Specific degradation of troponin T and I by μ -calpain and its modulation by substrate phosphorylation

Fabio DI LISA,*|| Roberta DE TULLIO,† Franca SALAMINO,‡ Roberta BARBATO,* Edon MELLONI,† Noris SILIPRANDI,* Stefano SCHIAFFINO,§ and Sandro PONTREMOLI†

*Dipartimento di Chimica Biologica and Centro Fisiologia Mitochondriale, CNR, Università di Padova, Via Trieste 75, 35121 Padova, Italy,

†Istituto di Chimica Biologica, Università di Genova, Viale Benedetto XV 1, 16132 Genova, Italy, ‡Istituto di Fisiologia Generale e Chimica Biologica,

Via Muroni 25, 07100 Sassari, Italy, and §Dipartimento Scienze Biomediche and Centro Biologia e Fisiopatologia Muscolare, CNR, Università di Padova,

Via Trieste 75, 35121 Padova, Italy

The degradation of troponin (Tn) subunits by calpain was studied by incubating either isolated cardiac Tns or myocardial cryosections with two different calpain isoenzymes isolated from rat skeletal muscle. Western-blot analysis with monoclonal antibodies against TnI and TnT showed that μ -calpain was at least ten times more active than m-calpain in degrading TnI and TnT both *in vitro* and *in situ*. TnC was completely resistant to both proteinase forms. Phosphorylation by cyclic AMP-dependent protein kinase (PKA) isolated from rat skeletal muscle reduced the sensitivity of TnI to degradation. This effect in combination with an increased efficiency of the endogenous inhibitor [Salamino, De Tullio, Michetti, Mengotti, Melloni and

Pontremoli (1994) *Biochem. Biophys. Res. Commun.* **199**, 1326–1332] probably reduces the proteolytic activity of calpain in cells on PKA stimulation. Conversely, phosphorylation by protein kinase C (PKC) resulted in a twofold increase in the degradation of TnI. Degradation by m-calpain was not modified by Tn phosphorylation. The different sensitivity to μ -calpain might be related to changes in TnI oligomeric structure. Indeed, on PKC phosphorylation, the apparent molecular mass of TnI calculated from the distribution coefficient of Tn complex in Sephadex G-100 matrix was reduced from 90 to 30 kDa suggesting dissociation of the Tn complex.

INTRODUCTION

All mammalian cells contain a soluble Ca^{2+} -dependent proteolytic system. This system is composed of different proteinase isoenzymes known as calpains and a natural protein inhibitor named calpastatin (for reviews see refs. [1–4]).

In muscle cells, two isoenzymes which differ on the basis of their Ca^{2+} requirement are expressed [1–4]. μ -Calpain is active at low, micromolar, Ca^{2+} concentrations, whereas m-calpain requires millimolar concentrations of Ca^{2+} . Although the molecular properties of these proteins have been characterized, little information about the physiological role of this proteolytic system is available. Nevertheless, myofibrillar protein degradation, disassembly of myofibrils, and removal of Z disks have been claimed to be caused by calpain [5,6].

Among the myofibrillar proteins, troponin (Tn) appears to be especially susceptible to calpain digestion [5–9]. It consists of three subunits: TnC, TnI and TnT. The binding of Ca^{2+} to TnC is thought to activate muscle contraction by releasing the inhibitory action of TnI–TnT–tropomyosin on actin–myosin interaction [10]. The initial demonstration of the involvement of calpains in the degradation of Tns was obtained with a crude preparation of both enzymes and substrates [7]. Further evidence was provided by using either heterologous calpain [8] or a single isoenzyme [9]. In addition, sensitivity to calpain digestion has been reported to be modified by phosphorylation of Tn molecules in experiments carried out using a heterologous source of cyclic AMP-dependent protein kinase (PKA) [7].

It has been established that Tn is also phosphorylated by protein kinase C (PKC) [11]. As the phosphorylation sites of the two kinases are different [12], variations in sensitivity to calpain digestion could possibly result from differences in structural modification. Thus several aspects of the calpain–Tn interaction remain to be elucidated.

In this paper we have compared the effectiveness of the two different calpain isoenzymes isolated from rat skeletal muscle on the degradation of myocardial Tns. We show that μ -calpain is much more active than m-calpain in degrading TnI and TnT both in purified preparations and in tissue slices. Phosphorylation of Tn molecules by PKC enhances the rate of their proteolysis, which, in contrast, is reduced when phosphorylation is catalysed by PKA.

MATERIALS AND METHODS

Materials

DEAE-cellulose was obtained from Whatman. Phosphatidylserine (bovine brain), dioleoylglycerol, histone type III-S, staurosporine (*Streptomyces* sp.), leupeptin, pepstatin A, phenylmethanesulphonyl fluoride, Triton X-100, butyl agarose and the various nucleotides were purchased from Sigma Chemical Co. [γ - ^{32}P]ATP (3000 Ci/mmol) was obtained from Amersham. All other chemicals were reagent grade. Monoclonal antibodies (mAbs) specific for TnT, TnI and TnC (respectively RV-C2, TI-1, TC-1) were prepared as previously described [13,14].

Abbreviations used: Tn, troponin; PKA, cyclic AMP-dependent protein kinase; PKC, protein kinase C; PKind, independent protein kinase; mAb, monoclonal antibody.

|| To whom correspondence should be addressed.

Isolation of Tn complex from rat heart

The Tn complex was partially purified from 40 g of rat cardiac tissue, following essentially the procedure described by Tobacman et al. [15], but omitting the steps that involve the use of potentially denaturing agents. Briefly, the Tn sample was loaded on to a DEAE-cellulose DE-52 column (1 cm × 12 cm) equilibrated with 50 mM sodium borate buffer, pH 7.5, containing 0.5 mM 2-mercaptoethanol and 0.1 mM EDTA. The column was washed with the same buffer until A_{280} was below 0.02. The adsorbed proteins were eluted with a linear gradient of NaCl from 0 to 0.3 M. The flow rate was 0.3 ml/min and fractions of 1 ml were collected. The different protein peaks were collected separately, concentrated by ultrafiltration (on an Amicon Y10 membrane) and subjected to SDS/PAGE using 12.5% minislab gels [16]. Electrophoresis was followed by Western-blot analysis [17], and the samples containing Tn were identified by the three mABs using the horseradish peroxidase-conjugate reaction [18].

Purification of calpain isoenzymes from rat skeletal muscle

μ - and m-Calpain were isolated from rat skeletal muscle and purified as previously reported [19].

Purification of kinases from rat skeletal muscle

PKA and PKC were purified as previously described [20]. Briefly, homogenates from rat skeletal muscle were loaded on a DEAE-cellulose DE-32 column, and three peaks of kinase activity were eluted with a linear gradient from 0 to 0.3 M NaCl. The first peak, eluted at the very beginning of the gradient (0.05 M NaCl), contains a protein kinase activity requiring cyclic AMP, named PKA; the second, eluted at an NaCl concentration of 0.08–0.11 M, was identified as PKC, because it requires Ca^{2+} , phosphatidylserine and diacylglycerol for catalytic activity; the third peak, eluted at 0.2 M NaCl, contains a histone-phosphorylating protein kinase, named independent PK (PKind), and is active in the absence of any effector. The three protein kinase activities were collected separately and further purified by affinity chromatography on a threonine-Sepharose column equilibrated in 50 mM sodium borate buffer, pH 7.5, containing 0.1 mM EDTA, 10 mM 2-mercaptoethanol and 10% glycerol [20]. Protein kinase activity was assayed in 0.2 ml of 50 mM sodium borate, pH 7.5, containing 0.4 μCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, 10 μM ATP, 0.1 mg of type III-S histone in the presence or absence of specific effectors: 20 μM cyclic AMP for PKA and 0.5 mM Ca^{2+} and phospholipids for PKC. The amount of ^{32}P incorporated into the substrate was evaluated as previously described [20]. One unit of protein kinase activity is defined as the amount of enzyme that catalyses the incorporation of 1 nmol of ^{32}P /min into histone under the assay conditions.

Phosphorylation of Tns

The Tn preparation (90 μg of protein) was incubated for 10 min at 30 °C in a volume of 150 μl containing 50 mM sodium borate buffer, pH 7.5, 5 mM MgCl_2 and 10 μM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (250 $\mu\text{Ci}/\mu\text{mol}$). Phosphorylation by the different purified kinases was studied in the presence of each of the following: (i) 1200 units of PKA and 20 μM cyclic AMP, (ii) 160 units of PKC, 0.5 mM CaCl_2 , 20 $\mu\text{g}/\text{ml}$ phosphatidylserine and 5 $\mu\text{g}/\text{ml}$ dioleoylglycerol; (iii) 2000 units of pKind. All reactions were stopped by the addition of 20 μM staurosporine and cooling the mixture in an ice-bath.

Degradation of Tns with μ - or m-calpain

The isolated Tn preparation (90 μg of protein) was incubated at 37 °C in a volume of 360 μl of 50 mM sodium borate buffer, pH 7.5, containing 17 units of μ - or m-calpain and 1 mM CaCl_2 . At the indicated times, 40 μl aliquots were collected. The reaction was stopped with 10 μl of 0.25 M Tris/HCl, pH 6.8, containing 10% SDS, 25% 2-mercaptoethanol and 25% glycerol and heated for 3 min at 100 °C. The samples were then submitted to SDS/PAGE (12.5% acrylamide) and transferred to a nitrocellulose sheet, and Tns were immunodetected with the specific mABs. Band intensity was measured with a Shimadzu flying-spot scanner. One unit of calpain activity is defined as the amount of enzyme that catalyses the digestion of 1 nmol of $-\text{NH}_2$ groups/h under the assay conditions.

Degradation of Tn by μ - or m-calpain *in situ*

Myocardial cryosections were prepared from frozen ventricles of freshly excised rat hearts. Four cryosections (7 μm each) per Eppendorf tube were exposed to calpains under the same conditions as described above. However, as the total protein content of the sample was 900 μg , approx. 10-fold higher than in the experiments with purified Tns, the calpain concentration was also increased 10-fold.

Determination of the distribution coefficient of Tn complexes

The distribution coefficient into Sephadex G-100 matrix was determined by the procedure of Ackers [21], modified by Pontremoli et al. [22]. Unphosphorylated or phosphorylated Tn complexes (30 μg), in 0.47 ml of 50 mM sodium borate/1 mM CaCl_2 , pH 7.5, were added to 0.53 ml of wet Sephadex matrix, previously equilibrated in 50 mM sodium borate, pH 7.5.

The suspensions were mixed end-over-end for 1 h at 20 °C and then solid matrix was packed by centrifugation at 10 g (200 rev./min) for 2 min. Aliquots of the clear supernatants (200 μl) were removed and the amount of Tn was determined by subjecting the samples to SDS/PAGE followed by detection of the TnI band with the specific mAb (TI-1).

The distribution coefficient (K_D) was calculated by using the equation

$$K_D = (V_t - V_a)/(V_{\text{tot}} - V_a)$$

where V_t is the ratio between the amount of protein added in the sample and that remaining in the supernatant after gel penetration, V_{tot} is the total volume of the system (1 ml) and V_a , the volume of the aqueous phase calculated from the dilution of a solution of Blue Dextran 2000 treated in the same conditions. The apparent molecular mass of the different Tn preparations was calculated by comparing the distribution coefficient of standard proteins with their molecular masses (transferrin, 78.5 kDa; BSA, 68 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; chymotrypsinogen A, 25 kDa).

RESULTS

The susceptibility of the Tn complex to degradation by calpain isoenzymes was determined by following the disappearance of the native protein band in SDS/PAGE and immunoblots probed with TnT, TnI or TnC mAB. Isolated Tn complex was incubated with purified μ -calpain or m-calpain at different incubation

Figure 1 Degradation of isolated TnI by μ - or m-calpain

μ -Calpain (●) or m-calpain (○) was incubated in the presence of Tn preparation as described in the Materials and methods section. The heated samples (50 μ l) were submitted to a minigel SDS/PAGE, transferred to a nitrocellulose sheet and TnI was immunodetected with its specific mAb. The intensities of the bands were measured with a Shimadzu flying-spot scanner.

Figure 2 Degradation of isolated TnI by different amounts of μ -calpain (●) or m-calpain (○)

Each incubation mixture (40 μ l) contained 10 μ g of partially purified Tn, 50 mM sodium borate buffer, pH 7.5, 1 mM CaCl_2 and the indicated amounts of μ - or m-calpain. After 10 min at 37 °C the reaction was stopped, the samples were heated and treated as described in the legend to Figure 1 and the Materials and methods section.

times. When Tn complex was incubated with μ -calpain and aliquots of the mixtures were analysed, after various times of incubation, for TnI content by immunoblotting (Figure 1), the amount of the native protein band rapidly disappeared, with a decrease of 50 % at 5 min and more than 90 % after 30 min. The antibody used in these experiments revealed the native protein band (Figure 1, inset), but the fragments produced by calpain degradation were not visualized.

In contrast, when the Tn preparation was incubated with the same amount of m-calpain, no degradation was observed. Thus the disappearance of Tn immunoreactivity on addition of μ -calpain must be considered as an authentic pattern of proteolysis. The difference in efficacy of the two isoforms rules out

Figure 3 Degradation of TnT (a) and TnC (b) by different amounts of μ -calpain (●) or m-calpain (○)

Aliquots of the same samples as obtained in Figure 2 were submitted to the same procedures, and then TnT (a) and TnC (b) were identified with the appropriate mAb, as described in the Materials and methods section.

the involvement of an artifactual conformational change which could result in a lack of immunoreactivity.

To confirm the selectivity of the two calpain forms, we repeated the experiment but this time incubating the Tn preparation with increasing concentrations of μ - or m-calpain. As shown in Figure 2, μ -calpain was very effective with TnI, digestion being complete when 3–5 units of μ -calpain was added. TnI was much more resistant to degradation by m-calpain, in fact no appreciable digestion occurred until the amount of proteinase was greater than 30 units. This observation indicates that m-calpain is about ten times less active than μ -calpain on TnI.

Similar patterns of degradation were observed with TnT which is highly sensitive to digestion by μ -calpain and almost resistant to degradation by m-calpain (Figure 3a). TnC, however, was found to be fully resistant to both proteinase forms (Figure 3b).

In a separate set of experiments, the effect of calpain isoenzymes was examined *in situ* on myocardial cryosections, where the ultrastructural myofibrillar pattern is maintained. In this case also, μ -calpain was more effective than m-calpain in TnI and TnT degradation (Figure 4). The addition of 80 units of μ -calpain induced a large decrease in both TnI and TnT, whereas m-calpain had no effect.

It is well established [11,12,23] that Tn can be phosphorylated by different protein kinases. It has been reported that PKA is involved in the phosphorylation of TnI [23], whereas PKC promotes phosphorylation of both TnI and TnT [11]. The two kinases identify different sites, modifying TnI in different regions [12]. In order to investigate whether the susceptibility of Tns to calpain digestion was altered by phosphorylation, we first isolated the different protein kinases (PKC, PKA, and PK ϵ) from rat skeletal muscle (see the Materials and methods section). As shown in Figure 5, both PKA and PKC promoted incorporation of ^{32}P into the Tn preparation, whereas the independent protein kinase (PK ϵ) was ineffective. ^{32}P incorporation reached a plateau after 10 min of incubation at 30 °C, indicating that all the sites of phosphorylation were then saturated with the protein kinase.

We then examined whether phosphorylation by PKA or PKC determines different patterns of TnI digestion by μ -calpain. As

Figure 4 Degradation of TnT (b) and TnI (a) in myocardial cryosections exposed to different amounts of μ -calpain (○) or m-calpain (●)

Data, expressed as percentage of control, were normalized with respect to TnC which was unaffected by calpain degradation. Aliquots obtained in the same way as the samples in Figure 2 were subjected to the same procedures, and then TnI (a) and TnT (b) were identified with the appropriate mAb.

Figure 5 Tn phosphorylation by PKA (●), PKC (○) or PKInd (■)

Tn preparation (90 μg) was phosphorylated in a volume of 200 μl with PKA, PKC or PKInd in the presence of the appropriate effectors as described in the Materials and methods section. At the indicated times, aliquots of incubation mixtures were removed and the reaction was stopped with 8% (final concentration) trichloroacetic acid; the amount of ^{32}P incorporated into acid-insoluble material was evaluated with a scintillation counter after filtration on a 25 μm glass-fibre filter.

shown in Figure 6, phosphorylation of the Tn preparation by PKA produced a decrease in the sensitivity of TnI to degradation by μ -calpain. Within the first 10 min of incubation more than 60% of unphosphorylated TnI had been degraded by μ -calpain, whereas in the phosphorylated state it was much more resistant to digestion. Also the rate of degradation of phosphorylated TnI was lower than that of the unphosphorylated protein. In contrast degradation of the TnI preparation phosphorylated with PKC was increased approximately twofold (Figure 6). Both treatments (with PKA and PKC) had no effect on the sensitivity of the Tn preparation to digestion by m-calpain (results not shown).

We also analysed whether these differences in Tn sensitivity to degradation by calpain were the result of changes in

Figure 6 Degradation of TnI after phosphorylation by PKA or PKC

Partially purified Tn preparation was incubated with either PKA (▲) or PKC (◆) for 10 min at 30 °C as described in the Materials and methods section. The phosphorylation reaction was stopped with staurosporine, then μ -calpain was added in the presence of 1 mM CaCl_2 . A time-course experiment similar to that described in Figure 1 was performed. The sample containing unphosphorylated Tn was treated in the same way but without ATP (●).

Table 1 Changes in apparent molecular mass of TnI after phosphorylation of isolated Tn complex by different rat muscle protein kinases

Tn preparations were incubated with PKA or PKC in the presence of the appropriate effectors as described in the Materials and methods section. After 10 min, the phosphorylation reaction was stopped with staurosporine, and aliquots (100 μl) of incubation mixture were submitted to gel permeation as described in the Materials and methods section. The K_D values for the standard proteins were respectively 0.12 for transferrin, 0.36 for BSA, 0.59 for ovalbumin, 0.8 for carbonic anhydrase and 0.94 for chymotrypsinogen A.

Protein kinase used to phosphorylate Tn complex	Distribution coefficient (K_D)	Apparent molecular mass of TnI (kDa)
None	0.083	90
PKA	0.38	60
PKC	0.79	30

the oligomeric structure of the Tn complex induced by phosphorylation. We examined whether phosphorylation with PKA and PKC produced changes in the apparent molecular mass of the Tn complex preparation. Molecular-mass changes were determined by calculating the coefficient for Tn distribution into Sephadex G-100 matrix, using TnI as the protein marker of the complex. As shown in Table 1, in the native unphosphorylated Tn complex, TnI protein had a K_D of 0.083 corresponding to a molecular mass of approx. 90 kDa. Treatment with PKA resulted in a change in the K_D from 0.083 to 0.38 which corresponds to a molecular mass of about 60 kDa. A different effect was observed on phosphorylation with PKC: the apparent molecular mass decreased to approx. 30 kDa which is close to that of the monomer. These results suggest that PKC phosphorylation causes an almost complete dissociation of TnI from its complex which is accompanied by an increase in its sensitivity to degradation by calpain.

DISCUSSION

The present results indicate that differences exist between the two calpain isoforms as far as Tn degradation is concerned. Confirming previous reports [7–9], TnI and TnT were found to be good substrates for calpain, whereas TnC proved to be resistant under all our experimental conditions. The degradation process was observed using μ -calpain, whereas m-calpain was much less active on both TnI and TnT. In addition, the disappearance of TnT and TnI induced by μ -calpain was completely prevented by the addition of 2 μ M leupeptin to the incubation mixture (results not shown).

Similar degradative patterns were observed when calpains were added to isolated purified Tns and when the proteinase preparation was layered on tissue slices. These findings further support the concept that μ -calpain is preferentially involved in the digestion of Tn both *in vivo* and *in vitro*. The large differences in the affinities for Tns between the two calpain isoenzymes further support a selective role for the two proteinases in muscle cells. In addition, the present data, together with a high Ca^{2+} requirement, seem to exclude the involvement of m-calpain in the degradation of Tns *in situ*.

Tn proteolysis has been proposed to play a role in functional alterations such as myocardial stunning. However, before extending our observations to situations *in vivo*, it should be pointed out that several factors can modulate the interaction between calpains and their substrates. It is well known that intracellular calpain activity is limited by the presence of a specific inhibitor, calpastatin. Furthermore, it is difficult to predict the extent and the degree of the increase in intracellular Ca^{2+} necessary to achieve calpain activation, particularly with the m-isoform, in order to promote Tn degradation.

Changes in protein conformation induced by phosphorylation as well as other covalent modifications have been shown to modulate sensitivity to proteolysis [24,25]. Tn molecules can be phosphorylated at different sites [12] by either PKC or PKA and the consequent protein modifications promote two different protein states which are reflected by different Ca^{2+} sensitivities of the myofibrillar actomyosin Mg-ATPase [26]. Similarly, phosphorylation by PKA or PKC appears to modulate the sensitivity of Tn to digestion in opposite directions.

Besides the present observation of a decreased Tn sensitivity to calpain digestion on phosphorylation by PKA, we have previously shown that activation of PKA results in changes in calpain-regulatory mechanisms, through the phosphorylation of calpastatin [20]. Thus the combination of a decrease in the sensitivity of substrate to proteolysis, together with an increase in the effectiveness of the calpain endogenous inhibitor, probably reduces the total Ca^{2+} -dependent proteolytic activity in cells on PKA stimulation.

It has been previously shown [7] that phosphorylation of TnI by PKA results in an increased sensitivity to calpain degradation.

We were not able to confirm these results, but the discrepancy could be attributed to differences in the experimental procedures used during the isolation of myofibrillar proteins, which imply the use of denaturing agents, and particularly the source of the enzymes. In our experimental conditions, isolated purified homogeneous μ -calpain, devoid of m-calpain activity and purified protein kinases activities were used.

In conclusion, we report that TnI and TnT are selectively degraded by μ -calpain, suggesting that this particular proteinase form is the one involved in degradation of these myofibrillar proteins *in vivo*. Phosphorylation of the Tns modifies their sensitivity to degradation, which can be explained on the basis of changes in their dissociation from the native complex.

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