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Activity profile of calpains I and II in chronically infarcted rat myocardium – influence of the calpain inhibitor CAL 9961

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1 The calpains have been proposed to be activated following cardiac ischaemia and to contribute to myocyte damage after myocardial infarction (MI). In this study, the activity of calpains I and II in the infarcted and non-infarcted rat myocardium and the action of the selective calpain inhibitor, CAL 9961, has been investigated.

2 MI was induced by permanent ligation of the left coronary artery. One, 3, 7 and 14 days post MI, the enzymes calpain I and II were separated from homogenates of the interventricular septum (IS) and left ventricular free wall (LVFW) by chromatography on DEAE-Sepharose. The activity of the calpains was measured in sham-operated and MI animals chronically treated with placebo or CAL 9961 (15 mg kg^{-1} d^{-1} s.c.) in a synthetic substrate assay. Treatment was started 3 days before MI induction.

3 Calpain I activity reached highest values in IS 14 days post MI, whereas maximum activity of calpain II was measured in LVFW 3 days post MI. In experiments *in vitro*, CAL 9961 completely inhibited both calpains. *In vivo*, chronic treatment of MI animals with CAL 9961 partially prevented the increase in calpain I activity in IS and reduced calpain II activity in LVFW to sham levels.

4 Our findings demonstrate that calpains I and II are activated after MI, however, both enzymes differ in their regional and temporal activation within the infarcted myocardium. Chronic inhibition of these enzymes with CAL 9961 might limit the calpain-induced myocardial damage and preserve cardiac structural integrity post MI.

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Abbreviations: AMC, 7-amino-4-methyl-coumarin; BW, body weight; $[Ca^{2+}]_i$, intracellular calcium concentration; ECG, electrocardiogram; IS, interventricular septum; LVFW, left ventricular free wall; MI, myocardial infarction; SR, sarcoplasmatic reticulum; THW, total heart weight

Introduction

Intracellular calcium ($[Ca^{2+}]_i$) plays a central role as a second messenger in regulating various cellular functions such as mechanical, metabolic and regulatory processes (Clapham, 1995; Berridge *et al*., 1998). In the myocardium, disturbances in the precise control of $[Ca^{2+}]_i$ result in dysregulation of various myocardial functions and compromise cell viability. During myocardial ischaemia or hypoxia, a characteristic rise in the myocardial $[Ca^{2+}]_i$ occurs (Arthur & Belcastro, 1997). This has been shown to be a pivotal event in myocardial cell injury and results in the activation of intracellular Ca^{2+}-dependent enzymes. Calpains, a family of Ca^{2+}-activated proteases found in the cytosol of many cell types (Murachi, 1983), have been implicated in a wide array of cellular pathological states associated with proteolysis and cell death. However, their precise function during myocardial ischaemia still remains to be established.

Ubiquitous calpain is composed of two distinct isoforms, calpain I (μ-calpain) and calpain II (m-calpain), which are similar in substrate specificity but differ in their requirements for Ca^{2+}: Calpain I is activated by micromolar $[Ca^{2+}]_i$ whereas calpain II requires millimolar $[Ca^{2+}]_i$ for half maximal activation (Mellgren, 1989; Suzuki *et al*., 1995). The calpains exist as inactive enzymes which are inhibited by the endogenous inhibitor, calpastatin. In the presence of Ca^{2+}, calpastatin dissociates yielding active calpain. When activated, both calpain isoforms degrade cytoskeletal proteins (e.g. fodrin, spectrin and microtubule-associated proteins), membrane receptors (e.g. growth factor receptors, adhesion molecules and ion transporters), enzymes (e.g. kinases, phosphatases and phospholipases) as well as signalling molecules (e.g. transcription factors) (Saido *et al*., 1994). Thus, during cardiac ischaemia, the increase in myocardial $[Ca^{2+}]_i$ can activate the calpains causing damage to myocardial proteins (Yoshida *et al*., 1995a) leading to myocyte death and, consequently, to loss of myocardial structure and function (Reimer & Jennings, 1986). Indeed, evidence suggests the involvement of the calpains in myocardial ischaemia-reperfusion injury (Iizuka *et al*., 1993; Yoshida *et al*., 1995b), myocardial stunning (Arthur & Belcastro, 1997) and cardiac hypertrophy (Maki *et al*., 1990). However, the actual functions of the calpains *in vivo* during chronic myocardial ischaemia and their contribution to the regulation of the cardiac structural remodelling process following myocardial infarction (MI) remain to be clarified.

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Several studies have reported that calpain inhibitors reduced proteolysis of myocardial proteins and, therefore, prevent contractile dysfunction during ischaemia and hypoxia, suggesting the involvement of the calpains in these processes (Yoshida *et al*., 1995b). However, in these studies, neither the temporal and regional differences in the activation of cardiac calpains following ischaemia were investigated nor the quantitative and qualitative differences of these agents in the inhibition of calpain I and/or II were considered. Additionally, problems of several calpain inhibitors are their low specificity for calpain, limited capability to penetrate the cell membrane, toxicity to living cells and difficulties in their administration.

Therefore, the aim of this study was to examine the temporal and regional differences in the activity of the calpains in the infarcted rat heart. In particular, we measured the enzymatic activity of the calpains I and II in the infarcted and non-infarcted myocardium in the acute (1 and 3 days) and chronic (7 and 14 days) phases after induction of MI. To achieve this, we established a method for the measurement of cardiac tissue calpains I and II activity using a chromatographic separation of both enzymes combined with a fluorimetric assay using a synthetic substrate. Finally, we investigated the effects *in vitro* and *in vivo* of a selective, cell permeant calpain inhibitor, CAL 9961, on the activity of the calpains I and II in the ischaemic and non-ischaemic rat myocardium in order to assess the ability of this substance to inhibit calpains I and II following MI.

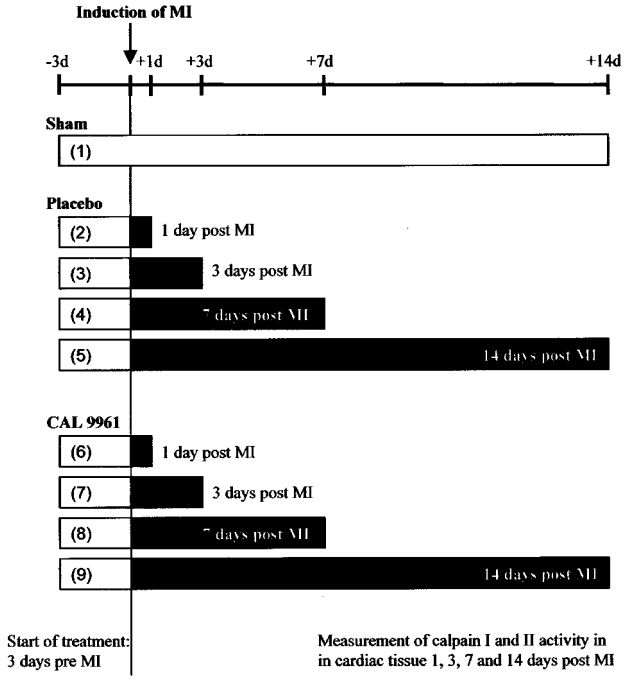

Figure 1 Representation of the experimental protocol (study design).

Methods

Study design

Male, normotensive Wistar rats (Charles River Viga GmbH, Sulzfeld, Germany), initially weighing 230–270 g, were used throughout the study. All experiments were performed in accordance with the German laws on animal protection as revised in 1993. The animals were housed individually at controlled temperature and humidity under a 12 h light/dark cycle. Rats had free access to a standard diet (Altromin® Altromin GmbH u. Co.Kg., Lage-Lippe, Germany) and to drinking water.

The animals were randomly divided into nine groups: Group 1: sham-operated without treatment; Group 2–5: myocardial infarction (MI) subjected to placebo treatment (0.9% saline); Group 6–9: MI subjected to calpain inhibitor (CAL 9961) treatment (15 mg kg^{-1} d^{-1}). CAL 9961 is a new selective, cell permeant calpain inhibitor with a K_i value for calpain I of 200 nM and for calpain II of 25 nM. The substance was kindly provided by BASF-Knoll AG (Ludwigshafen, Germany) and was administered daily as a solution in water *via* subcutaneous injection. Treatment was initiated 3 days prior to induction of MI (3 days pre MI) in all groups and continued until sacrifice. The activity of calpain I and calpain II was investigated in cardiac tissue samples 1, 3, 7 and 14 days after surgery, according to the study design indicated in Figure 1. The number of animals per group was 6–8.

After 1 week in individual cages, rats were anaesthetized by injection of Ketamin-Xylazin (35 mg 2 mg^{-1} kg^{-1} i.p.) and artificially ventilated (70 ventilations min^{-1}, 200 mm H_2O, 2.5 ml $ventilation^{-1}$) to perform a left thoracotomy. Rats in group 1 underwent a sham operation. In the remaining animals, MI was induced by permanent ligation of the left coronary artery. Body weight (BW) was measured daily before induction of MI and during the 14 days post MI to adjust the individual dose of CAL 9961.

On days 1, 3, 7 and 14 post MI, the hearts were excised from the sham-operated and infarcted animals with placebo or CAL 9961 treatment, weighed and dissected into the cardiac tissue regions, interventricular septum (IS) and left ventricular free wall (LVFW) including scar tissue and area at risk. This procedure was performed within 2 min to minimize possible postmortem artifacts. The degree of cardiac hypertrophy was determined by measurement of the ratio of total heart weight (THW) to BW (THW/BW). Calpains I and II were separated from the cardiac tissue regions IS and LVFW using anion exchange chromatography. The activity of both enzymes was measured using a synthetic fluorogenic substrate.

The inhibitory action of CAL 9961 on calpains I and II activity was measured in experiments *in vitro* by addition of the inhibitor (226 nM) to the activity assay containing either commercially available purified calpain I and II (0.5 units ml^{-1}, Calbiochem, Schwalbach, Germany) or calpain I and II separated from cardiac tissue samples of animals used in this study. To investigate the effect *in vivo* of the protease inhibitor on calpains I and II activity of cardiac tissue samples, infarcted animals were chronically treated with CAL 9961.

Chromatographic separation of calpains I and II

Each cardiac tissue sample (approximately 0.6–1.2 g) was homogenized in five volumes of homogenization buffer (25 mM imidazole/HCl containing 5 mM cysteine and 1 mM

EDTA, pH 7.5) for 1 min using an IKA Ultra-Turrax (20,500 r.p.m., T25, Janke and Kunkel, Germany). The homogenates were centrifuged at $100,000 \times g$ for 1 h (Beckman Ti 70.1 rotor, 37,000 r.p.m.), and the supernatants were applied to a 1×10 cm DEAE-Sepharose column (Amersham-Pharmacia Biotech, Uppsala, Sweden) equilibrated in homogenization buffer. All procedures were performed on ice or at 4°C using pre-cooled buffers and centrifuges. The elution was performed with a 50 ml linear gradient of 0–500 mM NaCl in homogenization buffer at a flow rate of 1 ml min^{-1}, giving 25×2 ml fractions. As the calpains rapidly autolyse in the presence of Ca^{2+}, homogenization and separation procedures were performed in Ca^{2+}-free buffers. In preliminary experiments, a reduction of the activity of both calpain isoforms as well as protein aggregation following freezing of the homogenates at −80°C as well as at −20°C was observed. The inactivation and precipitation were overcome by the addition of glycerol to a final concentration of 16.7% (v v^{-1}) prior to freezing. Following chromatography, NaCl present in the fractions caused a slight depression of calpain activity. This was, however, at most 20% inhibition and was not relevant since differences in calpain activity in the same region of the chromatogram were being considered.

To identify calpains I and II in the fractions, SDS electrophoresis using a 10% polyacrylamide resolving gel was carried out according to Laemmli (1970). In the blotting subsequent procedure, proteins were transferred from the gel onto Immobilon-P transfer membranes (Millipore, Bedford, MA, U.S.A.) for 1.5 h at 0.8 mA cm^{-2}. Protein transfer was confirmed by the staining intensity of a standard protein (β-actin, Amersham-Pharmacia Biotech, Uppsala, Sweden) with Ponceau-Red (Sigma, Deisenhofen, Germany). After washing the membranes three times in TTBS (0.1% Tween 20, 100 mM Tris-HCl, 150 mM NaCl, pH 7.5), they were blocked for 1 h in 5% non-fat milk/TTBS and incubated for 12 h at 4°C with one of the following mouse monoclonal primary antibodies (Chemicon, Hofheim, Germany): Anti-large and anti-small subunit of calpain I, and anti-large and anti-small subunit of calpain II, all in a 1 : 1000 dilution in TTBS. Following three washes in TTBS, the membranes were incubated with a 1 : 1000 dilution of the horseradish-peroxidase-coupled secondary rabbit anti-mouse IgG antibody (Amersham-Pharmacia Biotech, Uppsala, Sweden) for 30 min at room temperature. The membranes were then washed extensively in TTBS and bound calpains were visualized using the chemiluminescence detection system based on the ECL-reagent (Amersham-Pharmacia Biotech, Uppsala, Sweden) and exposed to ECL-film according to the manufacturer's instructions.

Enzyme assay

Calpains I and II activity was measured using the synthetic fluorogenic substrate for calpain, Suc-Leu-Tyr-AMC (Calbiochem, Darmstadt, Germany and Affinity Research, Mamhead, U.K.) (Sasaki *et al*., 1984). Specific proteolysis of the substrate by calpain liberates the AMC group, leading to an increase in fluorescence at 470 nm (excitation 385 nm). The enzyme activity for calpains I and II was measured in all samples in a total volume of 200 μl, containing 170 μl of the enzyme fraction, 10 μl Suc-Leu-Tyr-AMC (final concentration 500 μM) and 20 μl $CaCl_2$ (final concentration 5 mM to optimally activate both calpain isoforms, Ca^{2+}-dependent activity). Details for the Ca^{2+}-titrations and the relationships between hydrolysis of Suc-Leu-Tyr-AMC and Ca^{2+}-requirements have been described previously by Elce *et al*. (1997b). To measure Ca^{2+}-independent activity, $CaCl_2$ was replaced by 20 μl EDTA (neutralized to pH 7.5 with NaOH) to give a final concentration of 10 mM. The reaction was performed at 30°C and started by addition of substrate. After 1 h, the reaction was stopped by addition of 200 μl alkali buffer (250 mM glycine, 85 mM Na_2CO_3, 120 mM NaCl, pH 10.7) and the fluorescence was measured in 220 μl of the stopped reaction mixture in a 96 well microtiter plate using a spectrofluorometer (Fluorolite 1000, Dynatech Laboratories, lamp voltage 6V). An AMC standard curve was included in each experiment. In this assay, substrate hydrolysis was linear with respect to time and the activity was generally proportional to the amount of protein added, indicating that the test was quantitative under these conditions. The protein concentration of each fraction was determined by the method of Bradford (1976) using bovine serum albumin as a standard. The activity of calpains I and II was determined as the difference between the Ca^{2+}-dependent and the Ca^{2+}-independent fluorescence per minute.

Statistical analysis

Statistical evaluation was performed with SYSTAT software using one-way analysis of variance with repeated measurements (ANOVA). Means shown to be different between individual groups were compared using the *post hoc* unpaired Student's *t*-test or the Bonferroni test when appropriate. A probability of $P < 0.05$ or less was considered as significant. Results are expressed as mean ± s.e.mean. Further details of statistical analysis are given in the legends to the figures.

Results

Calpain separation and assay

In the present study, the activity of calpains I and II in the infarcted and non-infarcted rat myocardium was quantified at various time intervals after the induction of MI. In crude supernatants, the calpains were separated by anion exchange chromatography on DEAE-Sepharose using a linear NaCl gradient from 0–500 mM. In accordance with the findings by Spalla *et al*. (1985), we observed two peaks of Ca^{2+}-dependent activity (5 mM). The first peak of activity eluted at 180–240 mM NaCl, and the second peak of activity emerged at 260–320 mM NaCl (Figure 2a,b). Using an immunoblotting technique, we identified predominantly calpain I in the first peak, whereas calpain II was present in the second peak (Figure 3). For both calpain isoforms, the 80 kDa subunit was detected. Although a number of primary antibody concentrations were tested no 30 kDa subunits could be detected in the calpain fractions.

Activity of calpains I and II in the infarcted myocardium

The activity of calpains I and II was measured in the interventricular septum (IS) and the left ventricular free wall (LVFW) of the rat myocardium at 1, 3, 7 and 14 days after

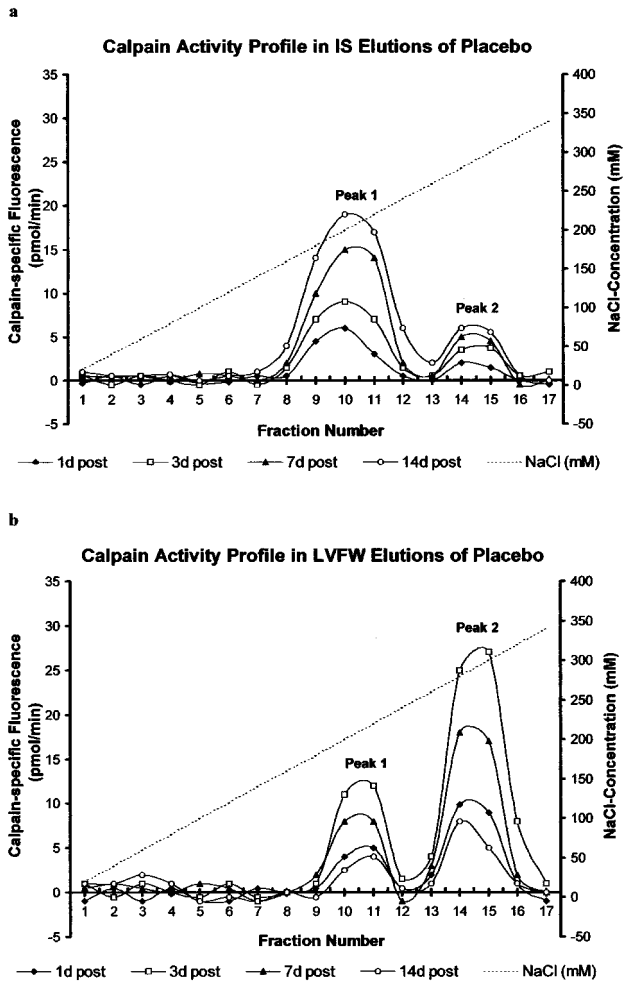

Figure 2 The elution profile of calpain specific activity ($Fluorescence_{Ca2+}$-$Fluorescence_{EDTA}$; pmol min^{-1}) following anion exchanger chromatography on DEAE-Sepharose of supernatants of the interventricular septum (IS) (a) and of the left ventricular free wall (LVFW) (b) of placebo-treated MI animals 1, 3, 7 and 14 days post MI. Two peaks of activity were observed occurring in fractions 8–12 (180–240 mM NaCl) and in fractions 13–16 (260–320 mM NaCl). Peaks in IS elutions increased in the time course following MI showing highest values 14 days post MI. Maximum peak values in LVFW elutions were measured 3 days post MI. $n=6-8$.

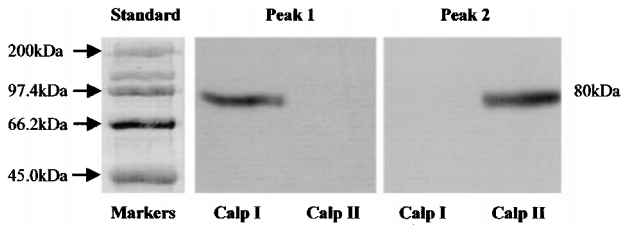

Figure 3 Representative experiments showing Western blots of the calpains I and II in elutions of the infarcted rat myocardium following anion exchanger chromatography on DEAE-Sepharose. For both calpain isoforms, the 80 kDa subunit was detected. Peak 1 was identified to contain calpain I, whereas peak 2 was identified as calpain II, respectively.

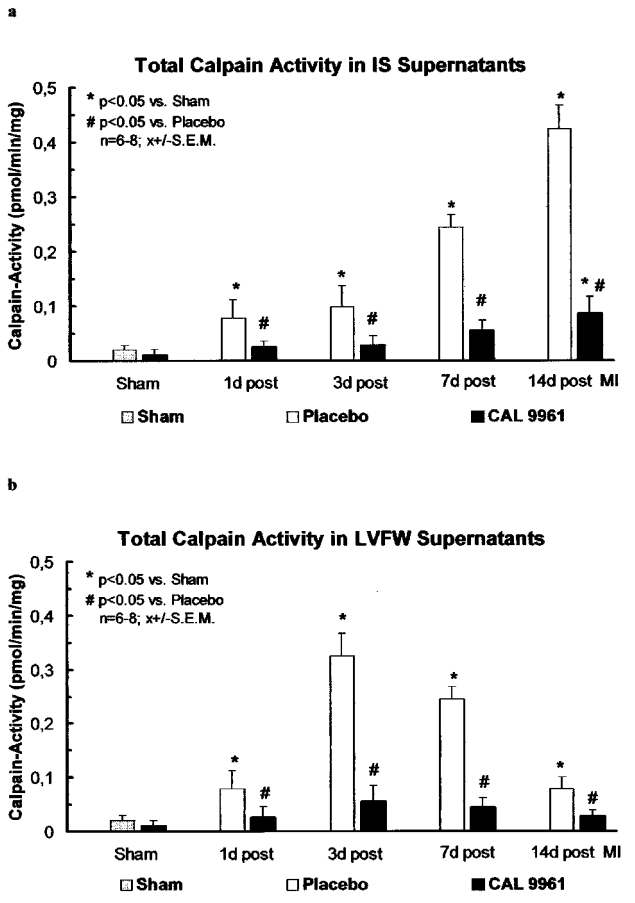

Figure 4 Total calpains activity (pmol AMC min^{-1} mg^{-1} protein) in supernatants of homogenates of the interventricular septum (IS) (a) and of the left ventricular free wall (LVFW) (b) of sham-operated and infarcted animals with placebo and CAL 9961 treatment (15 mg kg^{-1} d^{-1} s.c.), measured 1, 3, 7 and 14 days post MI; $*P<0.05$ compared with sham; $\#P<0.05$ compared with placebo; Data represent mean ± s.e.mean; $n=6-8$.

infarction, in order to investigate regional and temporal alterations of the calpains activity. Following homogenization and centrifugation of the cardiac tissue samples, the high-speed supernatants contain both calpain isoforms allowing the measurement of the total calpain activity. As shown in Figure 4a, the activity of the calpains was significantly increased 1 day post MI in IS supernatants of placebo-treated MI animals compared to sham-operated animals. In the time course post MI, the activity of the calpains further increased after 3 and 7 days and exhibited highest activity 14 days post MI in IS of placebo-treated MI animals. In contrast, the calpains activity of LVFW supernatants was increased on day 1 post MI, reached maximum activity on day 3 and decreased strongly during days 7 and 14 in placebo-treated MI animals compared to sham-operated animals (Figure 4b).

To identify quantitative differences in the activity between both calpain isoforms within the infarcted (LVFW) and non-infarcted (IS) rat myocardium and at different time points post MI, calpain I and calpain II were separated from the cardiac tissue homogenates using DEAE-Sepharose chromatography. In elutions of the IS (Figure 2a) and of the LVFW (Figure 2b) of placebo-treated MI animals, two peaks of calpains activity within the same fraction range, representing calpain I in fractions 8–12 and calpain II in fractions 13–16,

were observed. Since peak widths were approximately the same, peak height can be viewed as semiquantitative measure of the activity of the individual calpain isoforms. As shown in Figure 2a, the calpain activity in IS elutions of placebo-treated MI animals was 3 fold higher for calpain I (peak 1) compared with calpain II (peak 2). Despite differences in the activity, both peaks increased within the time after induction of MI so that the highest activities for calpains I and II were detectable 14 days post MI in fractions of IS elutions. In contrast to IS elutions, significantly more calpain II activity (peak 2) was measured relative to calpain I (peak 1) in LVFW elutions (Figure 2b). However, this maximum activity for both calpains was measured 3 days post MI and remained elevated for 7 and 14 days post MI in LVFW.

Effects of CAL 9961 on calpains activity post MI in vitro *and* in vivo

To investigate the action of the calpain inhibitor CAL 9961 *in vitro*, the calpain activity assay was performed using either commercially available purified calpains I and II or crude calpains I and II from cardiac tissue samples. Following dose-findings studies, a dosage of 226 nM CAL 9961 completely suppressed the activity of commercial calpains I and II (0.5 units ml^{-1}) as well as of calpains I and II in homogenates of cardiac tissues (IS, LVFW).

In experiments *in vivo* the effects of chronic treatment with CAL 9961 (15 mg kg^{-1} d^{-1}), started 3 days prior to induction of MI, on the total calpain activity in IS and LVFW were studied. In these experiments, chronic treatment of MI animals with CAL 9961 attenuated the MI-induced increase in the total calpain activity in supernatants of IS 1, 3 and 7 days post MI to values not significantly different from those in sham-operated animals (Figure 4a). Fourteen days post MI, CAL 9961 treatment reduced the total calpain activity relative to placebo treatment, however, this activity was significantly elevated in comparison with sham-operated animals (Figure 4a). The post-MI elevation in total calpain activity in LVFW supernatants was abolished in CAL 9961-treated compared with placebo-treated MI animals as measured at all four time points post MI (not significantly different from sham-operated animals) (Figure 4b). These results suggest a partial inhibition of total calpain activity by CAL 9961 in the non-infarcted myocardium during the chronic state post MI whereas total calpain activity was reduced to sham levels in the infarcted myocardium during the acute phase post MI.

To investigate which calpain isoform was inhibited by CAL 9961, calpains I and II were separated from the cardiac tissue samples of sham-operated and MI animals with placebo and CAL 9961 treatment. Calpains I and II activity separated by DEAE-Sepharose chromatography of IS and LVFW high-speed supernatants was suppressed in CAL 9961-treated MI animals (Figure 5a,b). As shown in Figure 5a, calpain I activity was decreased (63%) and calpain II activity was reduced to sham levels (69%) by CAL 9961 in IS elutions 14 days post MI compared with placebo-treated MI animals. In contrast, the post-MI increase in activity of both calpain isoforms was completely attenuated (57% for calpain I, 81% for calpain II) by chronic CAL 9961 treatment in LVFW 3 days post MI compared with placebo treatment (Figure 5b).

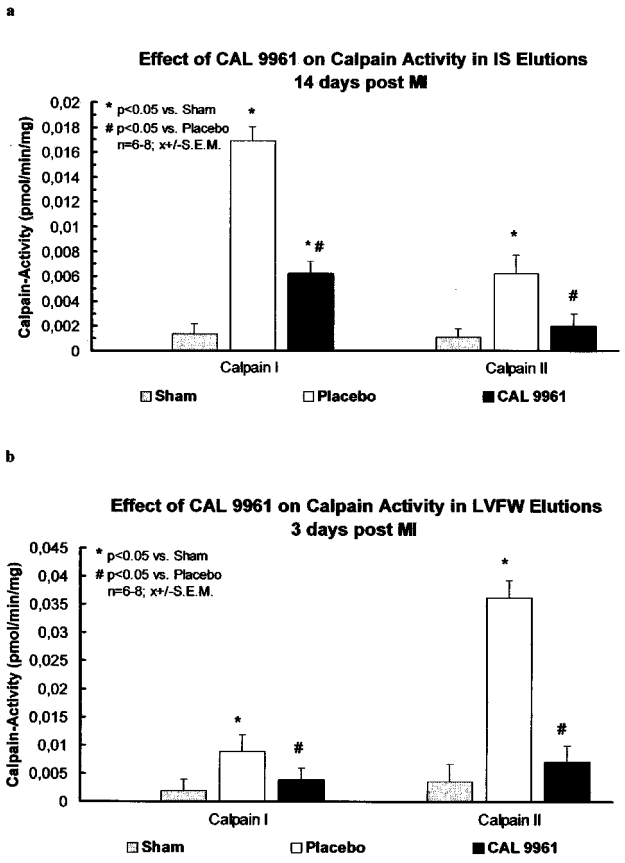

Figure 5 Effects of chronic CAL 9961 treatment (15 mg kg^{-1} d^{-1} s.c.) on calpains I and II activity (pmol AMC min^{-1} mg^{-1} protein) in fractions following anion exchanger chromatography on DEAE-Sepharose of the interventricular septum (IS) of infarcted rat hearts 14 days post MI (a) and of the left ventricular free wall (LVFW) of infarcted rat hearts 3 days post MI (b) compared to fractions of sham-operated and placebo-treated infarcted rat hearts. $*P<0.05$ compared to sham; $\#P<0.05$ compared to placebo; Data represent mean $\pm$ s.e.mean; $n=6-8$.

To investigate the effects of CAL 9961 on cardiac hypertrophy post MI, we measured the ratio of total heart weight (THW) to body weight (BW). Fourteen days post MI, THW/BW of placebo-treated MI animals (0.314 ± 0.01) was significantly increased compared to sham-operated animals (0.256 ± 0.01). In contrast, THW/BW of CAL 9961-treated MI animals (0.298 ± 0.01) was reduced when compared to placebo-treated MI animals but significantly increased compared to sham-operated animals. These results suggest an effect of CAL 9961 on cardiac hypertrophy post MI.

Discussion

Detection and separation of calpains I and II

In this work we have established a simple and specific fluorimetric assay for calpains in crude chromatographically fractionated high-speed supernatants of rat heart. Using DEAE-Sepharose chromatography, two peaks of calpains activity, corresponding to isoform I and II, could be separated and quantified (Figure 2a,b). Using an immunoblotting technique, we identified the large 80 kDa subunit of

calpain I in peak 1 and the large 80 kDa subunit of calpain II in peak 2 (Figure 3). These results suggest that the small regulatory 30 kDa subunit was dissociated from the large catalytic subunit of both calpains following chromatography. A dissociation of the large and small subunit following separation of the calpains has been described previously by Elce *et al.* (1997a). In addition, the use of Ca^{2+}-free media in all steps of the preparation prevented the autolysis of the large 80 kDa subunit of calpains I and II into inactive fragments (Michetti *et al.*, 1996). In the work reported here, the Ca^{2+}-dependent proteolytic activity of the 80 kDa subunits was measured using the fluorimetric enzyme assay in fractions following DEAE-Sepharose chromatography in the presence of 5 mM Ca^{2+}, suggesting that the large catalytic subunits of both calpain isoforms were intact and active in the presence of Ca^{2+}. Using this method, the activity measured for both calpain isoforms represents the maximum activity of calpains I and II, and when extrapolating to the *in vivo* situation, the actual intracellular Ca^{2+}-concentration should be taken into account. Our results further suggest the hypothesis that the function of the small subunit is solely to assist folding of the large subunit and that the large subunit exerts its full catalytic activity as a monomer when Ca^{2+} is present (Yoshizawa *et al.*, 1995a, b). The loss of the regulatory 30 kDa subunit of both calpains might be explained by separation or degradation during chromatography. These results are in agreement with the findings of Croall & DeMartino, 1991) who showed that the small subunits were often apparently degraded or absent after liquid chromatographic separation of the calpains.

Calpain I in the non-infarcted myocardium post MI

Following the development of an enzyme assay for calpain I and II from the cardiac tissue samples, we measured the activity of both calpain isoforms in the non-infarcted (IS) and infarcted (LVFW) rat myocardium at day 1, 3, 7 and 14 post MI to clarify regional and temporal alterations in calpains I and II activity in the infarcted heart. Our results show that MI induced a steady increase in the activity of calpain I in IS, reaching highest values 14 days post MI, whereas calpain II showed less activity relative to calpain I in this cardiac region (Figures 2a and 4a). The increase in calpain I activity 14 days post MI was accompanied by a compensated cardiac hypertrophy as evidenced by an increase in THW/BW. Our results are in agreement with findings of previous studies showing a transcriptional and translational up-regulation of calpain I in the non-infarcted hypertrophied rat myocardium (Sandmann *et al.*, 2001). Additionally, studies on rats demonstrated an increase in diastolic myocardial $[Ca^{2+}]_i$ in ventricular myocytes of the failing heart which were associated with cardiac hypertrophy following pressure overload (Gwathmey & Morgan, 1985; Steenbergen *et al.*, 1987b) and MI (Sandmann *et al.*, 1999). This might be related to an increased Ca^{2+}-inward movement (Elliott *et al.*, 1989; Sipido *et al.*, 2000) and/or to a reduced Ca^{2+}-reuptake by the sarcoplasmatic reticulum (SR) (De la Bastie *et al.*, 1990; O'Rourke *et al.*, 1999; Szymanska *et al.*, 2000). Thus, an increase in myocardial $[Ca^{2+}]_i$ in IS during the late phase post MI might be responsible for activation of calpain I in addition to its up-regulation. As calpain II requires millimolar $[Ca^{2+}]_i$ for activation (Mellgren, 1989; Suzuki *et al.*, 1995) which can be observed during the loss of the structural integrity of myocytes (Steenbergen *et al.*, 1990) the increase in $[Ca^{2+}]_i$ in IS is probably insufficient to activate calpain II. Indeed, previous studies showed that calpain I is activated by a moderate rise of $[Ca^{2+}]_i$ in the hypertrophying myocardium of the failing heart (Mellgren, 1989). The fact that fodrin and tubulin, which have been shown to link the plasma membrane with the cytoskeleton (Sobue *et al.*, 1987), are substrates for calpain I (Hu & Bennett, 1991; Billger *et al.*, 1988) suggests that breakdown of these myocardial structural proteins might be the initial step in changing of shape and size of myocytes following ischaemia. Thus, calpain I seems to be involved in the structural remodelling process and cardiac hypertrophy of the non-infarcted myocardium.

Calpain II in the infarcted myocardium

In contrast, the present study shows that the activity of calpain II was maximally increased 3 days post MI in LVFW (Figures 2b and 4b). The experimental animal model of MI used in this study resulted in an ischaemia of the LVFW which is associated with an abnormal increase in myocardial $[Ca^{2+}]_i$ during the acute phase post MI (Steenbergen *et al.*, 1990). This increase in myocardial $[Ca^{2+}]_i$ has been proposed to be the major mediator of the structural deterioration of the myocardium leading to cardiac necrosis (Grinwald & Nayler, 1981). Thus, the Ca^{2+}-overload in the ischaemic myocardium activates calpain II, which has been shown to require millimolar Ca^{2+}-concentrations for optimal activity (Suzuki *et al.*, 1995). The lower activity of calpain I in the ischaemic myocardium might be explained by the fact that calpain II proteolytically degrades calpain I (Tompa *et al.*, 1996). One could speculate that activation of calpain I by an increase in myocardial $[Ca^{2+}]_i$ might lead to degradation of membrane proteins involved in Ca^{2+} transport and finally result in a further increase in the cytoplasmatic Ca^{2+}-concentration. The resulting Ca^{2+}-overload might activate calpain II which, subsequently, induces myocyte injury and myocardial necrosis. Additionally, in previous experiments, we demonstrated an up-regulation of calpain II in the infarcted region (Sandmann *et al.*, 2001) and Mellgren *et al.* (1988) reported a disappearance of the endogenous calpain inhibitor, calpastatin, in the ischaemic area within the first hours post MI. Thus, it can be speculated that the calpain-calpastatin-ratio might be shifted to more calpain II in the ischaemic myocardium, leading to abnormal protein degradation and increased myocardial damage following MI.

Action of the calpain inhibitor on cardiac calpains

A major aim of this study was to investigate the inhibitory action *in vitro* and *in vivo* of the cell permeant calpain inhibitor, CAL 9961, on the activity of the calpains and to assess the selectivity of the drug to calpain I and/or calpain II. The experiments *in vitro* showed that CAL 9961 potently inhibited the activity of both calpain isoforms when tested both with commercially available calpains I and II or with crude calpains I and II from cardiac tissue samples. Chronic treatment of MI animals with CAL 9961 reduced calpain I activity in IS 14 days post MI and suppressed the increase in

calpain II activity in LVFW 3 days post MI (Figure 5a,b). The fact that calpains I and II were inactive in chromatographically fractionated heart extracts of CAL 9961-treated MI animals suggests that this agent potently inhibited cardiac calpains. The results in Figure 5a,b indicate a higher selectivity of CAL 9961 to calpain II. Whereas some studies showed that several calpain inhibitors such as E-64, CI-1 and NCO-700 inhibit the activity of the calpains in the ischaemic-reperfused rat heart (Yoshida *et al*., 1995b; Toda *et al*., 1989; Cuzzocrea *et al*., 2000; Toyo-Oka *et al*., 1982), the present study has demonstrated for the first time the inhibitory action of the calpain inhibitor, CAL 9961, on the activity of the calpains in an animal model of permanent coronary occlusion in rats. Earlier studies demonstrated a contribution of activated calpains I and II in the proteolytic digestion of microtubules (Billger *et al*., 1988; Sato *et al*., 1993), actin-binding protein (Wencel-Drake *et al*., 1991), fodrin (Yoshida *et al*., 1995a) and vinculin (Steenbergen *et al*., 1987a) in the ischaemic myocardium which was associated with subsequent myocyte death and loss of myocardial structural integrity and, eventually, cardiac dysfunction. Additionally, the synthetic calpain inhibitor, NCO-700, has been shown to reduce the size of the acute myocardial infarcted region in rabbits with coronary artery ligation, indicating that calpain is involved in myocardial degradation in the ischaemic heart (Toyo-Oka *et al*., 1982; 1991). Thus, from our results, one might speculate that inhibition of the cardiac calpains by CAL 9961 protects the infarcted heart against calpain I-mediated structural cardiac remodelling in the late phase post MI and against calpain II-induced myocardial damage in the acute phase post MI. However, further experiments in permanent coronary ligated rats are needed to clarify a correlation between inhibition of cardiac calpains post MI by CAL 9961 and the effects on infarct size, myocardial remodelling and cardiac function. Finally, experiments using isoform-specific calpain inhibitors or calpains I and/or II knock-out mice are necessary to determine the individual role of calpains I and II during ischaemia.

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