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Calcium-supported calpain degradation rates for cardiac myofibrils in diabetes

Sulfhydryl and hydrophobic interactions

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Abstract

Objective: The purpose was to investigate the calcium required for calpain-mediated degradation of selected cardiac myofibril proteins modified by diabetes, sulfhydryl (SH) and hydrophobic reagents. **Methods**: After 20 weeks of streptozotocin-induced (55mg·kg^{-1}) diabetes, calcium sensitive calpain (1.5 U·ml^{-1}) degradation rates of purified cardiac myofibrillar proteins (1 mg·ml^{-1}) were measured, *in vitro*, and compared to degradation rates for N-ethylmaleimide (NEM) and 2-p-toluidinylnapthalene-6-sulfonate (TNS) treated samples. **Results**: Diabetes (blood glucose of 550 ± 32 mg·dl^{-1}) reduced the yield of purified myofibrillar protein with minimal change in fibril protein composition. Total SH group reactivities (nmol·mg^{-1}·30min) were 220 ± 21, 163 ± 17 and 156 ± 24 for control, diabetic and NEM-treated (0.5mM) myofibrils ($p \leq 0.05$). Calpain degradation rates were faster for all diabetic and SH modified myofibrillar proteins ($p \leq 0.05$), with a 45 and 35% reduction in the pCa_{50} for a 37 kDa protein of diabetic and NEM-treated fibril complexes. For control myofibrils, both 100 and 200 uM TNS, reduced calpain degradation rates to a similar extent for all substrate proteins. In contrast, diabetic and NEM-treated samples showed a further reduction in calpain degradation rates with increasing TNS from 100 to 200 uM. **Conclusion**: Our results support the hypothesis that in diabetes the calcium requirements for calpain degradation rates are reduced and dependent upon sulfhydryl group status and Ca^{2+}-induced hydrophobic interactions, implicating a 37 kDa myofbillar-complexed protein. (Mol Cell Biochem **135**: 51–60, 1994).

Key words: diabetic cardiomyopathy, proteolysis, calcium binding proteins, myofibrillar complexes, troponin

Introduction

The control of calpain activity in striated muscle is dependent upon: 1. the Ca^{2+} concentration, 2. autolyzed/unautolyzed state of the enzyme, 3. the level of inhibitor, calpastatin and presumably 4. the availability of digestible substrates. [1, 2] The relationships among these dynamic factors led Kapprell and Goll [3], as well as others [1, 2, 4], to speculate on the mechanism underlying calpain activation *in vivo*. Although autolysis of calpain reduces the Ca^{2+} concentration required for its binding to calpastatin, it does not eliminate calpastatins action on calpain [3]. The evidence that only 5% of th calpain is in the active form and calpastatin is distributed widely within muscle cells, makes it unlikely that the enzyme would ever be released from its inhibition *in vivo* [1]. In contrast sarcomeric Z-line disruption and sarcoplasmic reticulum and mitochondrial swelling reminiscent of calpain activation is associated with many pathophysiological conditions, such as diabetic cardiomyopathy [1, 5] and reperfusion-injury [6]. These data have made it necessary to speculate on how calpain's proteolytic activity may be regulated *in vivo*.

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One primary mechanism of calpain activation is the calcium concentration which in the micromolar and millimolar range play a central role in supporting calpain activities of the low and high Ca^{2+} requiring isoforms, u- and m-calpain, respectively. Since the cytosolic Ca^{2+} concentrations in the heart are well below those necessary to activate m-calpain, both under normal and pathological states, other mechanism(s) of Ca^{2+} induced calpain activation must exist. Sacchetta *et al.* [7] reported that glutathione disulfide (GSSG) and cystine reduced the Ca^{2+} requirement, by an order of magnitude, for erythrocyte calpain activation. Furthermore the oxidation of thiol groups and the formation of mixed disulfides within protein digestible substrates have been identified as a protein 'targeting' mechanism for proteases [8]. Reports showing diabetic cardiac myofibrils having a lower sulfhydryl group reactivity and content [9] (i.e. more oxidized), lead us to hypothesize a more rapid calpain mediated degradation of diabetic cardiac myofibrils linked to the oxidation-reduction status of protein substrates. To test this hypothesis we compared individual degradation rates of myofibrillar complexes from control and diabetic samples, together with those myofibrils undergoing irreversible SH modification (NEM).

What effect Ca^{2+} has on the calpain degradation rates of SH group modified myofibrillar complexes is unavailable in the literature. The increased myofibril fragmentation index of Hattori and Takahashi [10] (performed in the presence of calpastatin, the endogenous inhibitor of calpain activity) showed that intracellular Ca^{2+} can directly weaken myofibrillar complexes. Although the mechanism(s) in unknown, Ca^{2+} may promote physiologically relevant hydrophobic and conformational changes within intact myofibrillar complexes necessary for calpain's action. Because calcium's action on the calpain degradation of myofibrillar complexes is direct (i.e. acting on the myofibrillar structures) and not solely to activate calpain [11], we are proposing an enhanced proteolysis of SH modified myofibrils, due to a decrease in *their* Ca^{2+} requirements for calpain action. This direct effect of Ca^{2+} on protein substrates of calpain, exclusive of calpain effects, was assessed by:

1. using an exogenous source of the high calcium requiring isoform of calpain (i.e. skeletal muscle) so as not to bias the degradation results,
2. quantifying individual degradation rates of all groups across a range of calcium levels and
3. using TNS to lower the Ca^{2+}-induced hydrophobic interactions of myofibrillar proteins and quantify the calpain degradation rates.

Methods

Male Wistar-Firth rats (190 to 200g) (Biosciences, Edmonton, Alberta) were randomly assigned to either a control (N=15) or diabetic (N=20) group. Diabetes was induced with a 55 $mg \cdot kg^{-1}$ dose of streptozotocin (Upjohn Co., Kalamazoo, MI, USA) into the penile vein [12]. Control rats were injected with a comparable volume of citrate buffer under ether anaesthesia. Diabetes was assessed by a minimum blood glucose concentration of 350 $mg \cdot dl^{-1}$. All animals were maintained on a reversed day and night cycle (i.e. 6am to 6pm). All procedures were conducted in accordance with the regulations established by the Canada Council of Animal Care with an ethics certificate received from The University of British Columbia Animal Care Committee.

Animals were sacrificed by an injection of euthanol, i.p., following 20 weeks of diabetes. The ventricular muscle was dissected out, trimmed of all visible fat, connective tissue and frozen with tongs precooled in liquid nitrogen. This procedure was accomplished in 2 minutes for all animals, in an attempt to standardize possible postmortem artifacts.

Myofibrillar isolation and purification

Individual hearts were homogenized in a borate-KCI buffer (pH 7.1) containing 0.039 M sodium borate, 1 mM PMSF, 0.025 M KCI and 5 mM EGTA. The homogenate was centrifuged at 3,200 rpm for 12 minutes (Hermle 360Z, rotor VO2805) and the supernatant discarded [12, 13]. The resulting pellet was washed with 1.0% Triton-X100 for removal of membrane bound proteins and resuspended in 0.15 M KCI, 50 mM tris (pH7.0) and 1.0 mM dithiothreitol. The homogenate was filtered through a layer of gauze cloth and recentrifuged. The myofibrillar pellet was rewashed twice in a low salt buffer containing; 0.1 M KCI, 2 mM $MgCI^2$, 2mM EGTA, 0.01 M tris-maleate (ph 7.0) and 1.0 mM dithiothreitol. All procedures were performed at 4°C. Protein determinations were done according to the method of Lowry *et al.* [14].

Calpain purification

The purification of millimolar Ca^{2+} sensitive calpain from skeletal muscle was performed as described [15]. Muscle was homogenised in low salt buffer (LSB) containing 2 mM EDTA, 2 mM EGTA, 50 mM HEPES, 1 mM DTT (pH 7.4). Following ammonium sulphate precipitation (25 and 65%), the resulting precipitate was resuspended in 0.5 volumes (of starting muscle weight) of LSB and the suspended protein was dialysed overnight. The ammonium sulphate free protein suspension was diluted with LSB and subjected to DEAE-Sepharose CL-4B anion exchange column (2.6cm × 35cm) chromatography. The calpain isoforms were eluted with a salt gradient from zero to 400 mM KCI, with the high

calcium requiring calpain isoform eluting as a distinct peak between 250 and 330 mM KCl. Pooled fractions of calpain were subsequently subjected to Phenyl-Sepharose CL-4B column (1.6cm × 25cm) chromatography, followed by affinity chromatography (i.e. Hexylamine-agarose column (1.6cm × 12cm) at 75 mlh·r^{-1}. Active calpain fractions were pooled, concentrated (Amicon N_2 pressure cell) and loaded onto an Ultrogel AcA 34 gel filtration column (1.6cm × 80cm). A summary of the purification procedure is included in Table 1.

Table 1. Purification of the high Ca^{2+} requiring isoform of the calcium activated neutral protease, millimolar-calpain, from rabbit skeletal muscle

Fraction	Total protein (mg)	Specific activity (Units/mg)	Purity (fold)
Dialysate	15,000	0.021	1.0
DEAE-seph	240	1.84	8.8
Phenyl-seph	40	5.5	263.2
o-Hexylamine	18	11.4	544.4
Ultragel-AcA34	4	32.5	1555.1

Seph = sepharose

Caseinolytic activity

Calpain activity was standardized to the proteolysis of casein substrate and was assayed at 37°C in the presence of 2 mg·ml^{-1} casein, 5 mM DTT, 2.5 mM Ca^{2+} and 50 mM Hepes (pH 7.4) [15]. After 30 minutes, caseinolysis was quenched upon the addition of an equal volume of ice cold 5% TCA. A unit of calpain activity is defined as the amount of TCA soluble product resulting in an increase of 1 unit at 280nm after calpain digest of casein substrate. Estimates of calpain activity were performed in triplicate.

Myofibrillar proteolysis

Myofibrills prepared from each heart were individually digested (1 mg·ml^{-1} of myofibrils) with 40 ug·ml^{-1} of calpain, 10 mM DTT and 20 mM tris-maleate buffer (pH 7.0) in a final volume of 0.04 ml. This ratio (w/w) of myofibril:calpain protein was used for all digestion experiments in this study. Free calcium concentrations (see legends) present during the digestions were calculated by the method of Fabiato [16]. All myofibrillar proteolytic digestions were conducted at 22°C and terminated by the addition of leupeptin (40 ug·ml^{-1}). Digested myofibrillar proteins for each heart were resolved by 5–15% linear gradient SDS-polyacrylamide slab gel electrophoresis using the Laemmli buffer system [17]. Quantification of protein bands was accomplished with a LKB 2202 ULTROSCAN Laser Densitometer and a LKB Integrator (Model 2221). Since actin is not degraded by calpain, the proportions of alpha-actinin, desmin, troponin-tropomyosin subunit (Tn-T) and C-protein in undigested myofibril samples were standardized relative to actin and expressed as 100% (i.e. control levels). Calpain mediated degradation of these substrate proteins in the myofibrillar complexes were expressed as % degraded relative to their respective control value.

Sulfhydryl analysis

Total myofibrillar sulfhydryl group reactivities were estimated according to the procedure of Habeeb [18]. An aliquot of the preparations (0.5 mg) was dissolved in 3.0 ml of a solution containing 50 mM imidazole (pH 7.4) with or without 1% SDS. Following a 5 minute pre-incubation at 37°C, 0.1 ml of 3 mM DTNB was added to start the reaction, which was allowed to run to completion (i.e. 30 min.). The absorbances were recorded at 412nm and sulfhydryl group reactivity (i.e. plus-minus 1% SDS) determined directly from absorbance using a molar absorptivity value of 13,600 M/cm.

Myofibrillar complex modification

a) Irreversible sulfhydryl modification of myofibrillar complexes was achieved by pretreating with N-ethylmaleimide (NEM). Myofibrillar protein (5 mg) was incubated for 15 min at room temperature with 0.5 mM NEM in a total volume of 1.5 ml. These NEM-treated samples were washed free of NEM and digested with calpain (as described above).
b) Hydrophobic modification of myofibrillar complexes was achieved with 2-p-toluidinylnapthalene-6-sulfonate (TNS) [19]. Myofibrillar protein (1 mg) was incubated (15 min at 30°C) with TNS (100 and 200 uM) in a total volume of 1.5 ml, in the presence and absence of 1 mM free Ca^{2+}. The TNS-treated samples were washed free of any unbound TNS and digested with calpain, as described. To determine the Ca^{2+} specific effects of TNS-modification, the percent of protein degraded, by calpain, without Ca^{2+} present during the TNS pre-treatment was subtracted from the percent degraded with Ca^{2+} present during the TNS exposure.

Results

The injection of STZ induced chemical diabetes in 17 out of 20 rats as evidenced by the hyperglycemia and anthropometric changes. The control body and heart weights were 458 ± 31 and 1.19 ± 0.05 grams (N = 15) compared to 282 ± 21 and 0.85 ± 0.06 grams for the 20 wks diabetic group

($p \leq 0.05$). These changes increased the heart/body weight ratio to 3.01 ± 0.09 from 2.53 ± 0.08 $mg \cdot g^{-1}$ ($p \leq 0.05$). The 20 wks plasma glucose concentrations were 115 ± 4 and 550 ± 32 $mg \cdot dl^{-1}$ ($p \leq 0.05$) for control and experimental rats.

Myofibrillar yield, composition and proteolysis

The yield of purified myofibrillar complexes was reduced in the diabetic hearts by 8% (i.e. C = 45 ± 2 vs D = 41 ± 3 $mg \cdot g^{-1}$). SDS-PAGE analysis of myofibrils prepared from control (N = 15), diabetic (N = 17) and NEM-treated (N = 10) samples revealed no differences in Coomassie stained bands (Fig. 1). Densitometric analysis showed minimal differences across all preparations ($p \leq 0.05$) in the percentage of myofibrillar proteins, represented by myosin heavy chain (55 ± 3), actin (20 ± 1), alpha-actinin (1.5 ± 0.4), desmin (1.8 ± 0.5), C-protein (1.1 ± 0.3) and troponin-tropomyosin subunit (Tn-T) (2.1 ± 0.8). The total sulphydryl group reactivities ($nmol \cdot mg^{-1} \cdot 30min$) were different for control (220 ± 21) compared to diabetic (163 ± 17) and NEM-treated samples (156 ± 24) ($p \leq 0.05$). Proteolytic activity for 40 $ug \cdot ml^{-1}$ of m-calpain using native myofibrillar complexes (1 $mg \cdot ml^{-1}$) as substrate, measured in TCA extracts, was 0.73 ± 0.05 $A_{280} \cdot mg^{-1} \cdot 30min$. When myofibrillar complexes from diabetic and NEM-treated samples were substrates, calpain degradation rates increased significantly to 0.95 ± 0.10 and 1.06 ± 0.11 $A_{280} \cdot mg^{-1} \cdot 30min$., respectively. The possibility that endogenous contaminating calpain proteolysis during myofibril preparation, even in the presence of 5 mM chelator, contributed to any of these results was dismissed based on:

1. lack of any visible effects of protein degradation (i.e. compare Figures 1 and 2);
2. no detectable degradation of purified myofibrils from each group was observed when incubated for 120 minutes in 5 mM Ca^{2+} and no chelator.

Purified cardiac myofibrillar protein complexes treated with m-calpain resulted in a distinct pattern of degradation (or removal in the case of alpha-actinin) characterized by loss of Coomassie stained bands with molecular weights of 37, 55, 98 and 140 kDA, presumably Tn-T, desmin, alpha-actinin and C-protein (Fig. 2). When diabetic and/or NEM-treated myofibrils were used as substrates for calpain, the loss of these substrate proteins was more rapid ($p \leq 0.05$) (Fig. 3). These patterns and rates of degradation-removal can be directly attributed to calpain, since 'control' digests for all samples containing leupeptin and E-64 did not show any evidence of protein degradation (data not shown). Although proteolytic rates for individual protein substances grouped within the myofibril complexes varied, minimal differences in the percent degraded-removed for specific digestible proteins was observed after 120 minutes of calpain incubation. Autolysis of calpain did not limit the percent degraded for individual myofibrillar proteins, since 120 minute digests 'spiked' with an additional calpain after 60 minutes, were similar to those not supplemented.

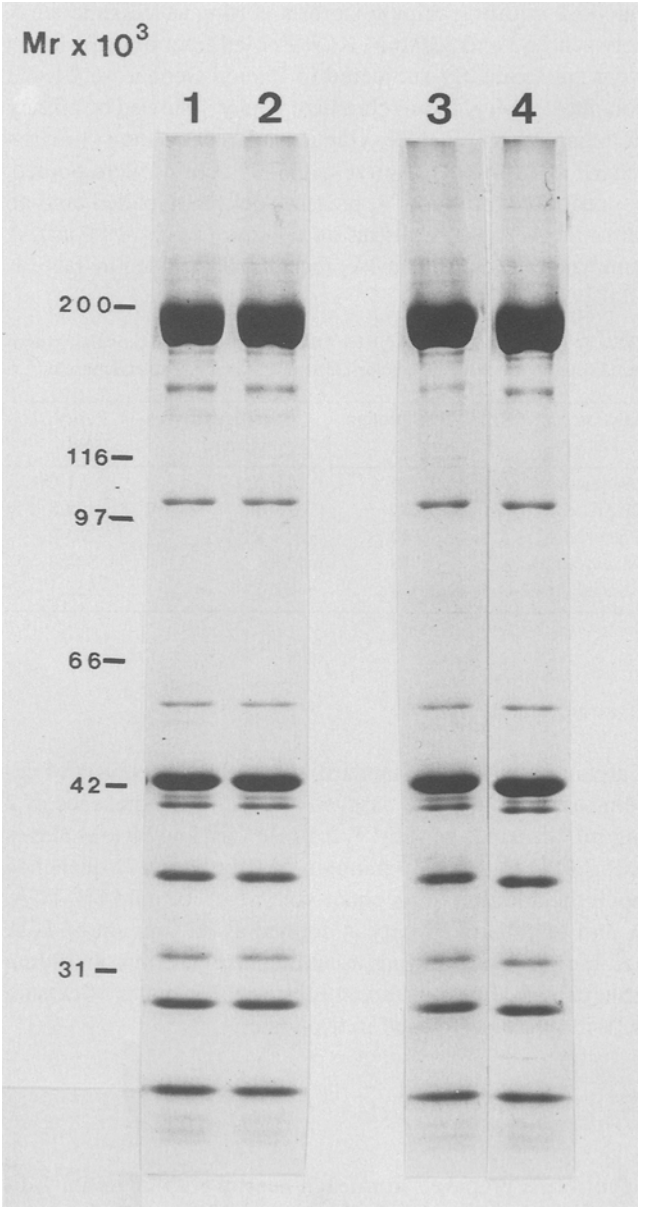

Fig. 1. Composition of myofibrils from rat heart resolved by 5–15% SDS-Page gradient gels. Lane 1, 40 ug of purified myofibrillar proteins from ventricular muscle, stained with Coomassie Brilliant Blue. Lane 2, myofibrillar proteins prepared from diabetic rat heart. Lanes 3 and 4 represent control and diabetic preparations which were treated with 0.5 NEM and subsequently used as calpain substrates. The molecular weight markers are as indicated.

Calcium requirement of calpain for myofibril degradation

Protein digestible substrates within the myofibrillar com-

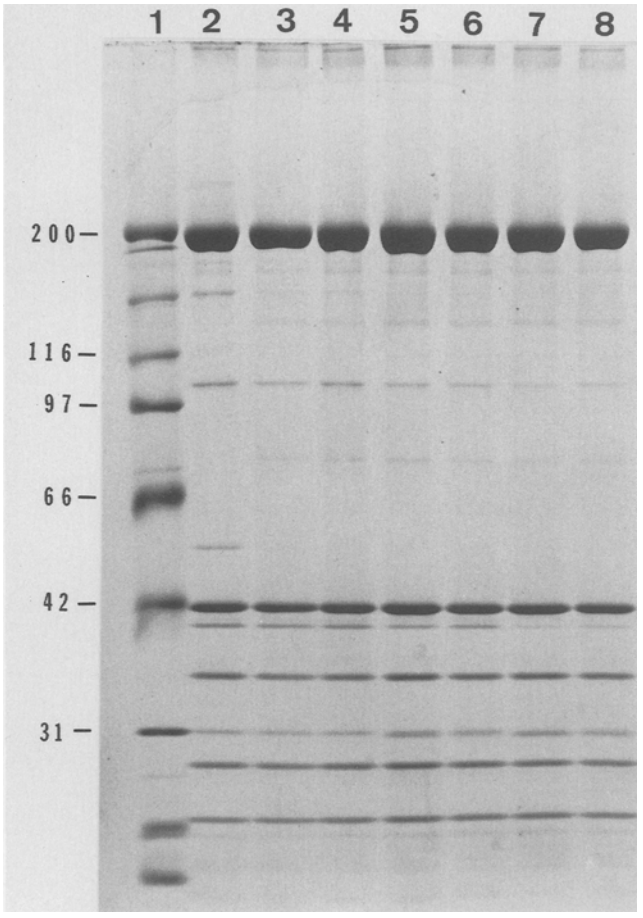

Fig. 2. Fragmentation of purified cardiac myofibrillar complex by calpain. SDS-polyacrylamide 5–15% linear gradient gels of purified myofibrils (1 $mg \cdot ml^{-1}$) from control hearts digested with 40 $ug \cdot ml^{-1}$ of calpain, stopped with 40 $ug \cdot ml^{-1}$ leupeptin at selected time intervals. Lane 1 loaded with 20 ug of molecular weight standards; lane 2 myofibrils in the absence of calpain; lane 3–8 represent digestion times of 0.5, 1, 5, 10, 20 and 30 minutes. Lanes 2–8 were loaded with 40 ug of protein per well. Assay conditions included 0.1 mM KCl, 20 mM tris, 1 mM free Ca^{2+} and 10 mM DTT at 22°C. Bands lost during digestion correspond to proteins with molecular weights of 37, 55, 98 and 140 kDA.

plexes required different free Ca^{2+} concentrations to elicit 50% degradation (removal) of each complexed protein (Fig. 4). For controls, free [Ca^{2+}] required for 50% maximal degradation of 37, 55, 98 and 140 kDA proteins were 201 ± 12, 330 ± 28, 212 ± 14 and 208 ± 15 uM. For diabetic myofibrillar substrate complexes, the Ca^{2+} requirements for 50% degradation-removal of the same proteins were 111 ± 13, 175 ± 18, 188 ± 16 and 204 ± 15 uM Ca^{2+}. For NEM-treated samples the same proteins required 131 ± 14, 213 ± 21, 197 ± 17 and 201 ± 15 uM Ca^{2+}. Regardless if the myofibrillar modifications were by diabetes or NEM, the largest shift in the free Ca^{2+} versus percent degradation curve occurred for the 37 and 55 kDA bands (Tn-T and desmin) (Fig. 4). Minimal differences between the native and modified samples existed in regard to the absolute amount of protein digested ($p \geq 0.05$) (Fig. 4).

StainsAll staining of myofibrils on 5–15% linear gradient SDS polyacrylamide gels revealed that the 37 kDa band, which disappeared upon calpain digestion, stained blue in all preparations (Fig. 5). The 55 kDa band stained red for all preparations. The relative amounts of TNS-treated myofibrils digested by calpain are presented in Table 2. There was a reduction in the amount of protein degraded for the TNS-treated control myofibrils with only that of Tn-T reaching statistical significance ($p \leq 0.05$). Doubling the TNS concentration did not change the magnitude of calpain degradation for the control preparations. A similar effect of TNS treatment (i.e. 100 uM) on diabetic and NEM-treated myofibrillar fractions was observed. In contrast, when treated with 200 uM TNS, the percent of Tn-T degraded was further reduced in the diabetic and NEM-treated myofibrils (Table 2). Although we attempted to slow down the degradation of desmin (i.e. by lowering the Ca^{2+} concentration and/or lowering the calpain activity) (data not shown), its digestion occurred much to quickly under our assay conditions to allow for any meaningful TNS comparison after seconds of digestion.

Discussion

The proteolysis experiments detailed in this investigation show that the high calcium requiring isoform of calpain

Table 2. Inhibition of calpain mediated myofibrillar complexed protein degradation by 2-p-toluidinylnaphthalene-6-sulfonate (TNS). Myofibril protein (1 $mg \cdot ml^{-1}$) degradation of selected substrates, pre-treated with 0, 100 and 200 uM TNS for 15 min in the presence of 1 mM Ca^{2+}, is expressed as percent degraded in 30 minutes by 40 $ug \cdot ml^{-1}$ of m-calpain. Each mean represents the average of 4 to 6 different preparations. Although minimal (<3%), the Ca^{2+}-independent TNS effects have been subtracted from the % degraded values for each protein

	TNS (uM)		
	0	100	200
Troponin-T:			
control	80 ± 9	59 ± 6 a	62 ± 10 a
diabetic	99 ± 8	63 ± 7 a	51 ± 6 a,b
NEM	99 ± 10	66 ± 10 a	60 ± 14 a
C-protein:			
control	78 ± 8	72 ± 10	70 ± 9
diabetic	97 ± 6	73 ± 8 a	68 ± 10 a
NEM	93 ± 9	70 ± 9 a	67 ± 11 a
alpha-actin:			
control	68 ± 8	61 ± 10	59 ± 9
diabetic	70 ± 9	55 ± 7 a	44 ± 9 a
NEM	68 ± 10	56 ± 11	50 ± 9 a

a = $p < 0.05$ for control vs TNS; b = $p < 0.05$ for 100 vs 200 uM TNS.

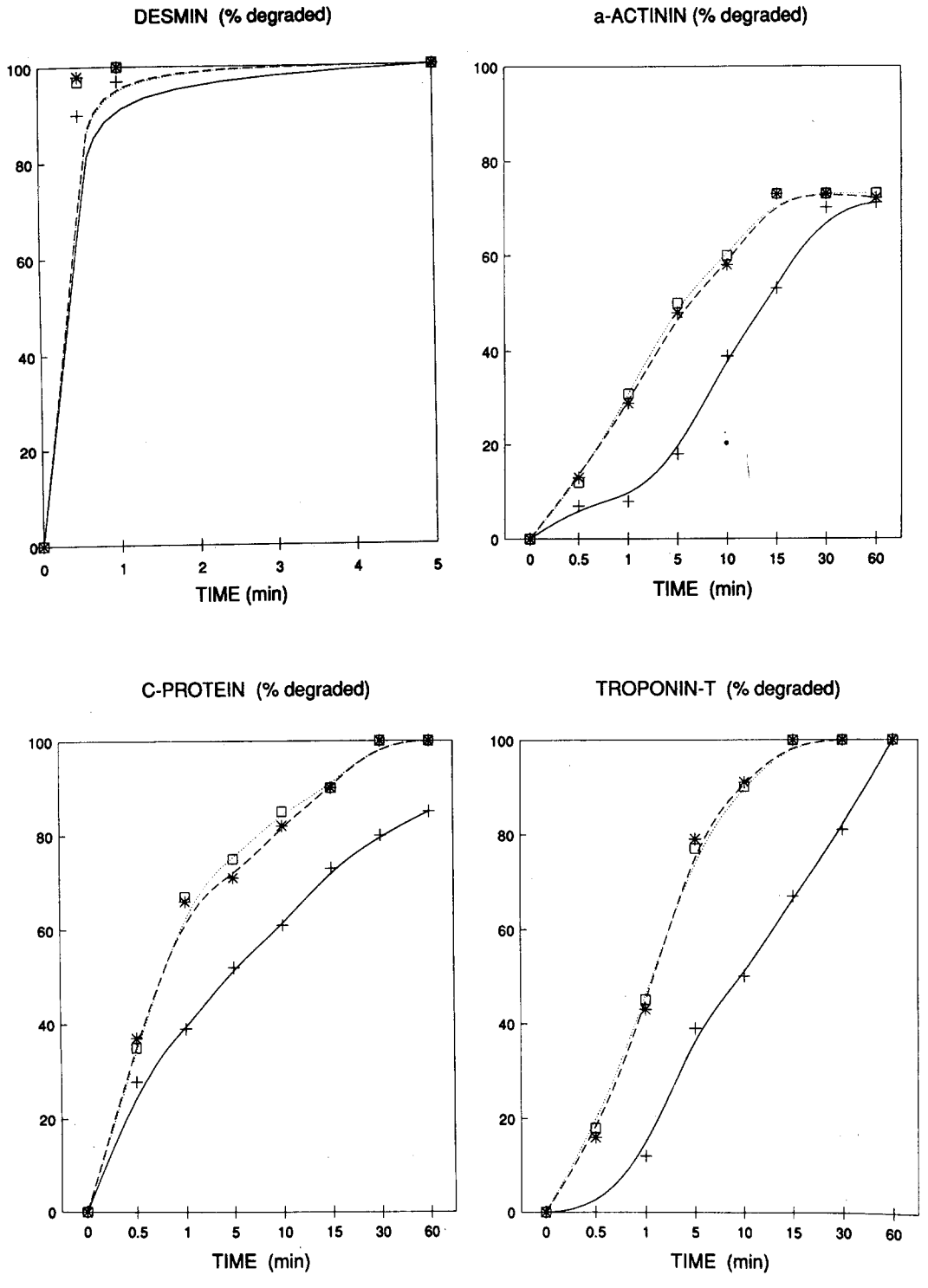

Fig. 3. Proportion of desmin, a-actinin, troponin-T and C-protein from control (+), diabetic (*) and NEM-treated myofibrils([]) degraded/removed by calpain (40 ug·ml^{-1}) in 60 minutes. The area represented by each protein was quanified by laser densitometry, normalized to actin and expressed as a percentage of the non-digested samples. Each point represents a mean of 5 different myofibrillar preparations, individually scanned, with a maximum standard error of ±9% for all points. Assay conditions were 0.1 mM KCl, 20 mM tris, 1 mM free Ca^{2+}, 10 mM DTT at 22°C for 60 min.

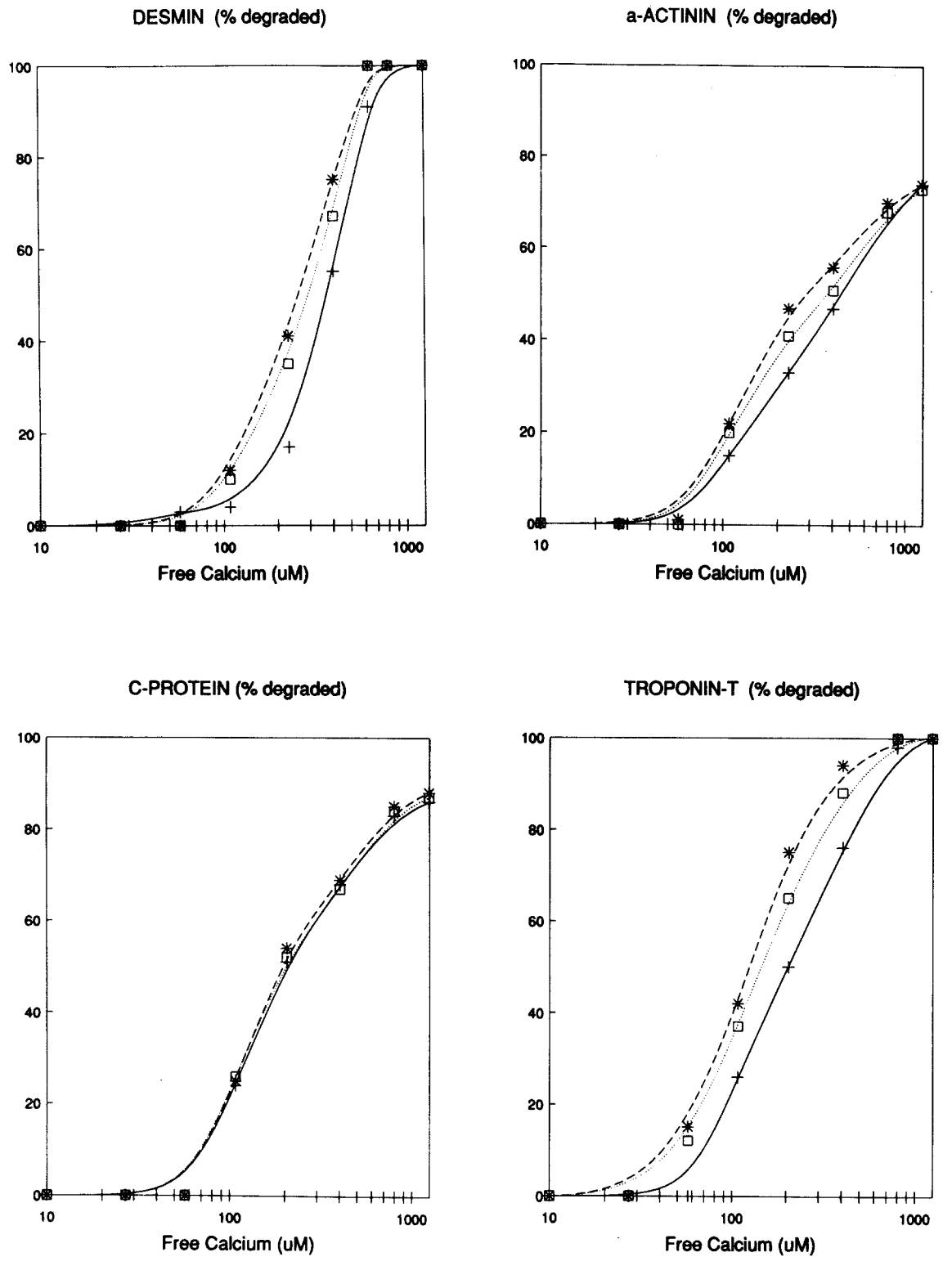

Fig. 4. The effect of Ca^{2+} concentration on the extent of calpain degradation/removal for desmin, a-actinin, troponin-T and C-protein from control (+), diabetic (*) and NEM-treated ([]) myofibrils. Free Ca^{2+} concentrations ranged from 0 to 1000 uM, as determined by the metal-ligand binding programs of Fabiato [16] using a K_{app} for CA-EGTA of 2.514×10^6 at pH 7.0 and 0.1 mM KCl. Other assay conditions were 20 mM tris, 10 mM DTT at 22°C for 60 minutes. Each point represents a mean from 4 to 6 different myofibrillar preparations with the largest standard error being ±12 percent.

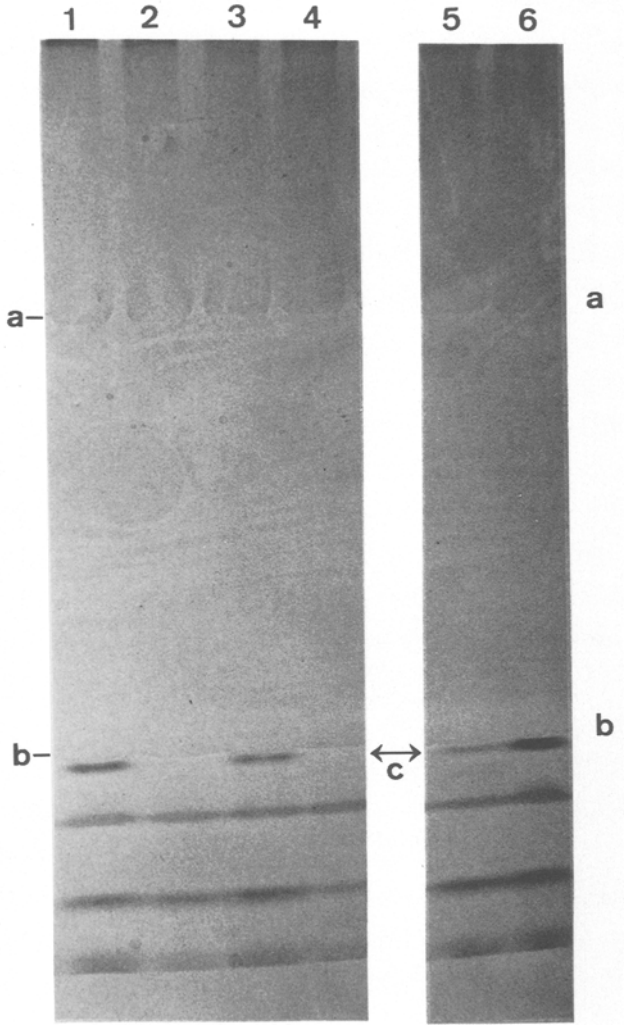

Fig. 5. Identification of proteins within myofibrillar complexes stained by StainsALL. Linear gradient (5–15%) SDS-PAGE gel loaded with 40 ug of purified myofibrils (1 mg·ml^{-1}) prepared from control rat heart in the absence (lane 1) and presence (lane 2) of 40 ug·ml^{-1} m-calpain for 60 minutes. Lanes 3 and 4 represent diabetic myofibrils incubated in the absence and presence of calpain, respectively. Lanes 1–4 were loaded with 40 ug protein/well. Lanes 5 and 6 are control myofibrils run with different protein concentrations, 40 and 80 ug/well, respectively. The letters a and b refer to reddish pink staining bands corresponding to myosin heavy chain and actin, while c refers to the Troponin-T band.

purified from skeletal muscle is capable of mediating degradation of 37, 55, 98 and 140 kDa proteins within the native cardiac myofibrillar complex. This pattern of degradation and/or removal is consistent with other reports employing striated muscle calpain on myofibrillar and Zeeline proteins [11, 20]. In all cases there was a more rapid degradation of substrates following either endogenous or exogenous sulfhydryl group modifications, which agrees with earlier observations of non-lysosomal proteases [7, 21, 22]. Factors contributing to this greater rate of proteolysis are unclear, but an altered oxidation-reduction status, has been suggested to be a targeting mechanism for selected protein degradation. For example, the oxidation of aldolase forms mixed disulphides which decreases its conformational stability and enhances the susceptibility of this enzyme to protease degradation [23]. Rivett [8] has found that several mammalian proteolytic enzymes including the calpain isoforms preferentially degrade the glutamine synthetase that was irreversibly inactivated by a mixed function oxidant system. Chung *et al.* [22] have reported that specific sulfhydryl residues in the sarcoplasmic reticulum ATPase, when modified, mark this enzyme for degradation. Since native cardiac myofibrillar proteins are in a more oxidized state in diabetes [9, 21], it is not surprising that their rates of degradation are increased. Confirmation of this modified SH group hypothesis was tested by subjecting native myofibrillar preparations to an irreversible SH group modifying agent M-ethylmaleimide (NEM). The similarity in proteolysis for these NEM-treated preparations provides indirect evidence that protein susceptibility of diabetic tissues is a function of its altered oxidation-reduction status which accompanies the disease.

The mechanism underlying the elevated rates of protein degradation for sulfhydryl modified myofibrillar proteins was studied by assessing calcium's role in the degradative process. That calcium is playing a central role in the degradation of myofibrillar complexes by acting on the substrate proteins supported in the literature [10, 11]. From the results of this study it is apparent that the 55 kDa and 37 kDa (presumably desmin and Tn-T) bands are associated with a reduced amount of Ca^{2+} necessary for 50% degradation by calpain in SH modified myofibrils. The physiological significance of this observation is uncertain, since the amount of Ca^{2+} necessary (i.e. > 100 uM) is higher than that normally reported for striated muscle. However, due to the dynamic nature of calpain activation, as suggested by Kapprell and Goll [3], this increased Ca^{2+} sensitivity may have physiological significance when the mechanism of calpain activation is fully elucidated. The Ca^{2+} sensitivity is not through the Ca^{2+} binding subunit of troponin, since Ca^{2+} activation of myofibrillar ATPase activity through Tn-C is unaffected by diabetic modification of sulfhydryl groups [9]. Similarily, this effect is independent of Ca^{2+} interaction with calpain, since not all substrate proteins responded in a similar manner (i.e. substrate specificity). The results of this study implicate Tn-T as potentially key protein element in the Ca^{2+} induced protein substrate changes for calpain mediated degradation of native myofibrils. The observation that Tn-T stained blue on StainsAll gels lends some indirect support for the suggestion that the acidic nature of Tn-T may indirectly participate in the Ca^{2+} sensitivity changes observed for myofibrillar degradation. This suggestion is not unfounded since other reports implicating Tn-T to myofibril

Ca^{2+} sensitivity have emerged. Reports showing that the Tn-T isoform affects the pCa-force relationship in striated muscle [24] and that calcium sensitivity of *cardiac* myofibrils is not exclusively a function of Tn-C, in native and Tn-C depleted myofibrils [25], in part supports the existence of Ca^{2+} responsiveness within the myofibrillar system to Tn-T. Although the mechanism of enhanced protein substrate degradation being linked to Tn-T indirectly by Ca^{2+} sensitivity changes remains speculative, it appears from our TNS results to involve Ca^{2+}-induced hyrophobicity changes within the myofibrillar complexed proteins. A similar mechanism involving a Ca^{2+}-induced exposure of hydrophobic regions on other proteins is well documented [26]. The observation that Tn-T degradation is further reduced, by higher TNS exposure of SH modified myofibril preparations and not those of controls, implicates it in a necessary hydrophobicity change for calpain action. Because calpains are attracted to hydrophobic regions [1, 2, 3], this theory of an indirect Ca^{2+}-induced increase in Tn-T hydrophobicity underlying calpain mediated myofibrillar degradation is pausible and requires further investigation.

The observation that C-protein and alpha-actinin did not have an altered Ca^{2+} sensitivity but did have faster rates of degradation for SH modified samples raises interesting possibilities. This implies that the SH modifications not only influence Ca^{2+}-induced hydrophobic changes but also some other, as yet undetermined process. Of particular significance in this regard is the report that SH group status influences the release of a myofilament pool, referred to as easily releasable myofilaments, which are devoid of alpha-actinin, C-protein and titin [27]. When SH groups are modified (i.e. NEM) the proportion of these easily releasable myofilaments are reduced (i.e. more bound to the myofibrillar fraction), possibly providing more substrate and/or binding sites for calpain in the region of the Z-line. This mechanism could underlie the increased rates of calpain degradation observed in this study, in the absence of any Ca^{2+} senisitivity change for alpha-actinin and C-protein.

In conclusion the results of this study show that

1. myofibril proteins from diabetic hearts have faster calpain-mediated degradation rates attributable to their SH group status;
2. the calcium requirements for calpain-mediated degradation are reduced;
3. the calcium sensitivity changes for calpain action are mediated through essential hydrophobic interactions which appear linked to troponin-tropomyosin subunit of the myofibrillar complex.

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