-PM proteolysis by CaDP (Fig. 3, lane 6).

We also examined the time course of β-PM degradation by CaDP. As can be seen in Fig. 4, in the initial stages (30 s and 1 min), limited proteolysis of β-PM occurred (lanes 4 and 5), whereas prolonged proteolysis

Fig. 3 Changes in the electrophoretic pattern of β-paramyosin (PM) after incubation with CaDP. A constant amount of paramyosin (10 μg) was incubated with CaDP and the ratios of proteinase to β-PM (EN/PM) tested were 1:10, 1:25, 1:50, 1:100, 1:200, and 1:300 (*lanes 7–12*, respectively). *Lanes: 1* protein molecular mass markers; *2* intact CaDP; *3* intact β-PM; *4* β-PM incubated with Ca^{2+}, in the absence of CaDP; *5* β-PM incubated with CaDP, in the presence of 10 mM EDTA instead of $CaCl_2$; *6* β-PM incubated with CaDP in the presence of Ca^{2+} plus 1 U of CIF

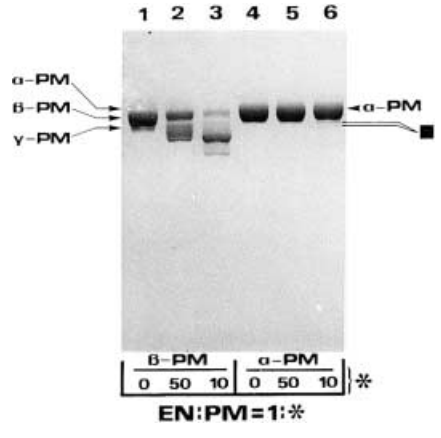

Fig. 2 Effect of CaDP on the β-paramyosin and α-paramyosin. β- or α-paramyosin (PM) was isolated from the arm muscle of *O. vulgaris* according to the methods described in the Materials and methods section. PM (10 μg) was incubated in the assay mixture with 0.2 μg of CaDP (*lanes 2 and 5*, for β- and α-PM, respectively) or with 1 μg of CaDP (*lanes 3 and 6*, for β- and α-PM, respectively). *Lanes 1 and 4*, 10 μg of intact β- and α-PM, respectively

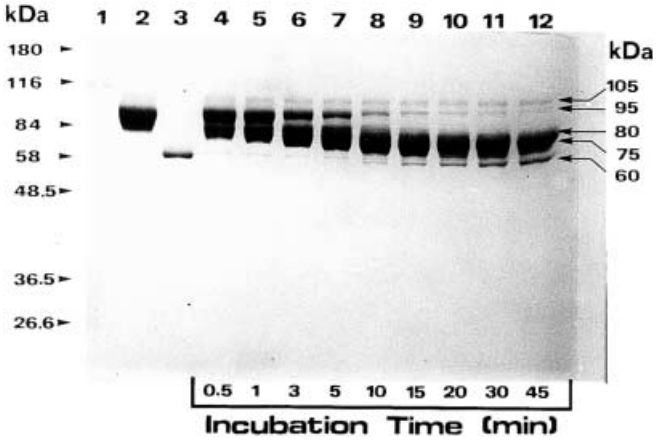

Fig. 4 Changes in the electrophoretic pattern of β-paramyosin during degradation by CaDP with respect to the incubation time. β-PM (10 μg) was incubated with CaDP (0.2 μg) in the assay mixture for 0.5, 1, 3, 5, 10, 15, 20, 30, and 45 min (*lanes 4–12*, respectively). *Lane 1* protein markers; *lane 2* intact β-PM; *lane 3* intact CaDP

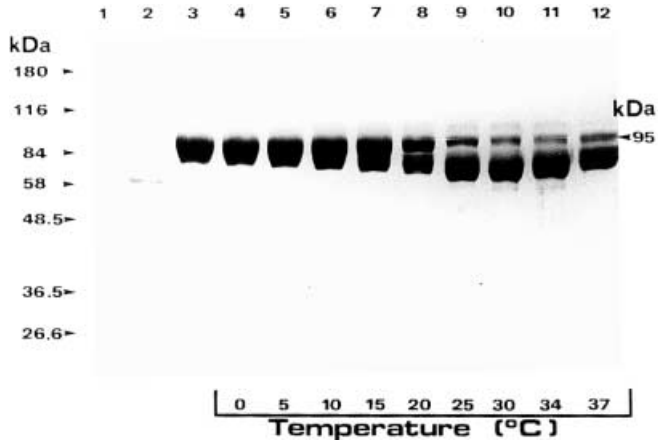

Fig. 5 Effect of temperature on the β-paramyosin degradation by CaDP. β-PM (10 μg) was incubated with CaDP (0.2 μg) in the assay mixture for 30 min at various temperatures: 0, 5, 10, 15, 20, 25, 30, 34, and 37 °C (*lanes 4–12*, respectively). *Lane 1* protein markers; *lane 2* intact CaDP; *lane 3* intact β-PM

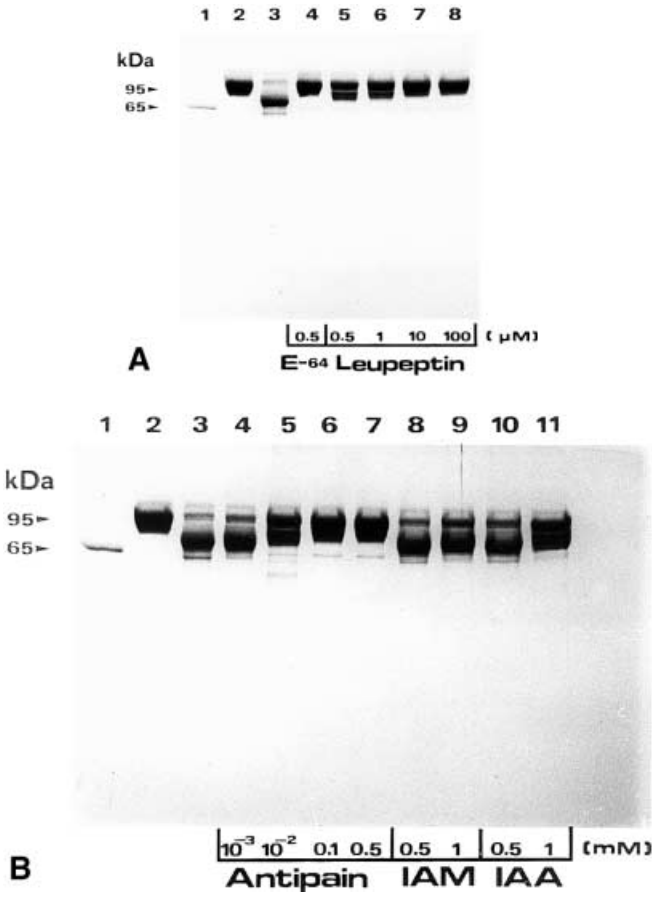

Fig. 6a, b Effect of various cysteine proteinase inhibitors on the degradation of β-paramyosin by CaDP. **a** β-PM (10 μg) was incubated with CaDP (0.2 μg) in the assay mixture in the presence of E-64 (0.5 μM) (*lane 4*) or leupeptin (0.5, 1, 10, and 100 μM) (*lanes 5–8*, respectively). **b** β-PM (10 μg) was incubated with CaDP (0.2 μg) in the assay mixture in the presence of antipain (10^{-3}, 10^{-2}, 0.1, and 0.5 mM) (*lanes 4–7*, respectively) or iodoacetamide (IAM) (0.5 and 1 mM) (*lanes 8 and 9*, respectively) or iodoacetic acid (IAA) (0.5 and 1 mM) (*lanes 10 and 11*, respectively). In both **a** and **b**: *lane 1* intact CaDP; *lane 2* intact β-PM; *lane 3* β-PM incubated with CaDP in the absence of any inhibitor

(up to 45 min) of β-PM by CaDP resulted in the complete disappearance of β-PM and the accumulation of the major polypeptides (lanes 6–12) described above. When we examined the effect of temperature on the β-PM proteolysis by CaDP, we found that this is a temperature dependent process (Fig. 5). In particular, at low temperatures (0 and 5 °C), no β-PM proteolysis occurred, whereas at higher temperatures (10–34 °C) the β-PM proteolysis rate was relative to the temperature tested. Optimal temperature for the β-PM proteolysis seemed to be 34 °C, a result quite similar to that obtained for the maximal caseinolytic activity of CaDP (Hatzizisis et al. 1996).

In Fig. 6 (a and b) the effect of various proteinase inhibitors and alkylating agents on the β-PM proteolysis by CaDP is shown. As can be seen, both E-64 and leupeptin, two known cysteine proteinase inhibitors, completely inhibited this proteolysis at low concentrations (0.5 μM for E-64, and 10 μM for leupeptin, respectively) (Fig. 6a). Antipain exhibited its inhibitory effect at higher concentration (0.5 mM), whereas iodoacetamide and iodoacetate, two known alkylating agents, had a slight inhibitory effect only at a concentration of 1 mM (Fig. 6b).

Degradation of myosin by CaDP

Isolated myosin from the arm muscle of *O. vulgaris* was also used as an endogenous substrate of CaDP. In a series of experiments, intact myosin was incubated with CaDP at various ratios of CaDP to myosin (w/w), and for increasing incubation times. In all experiments, intact myosin, myosin incubated with CaDP in the absence of Ca^{2+}, and myosin incubated with CaDP and Ca^{2+} in the presence of 1 U CIF were used as controls. The results revealed that: (1) myosin heavy chain was degraded by CaDP in the presence of 5 mM Ca^{2+} at all proteinase to substrate ratios tested, even at a ratio of 1:300 (Fig. 7, lanes 7–12); (2) myosin light chains were more resistant to degradation by CaDP (Fig. 7, lanes 7–12); (3) the proteolytic degradation of myosin heavy chain by CaDP resulted in the accumulation of four groups of polypeptides with molecular masses of approximately 155 kDa, 125 kDa, 115 kDa, and 102 kDa, respectively; and (4) CIF completely inhibited the myosin degradation by CaDP (Fig. 7, lane 6).

We also examined the time course of myosin degradation by CaDP. As can be seen in Fig. 8, when the incubation time increased up to 5 min, the MHC (200 kDa) became progressively less intense, while the four groups of polypeptides described above accumulated. However, prolonged incubation of myosin with CaDP (10–45 min) resulted in the almost complete disappearance of MHC without a further accumulation of the large proteolytic fragments, possibly indicating that

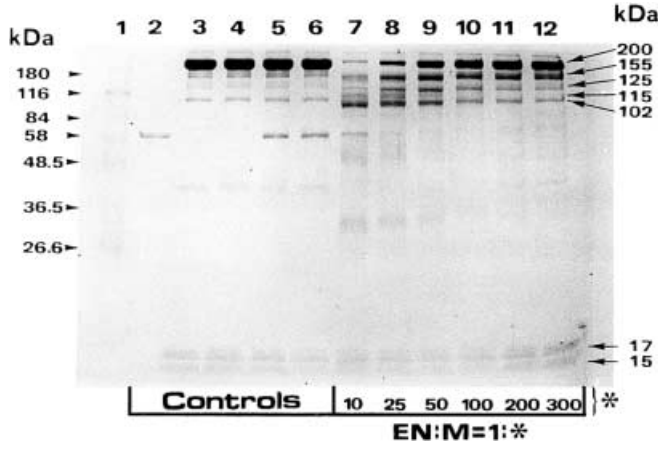

Fig. 7 Changes in the electrophoretic pattern of myosin during degradation by CaDP in the presence of Ca^{2+}. Purified myosin (10 μg) was incubated with increasing quantities of CaDP in the assay mixture which contained 50 mM imidazole/HCl buffer, pH 7.0, 5 mM 2-mercaptoethanol and 5 mM $CaCl_2$ in a total volume of 30 μl, for 30 min, at 30 °C. Enzyme to substrate ratios (wt/wt) tested were: 1:10, 1:25, 1:50, 1:100, 1:200, and 1:300 (*lanes 7–12*, respectively). *Lanes: 1* protein markers; *2* intact CaDP; *3* intact myosin; *4* myosin incubated with Ca^{2+} in the absence of CaDP; *5* myosin incubated with CaDP in the presence of 10 mM EDTA instead of $CaCl_2$; *6* myosin incubated with CaDP in the presence of Ca^{2+} plus 1 U of CIF

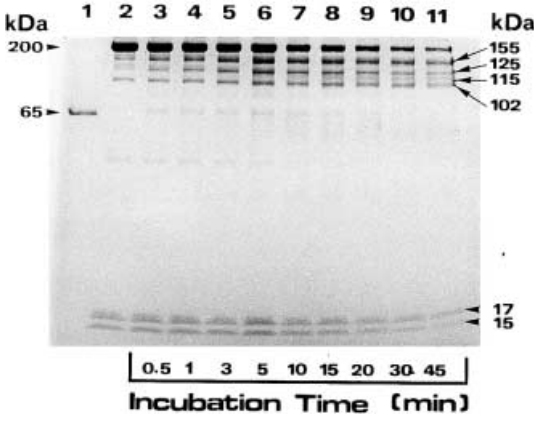

Fig. 8 Changes in the electrophoretic pattern of myosin during degradation by CaDP with respect to the incubation time. Myosin (10 μg) was incubated with CaDP (0.2 μg) in the assay mixture for 0.5, 1, 3, 5, 10, 15, 20, 30, and 45 min (*lanes 3–11*, respectively). *Lane 1* intact CaDP; *lane 2* intact myosin

a further proteolysis to smaller products that are not resolved in the 10% SDS-polyacrylamide gels occurred.

Degradation of G-actin by CaDP

G-actin was isolated to apparent homogeneity with a molecular mass of 43 kDa (Fig. 1, lane 11) from the arm muscle of *O. vulgaris* and tested as a substrate of CaDP. The electrophoretic pattern obtained showed that G-actin was not degraded at all by CaDP, even at a CaDP to G-actin ratio of 1:10 (w/w) (data not shown). This result contradicts the result shown in Fig. 1 (lanes 7 and 8) and possibly represents the differential degradation of F-actin and/or G-actin by CaDP.

Autolysis of CaDP in the presence of substrate

In our previous studies on the autolysis of CaDP, we showed that this proteinase isolated from the arm muscle of *O. vulgaris* undergoes an autolytic degradation in the absence of substrate. In particular, the conversion of the 65 kDa subunit to the 50.6 kDa and 31.5 kDa fragments is completed slowly within 90 min (at 10 mM Ca^{2+}) or 3 h (at 5 mM Ca^{2+}). Furthermore, the susceptibility to autolytic degradation of CaDP seems to be identical for all subunits in the presence of Ca^{2+} (Hatzizisis et al. 1996).

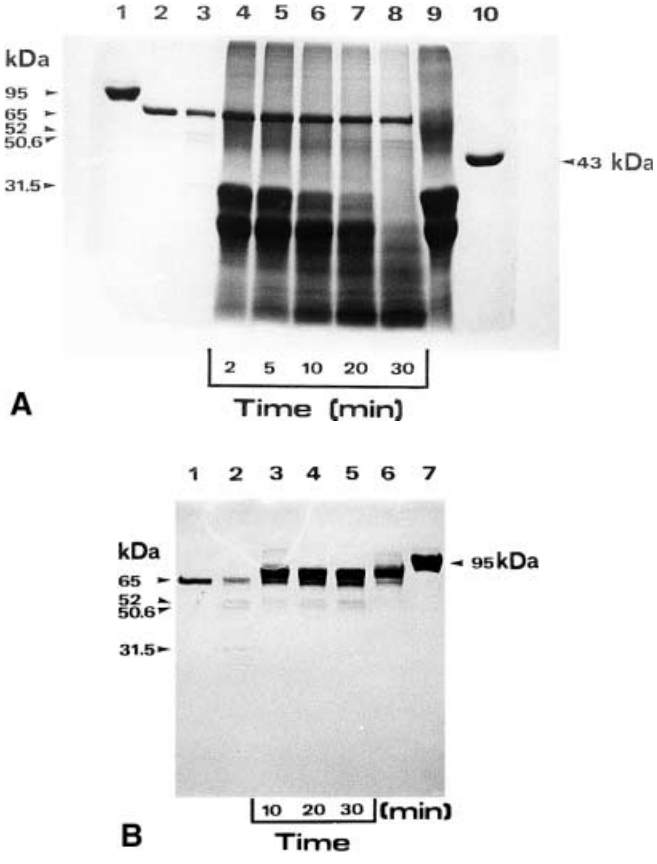

Fig. 9 **a** Time course of CaDP autolysis in the presence of casein. *Lanes 4–8* autolysis of CaDP (7 μg) in the presence of alkali-denatured casein (20 μg) for 2, 5, 10, 20, and 30 min, respectively. *Lane 1* intact β-PM; *lane 2* intact CaDP; *lane 3* CaDP incubated in the autolysis mixture in the absence of substrate; *lane 9* untreated casein; *lane 10* intact G-actin. **b** Time course of CaDP autolysis in the presence of the endogenous substrate β-PM. *Lanes 3–5* autolysis of CaDP (7 μg) in the presence of β-PM (10 μg) (10, 20, and 30 min, respectively); *lane 6* degradation of β-PM (10 μg) by CaDP (1 μg); *lane 1* intact CaDP; *lane 2* CaDP incubated in the autolysis mixture in the absence of substrate; *lane 7* intact β-PM. The *arrows* correspond to the molecular masses of β-PM (95 kDa), CaDP (intact and autolysed) (65, 52, 50.6 and 31.5 kDa), and G-actin (43 kDa), respectively

In Fig. 9 (a and b) the time course of CaDP autolysis in the presence of the exogenous substrate casein, and the isolated endogenous β-paramyosin, respectively, is shown. As can be seen, the degradation of both substrates was accompanied by the autolysis of the proteinase. In particular, limited autolysis of CaDP occurred after incubation for 10 min with the respective substrates and the 65 kDa native subunit was converted to two products with molecular masses of 52 kDa and 50.6 kDa (Fig. 9a, lanes 6–8 for casein, and Fig. 9b, lanes 3–5 for β-paramyosin, respectively). In contrast, in the absence of any substrate, the 65 kDa native subunit was additionally degraded to smaller polypeptides with molecular masses of ~31.5 kDa (Hatzizisis et al. 1996). This prolonged autolysis was completely inhibited by CIF isolated from the same tissue of *O. vulgaris*.

Reversible inhibition of CaDP autolysis by CIF

In order to examine if CIF inhibits reversibly or irreversibly the autolysis of CaDP, three samples of isolated CaDP (7 μg each) were preincubated with CIF in the presence of 5 mM Ca^{2+}, for 5 min at 30 °C. Two of the enzymatic samples were then dialysed for 6 h against 1 l of 50 mM imidazole/HCl buffer, pH 7.0. Then the enzymatic samples were incubated in the autolysis mixture in the absence or presence of 5 mM Ca^{2+}, for 60 min at 30 °C. As can be seen in Fig. 10, the autolytic degradation of CaDP occurred only after the removal of CIF by dialysis (lane 2), indicating that the binding of CIF to CaDP is reversible.

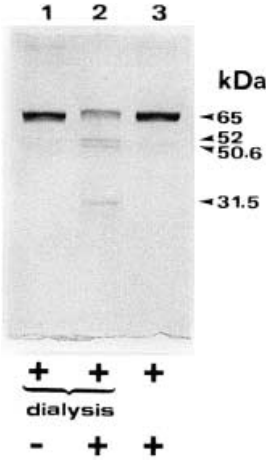

Fig. 10 Reversible inhibition of CaDP autolysis by CIF. CaDP (10 μg) was preincubated with CIF in the presence of Ca^{2+} and then, either with or without dialysis, was incubated with the autolysis mixture in the absence (−) or presence (+) of Ca^{2+} and subjected to SDS-PAGE. *Lane 1* after dialysis and incubation in the absence of Ca^{2+}; *lane 2* after dialysis and incubation in the presence of Ca^{2+}; *lane 3* without dialysis and after incubation in the presence of Ca^{2+}. The gel was stained with Coomassie brilliant blue

Discussion

In the present study, the proteolytic degradation of intact myofibrils and various myofibrillar proteins isolated from the arm muscle of *O. vulgaris* by CaDP was examined.

In the first step, intact myofibrils isolated from the arm muscle of *O. vulgaris* were used as possible endogenous substrates of CaDP. The results of this study revealed that CaDP isolated from the same tissue of *O. vulgaris* degrades myosin heavy chain, paramyosin and actin and, to a lesser extent, myosin light chains (Fig. 1). Our results are in accordance with those reported by several investigators with respect to the effect of CaDPs on crustacean myofibrillar proteins in both intact muscle and isolated myofibrils (Mykles and Skinner 1982a, 1983; Mykles 1990; Mattson and Mykles 1993).

On the contrary, in vertebrate skeletal muscle CaDPs do not degrade either myosin or actin, whereas troponins T and I, tropomyosin, α-connectin and nebulin represent "good" substrates for CaDPs (Dayton et al. 1976, 1981; Hara et al. 1983; Croall and DeMartino 1984; Inomata et al. 1985; Zeece et al. 1986; Barrett et al. 1991; Goll et al. 1991). The most characteristic property of vertebrate CaDPs is their specificity for Z-line removal from intact myofibrils (Dayton et al. 1976; Azanza et al. 1979; Zeece et al. 1986; Goll et al. 1991; Sargianos et al. 1996). This property has also been reported for striated invertebrate muscles (Mykles 1990).

It is widely known that paramyosin is easily degraded during extraction and that it is difficult to recover in its native form. In particular, native paramyosin (α-PM) consists of two identical subunits each of 105–115 kDa (Cohen et al. 1971; Stafford and Yphantis 1972; Mackenzie and Epstein 1980), while two proteolytic products of α-PM with molecular masses of 91–100, and 94–97 kDa, respectively, have been characterized as β- and γ-paramyosins (Stafford and Yphantis 1972; Elfvin et al. 1976; Cooley et al. 1979). The data reported by these investigators suggest that different paramyosin forms are recovered, in relation to the extraction method followed. In addition, α-PM is resistant to proteolysis in intact myofibrils, whereas it is more susceptible in the soluble form (Harris and Epstein 1977; Mackenzie and Epstein 1980).

Our experiments, on isolation from intact myofibrils showed that depending on the extraction method followed, α-paramyosin or β- and γ-paramyosins were recovered. In particular, we found that during the extraction in the presence of 10 mM EDTA and 1 mM 2-mercaptoethanol, α-paramyosin (105 kDa) was recovered whereas in the absence of EDTA and 2-mercaptoethanol β- (95 kDa) and γ-paramyosins (80 kDa) were recovered (Fig. 2, lane 1 for β-PM and γ-PM and lane 4 for α-PM, respectively). Our results are in accordance with those reported by almost all investigators for paramyosins isolated from various invertebrates

(Mackenzie and Epstein 1980; Epstein et al. 1985; Mykles 1985, 1988; Vinos et al. 1991; Ismail and Mykles 1992).

In the present study, we examined in detail the effect of *O. vulgaris* arm muscle CaDP on the degradation of α- and β-paramyosins isolated from the same tissue of the animal. The results of these experiments revealed that: (1) both isolated forms of paramyosin (α-, and β-) are partially degraded by this proteinase but each form exhibits a different sensitivity to proteinase (Fig. 2). The most obvious difference is that α-PM is not degraded at a ratio of CaDP to α-PM of more than 1:10 (Fig. 2), whereas β-paramyosin is degraded by CaDP even at a ratio of 1:300 (Fig. 3); (2) the β-PM degradation rate depends on the enzyme to substrate ratio (Fig. 3); (3) this proteolysis is both time dependent (Fig. 4), and temperature dependent (up to 34 °C) (Fig. 5); (4) the Ca^{2+} requirement of CaDP for the degradation of β-PM is higher than 2 mM (data not shown); (5) proteolysis is maximal at neutral pH (data not shown); and (6) proteolysis is strongly inhibited by endogenous CIF as well as by known cysteine proteinase inhibitors such as E-64, leupeptin, and antipain (Figs. 4, 6a, b), and is slightly inhibited by alkylating agents such as iodoacetamide and iodoacetate (Fig. 6b). In all cases, β-paramyosin degradation produces three groups of proteolytic fragments of lower molecular mass (80, 75, and 60 kDa, respectively).

Furthermore, our CaDP degrades isolated myosin from the arm muscle of *O. vulgaris* and the degradation rate: (1) depends on the proteinase to substrate ratio (Fig. 7), and (2) is time dependent (Fig. 8). In all cases, myosin heavy chain (200 kDa) degradation produces four proteolytic fragments of lower molecular mass (155, 125, 115, and 102 kDa, respectively) (Figs. 7 and 8).

By contrast, our experiments on isolated G-actin degradation by CaDP clearly show that G-actin is not degraded by CaDP even at a ratio of 1:10 (data not shown), suggesting that G-actin does not represent a "good" substrate for CaDP.

Paramyosin is a coiled-coil α-helical structural protein found in many invertebrates and is restricted to both striated and smooth muscles where it forms the core of the myosin-containing thick filaments. The detailed molecular packing of paramyosin in the core and the array of myosin on the surface of the paramyosin core remain unknown. However, it seems that myosin molecules are situated on the paramyosin core filament towards both ends, and they consequently have polarity towards both ends (Cohen et al. 1971; Castellani et al. 1983; Bennett and Elliot 1984; Gengyo-Ando and Kagawa 1991). Although studies of paramyosin structure are numerous, the precise function of paramyosin is still unknown, but it seems that this myofibrillar protein may play an important role in the invertebrate muscle contraction mechanism (Cohen et al. 1971; Szent-Gyorgyi et al. 1971; Dover and Elliot 1979; Castellani et al. 1983; Bennett and Elliot 1984; Kagawa et al. 1989; Gengyo-Ando and Kagawa 1991).

Muscle contraction in molluscs is characterised by a phenomenon termed "catch" in which tension is maintained over long periods with little energy expenditure. Two mechanisms have been proposed to account for catch. The most popular theory is that tension is maintained via cross-bridge interactions with actin and that these are either non-cycling or slowly cycling. The alternative theory is that contractile force is maintained via paramyosin–paramyosin interactions (Watabe and Hartshorne 1990). In molluscs, as in several other invertebrates, the major Ca^{2+}-dependent regulatory mechanism is due to binding of Ca^{2+} to the two regulatory light chains of myosin. Molluscan myosins are regulatory molecules and differ from the respective vertebrate myosins in that they have a specific binding site for Ca^{2+} which is required for the regulation of Mg^{2+}-ATPase (Kendrick-Jones et al. 1970).

The heterogeneity of paramyosin as well as of other myofibrillar proteins in fast and slow muscle fibers of crustaceans is of interest: paramyosin subunits have a molecular mass of 105 kDa in slow fibers, whereas in fast fibers the molecular mass is 110 kDa (Mykles 1985, 1988; Ismail and Mykles 1992). Furthermore, Epstein et al. (1976) have reported that native (105 kDa) and cleaved (94 kDa) paramyosins inhibit the Mg^{2+}-ATPase activity to different extents. The possible involvement of CaDP, via the paramyosin proteolysis, in regulatory mechanisms of paramyosin–myosin interactions could be of great interest with respect to the functional role of CaDP in invertebrate smooth muscle contraction.

Our experiments on the CaDP autolysis in the presence of substrate showed that, in parallel to the proteolysis of the substrate, the proteinase undergoes autolytic modification producing large active fragments (Fig. 9a and b). By contrast, in the absence of any substrate the proteinase undergoes extensive autolysis producing small inactive proteolytic fragments (Hatzizisis et al. 1996). The autolysis of CaDP is strongly inhibited by its endogenous inhibitory factor, and this inhibition is reversible (Fig. 10). Since our CaDP is a homopolymer, possibly consisting of eight similar if not identical subunits, the proteinase contains at least eight copies of each functional domain. Thus, there is a potential for co-operative interactions between active and calcium binding sites. Although the CaDP molecule isolated from the *O. vulgaris* arm muscle differs in subunit composition from the vertebrate calpains, it seems that the molecular mechanisms of its autolytic modification and substrate degradation are similar. Recent studies by Beyette and Mykles (1997) on the autolysis of a CaDP isolated from a lobster muscle have shown that this CaDP consists of two identical subunits each of 95 kDa and also undergoes autolytic modification in the presence of exogenous casein, producing large active fragments. According to these investigators, the effect of substrate on CaDP may constitute an effective activation/inactivation mechanism in which the enzyme is transiently active before it is autolytically inactivated.

Such effects are not unusual in proteinases with multiple catalytic sites.

In conclusion, the present study yielded information on myofibril degradation by CaDP in the arm muscle of *O. vulgaris*. Thus, in intact myofibrils, CaDP partially degrades all major myofibrillar proteins such as myosin, paramyosin and actin. Furthermore, CaDP degrades isolated β-paramyosin and myosin heavy chain in the presence of Ca^{2+} whereas this proteinase does not degrade isolated G-actin. These data support the suggestion that in the *O. vulgaris* arm muscle, CaDP preferentially degrades myofibrillar proteins of the thick filaments. The results of the present study also showed that isolated CaDP undergoes autolytic modification in the presence of substrate and that this modification is reversibly inhibited by its endogenous inhibitory factor. It seems that the proteolytic degradation of paramyosin and myosin in both intact myofibrils and in situ is accompanied by a CaDP autolytic modification in the presence of Ca^{2+}, which could represent an important regulatory mechanism of CaDP action and function in vivo in the arm muscle of *O. vulgaris*.

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