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Calcium channel blockade limits transcriptional, translational and functional up-regulation of the cardiac calpain system after myocardial infarction

Steffen Sandmann[a,*], Johannes Spormann[a], Freerk Prenzel[a], Lee Shaw[b], Thomas Unger[a]

[a] *Institute of Pharmacology, Christian Albrechts University of Kiel, Hospitalstrasse 4, 24105 Kiel, Germany*
[b] *Institute of Biochemistry, Christian Albrechts University of Kiel, Kiel, Germany*

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Abstract

Abnormal Ca^{2+} inward current through cardiac Ca^{2+} channels during ischemia has been shown to be an initial signal for activation of myocardial Ca^{2+}-dependent enzymes. This study investigated the contribution of cardiac L- and T-type Ca^{2+} channels in the calpain-mediated myocardial damage following myocardial infarction. Myocardial infarction was induced by permanent ligation of the left coronary artery. Infarcted rats were orally treated with placebo, amlodipine (L-channel blockade; 4 mg/kg/day) or mibefradil (L-/T-channel blockade; 10 mg/kg/day) beginning 7 days before induction of myocardial infarction. Gene expression, protein levels and enzyme activity of calpains I and II were measured 1, 3, 7 and 14 days post coronary occlusion in the noninfarcted and infarcted myocardium. Infarct size, left ventricular dilation and interstitial collagen volume fraction were determined in picrosirius red-stained hearts. Myocardial infarction induced an up-regulation of calpain I mRNA, protein and activity in the noninfarcted myocardium (maximum 14 days post infarction), whereas mRNA, protein and activity of calpain II were maximally increased in the infarcted myocardium 3 days post infarction. Fourteen days post infarction, infarct size was 49%, the left ventricle was dilated and interstitial collagen volume fraction was increased. Amlodipine inhibited mRNA, protein and activity up-regulation of calpain I decreased interstitial collagen volume fraction and infarct size. Mibefradil attenuated mRNA, protein and activity up-regulation of calpain II at all four time points measured and of calpain I at 7 and 14 days post infarction, reduced infarct size and prevented left ventricular dilation. Infarction-induced cardiac hypertrophy was accompanied by an up-regulation of calpain I, whereas calpain II was up-regulated in the infarcted myocardium. Cardiac L- and T-type Ca^{2+} channel blockade differentially reduced post infarction remodeling associated with selective inhibition of cardiac calpains I and II, respectively.
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1. Introduction

The calpain system is an intracellular, strongly Ca^{2+}-dependent, cysteine protease system existing in all mammalian and in some nonmammalian cells. The calpain family consists of ubiquitous and tissue-specific isoforms of calpains and of the naturally occurring endogenous inhibitor, calpastatin (Sorimachi et al., 1994; Suzuki et al., 1987). The best characterized calpains are the widely distributed isoenzymes, calpain I, requiring micromolar intracellular Ca^{2+} concentrations for half-maximum activity, and calpain II, which requires millimolar intracellular Ca^{2+} concentrations for activation. Although the precise intracellular functions of the calpains have not been fully defined, a number of studies indicate their potential importance in regulated proteolysis of key enzymes and structural proteins as well as in apoptotic and necrotic processes (Wang et al., 1989; Rechsteiner and Rogers, 1996). The calpains have also been demonstrated to play a harmful role in a variety of pathological states which are associated with a Ca^{2+} overload, such as Duchenne's muscular dystrophy (Reddy et al., 1986), Alzheimer's disease (Saito et al., 1993), multiple sclerosis (Hong et al., 1994) and development of cataract (Lampi et al., 1992). Recently, the calpains have been shown to participate in pancreatic β-cell damage and is the only known gene polymorphism of Type 2 diabetes mellitus in humans (Permutt et al., 2000) which is associated with cardiovascular

* Corresponding author. Tel.: +49-431-597-3520; fax: +49-431-597-3522.
E-mail address: s.sandmann@pharmakologie.uni-kiel.de (S. Sandmann).

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complications. Thus, studies investigating the regulation of the calpains are of importance to clarify the role of these en zymes as a cardiovascular risk factor.

A rise in intracellular Ca^{2+} in myocytes during myocardial ischemia (Steenbergen et al., 1990) has been considered to be a pivotal event in the activation of the calpains leading to cardiac cell death and structural damage of the myocardium by breakdown of myofibrillar proteins such as myosin heavy chain, troponin T and I, tropomyosin A and α-actinin (Toyo-oka and Ross, 1981; Steenbergen et al., 1987). However, no substantial effort has been made thus far to identify regional and temporal differences in the regulation of myocardial calpains I and II and their contribution to ischemia-induced cardiac damage. Additionally, no studies have been performed to clarify whether alterations in the activity of calpains I and II during myocardial ischemia were related to transcriptional and/or translational changes of the calpains.

Under the assumption that the Ca^{2+} entry into myocytes through cardiac Ca^{2+} channels during myocardial ischemia is the initiating signal in triggering Ca^{2+} overload, we investigated the effects of a chronic Ca^{2+} channel blockade on the Ca^{2+}-dependent cardiac calpain system. Ca^{2+} channel antagonists have been shown to exert beneficial myocardial effects via their inhibiting action on the slow Ca^{2+} inward current through L-type Ca^{2+} channels into cardiac cells. This effect has been thought to reduce the activation of intramyocardial Ca^{2+}-dependent enzymes (Nayler and Britnell, 1991) which are known to be harmful to cardiac myocytes and to contribute to cardiac dysfunction and fibrotic processes. Additionally, cardiac T-type Ca^{2+} channels have been shown to be up-regulated in the failing myocardium (Nuss and Houser, 1993), suggesting an influence of this channel type on intramyocardial Ca^{2+} levels. However, at present, the impact of L- and T-type Ca^{2+} channels on the cardiac calpain system-mediated structural remodeling process and tissue injury of the infarcted heart has not been addressed. Therefore, the present study in rats was performed to investigate the in vivo effects of chronic blockade of cardiac L-type Ca^{2+} channels by amlodipine and cardiac L- and T-type Ca^{2+} channels by mibefradil on transcriptional, translational and functional regulation of calpain I, calpain II and calpastatin in the infarcted and noninfarcted myocardium. To assess a time-dependent correlation between transcriptional, translational and functional regulation of the calpains and their contribution to the cardiac remodeling process following myocardial infarction, we investigated the action of long-term treatment with amlodipine and mibefradil on infarct size, left ventricular dilation and interstitial myocardial fibrosis.

2. Methods

2.1. Study design

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

The animals were randomly divided into 13 groups ($n = 7–9$ per group). Group 1: sham operation without treatment. Groups 2–5: myocardial infarction subjected to placebo treatment (0.9% saline). Groups 6–9: myocardial infarction subjected to amlodipine treatment (4 mg/kg/day). Groups 10–13: myocardial infarction subjected to mibefradil treatment (10 mg/kg/day). Amlodipine and mibefradil were kindly provided by Hoffmann-La Roche (Grenzach-Wyhlen, Germany). The Ca^{2+} channel antagonists were dissolved in water and given daily via gastric gavage. Treatment was begun 1 week prior to coronary occlusion (7 days pre) in all groups and continued until sacrifice. Investigation of gene expression, protein levels and enzyme activity of calpain I, calpain II and calpastatin were performed 1, 3, 7 and 14 days after surgery, and morphological studies were performed 14 days post infarction.

After 1 week in single cages, rats were anaesthetized by injection of methohexital-Na^{+} (10 mg/kg, i.v.) and artificially ventilated (70 ventilations/min, 200 mm H_2O, 2.5 ml/ventilation) to perform a left thoracotomy. Rats in Group 1 underwent a sham operation. In the remainder of the animals, myocardial infarction was induced by permanent ligation of the left coronary artery. Briefly, after anterior pericardectomy, the left coronary artery was ligated intrathoracally 2 mm distal of its origin using a sterile 6-0 suture material (Ethibond, Ethicon, Norderstedt, Germany) under a stereo-microscope. Successful ligation of the coronary artery was verified by the occurrence of arrhythmias in the ECG and by observing the color changes of the ischemic area. This method induced a transmural infarct in the left ventricular free wall, whereas the interventricular septum and the right ventricle remained noninfarcted. Body weight was measured daily before induction of myocardial infarction and during the 14 days post infarction to adjust drug doses to the individual body weight.

Preliminary experiments using different oral dosages of amlodipine (1–10 mg/kg/day) and mibefradil (5–15 mg/kg/day) had been performed to determine a dose of both drugs that did not affect peripheral hemodynamics and cardiac function in normotensive rats. In these experiments, the femoral artery was cannulated to measure mean arterial blood pressure and heart rate before induction of myocardial infarction (control conditions) during the surgery and the following 48-h time interval after infarction or sham operation beginning immediately after last oral application of the drugs (Sandmann et al., 2001a). In these experiments, the nonantihypertensive oral doses of 4 mg/kg/day amlodipine and of 10 mg/kg/day mibefradil administered daily and started 7 days before induction of infarction or sham oper-

ation were determined. Earlier experiments using isolated papillary muscle preparations from infarcted rat hearts demonstrated that chronic oral treatment with these doses of amlodipine and mibefradil significantly decreased the peak systolic and diastolic intramyocardial Ca^{2+} concentration (Min et al., 1999). In additional experiments, 4 mg/kg/day amlodipine or 10 mg/kg/day mibefradil equipotently reduced mean arterial blood pressure in spontaneously hypertensive rats, showing a comparable Ca^{2+} channel inhibiting effect of both drugs. Thus, the doses used ensured the investigation of the effects of a long-term L- and L-/T-type Ca^{2+} channel blockade on gene expression, protein levels and enzyme activity of the cardiac calpains in the infarcted rat heart independently of hemodynamic influences.

The study was divided into four sets of experiments. In the first set ($n=7$ per group), gene expression of calpain I, calpain II and calpastatin was investigated in three cardiac tissue regions: right ventricle, interventricular septum and left ventricular free wall of sham-operated and infarcted animals with placebo, amlodipine and mibefradil treatment on days 1, 3, 7 and 14 post surgery using reverse transcription-polymerase chain reaction. In these experiments, the left ventricle was separated from the atria and the right ventricle and then divided into the interventricluar septum, representing the noninfarcted, hypertrophed myocardium and the left ventricular free wall, representing the infarcted, ischemic myocardium, by an incision along the line where the right ventricle was cut off from the left ventricle. The left ventricular free wall consisted of scar tissue and area at risk. In the second set ($n=9$ per group), cardiac protein levels of the three calpains were investigated in paraffin fixed hearts of sham-operated and infarcted animals at the four time points post coronary occlusion via immunohistological staining of the calpains. In the third set ($n=9$ per group), myocardial remodeling was determined by measurement of infarct size, left ventricular dilation, septal thickness and interstitial collagen volume fraction in sham-operated and infarcted animals 14 days post coronary occlusion. In the fourth set ($n=7-8$ per group), enzyme activity of calpains I and II was measured in the right ventricle, interventricluar septum and left ventricular free wall of sham-operated and infarcted animals treated with placebo or Ca^{2+} channel antagonists following chromatographic separation of both enzymes.

2.2. Investigation of gene expression

Hearts of sham-operated and infarcted animals were rapidly excised on the day of sacrifice and placed on a preparation chamber at 4 °C. The left ventricle was separated from the atria and the right ventricle and further divided into the interventricular septum and the left ventricular free wall. Total RNA were isolated from the three ventricular tissue samples using a single-step isolation method described by Chomczynski and Sacchi (1987). Integrity of the RNA was confirmed by agarose gel electrophoresis, and the concentration was determined by densitometric measurement of UV absorption at 260 nm. Molecular size standards, Super Script Preamplification Systems and Taq Polymerase were purchased from Gibco BRL (Eggenstein, Germany).

Using reverse transcription-polymerase chain reaction, 5 μg of total RNA were amplificated with oligo (dT) primers using kit reagents according to the manufacturer's recommended protocols (Prime-It II Random Primer Kit, Strategen, Heidelberg, Germany). Primers were designed using software supplied by the German Cancer Research Center (DKFZ, Heidelberg, Germany) and were obtained from Pharmacia Biotech (Cambridge, UK). Details concerning primers and exact polymerase chain reaction protocols for the various amplification reactions used have been described previously (Sandmann et al., 2001b). For experiments with amplification of GAPDH mRNA as house-keeping gene, the reaction mixture was equally split into two tubes before specific primers and polymerase chain reaction reagents were added.

In order to measure mRNA levels, the gels were digitized using an optical scanner (EPSON GT-8000, Seiko, Tokyo, Japan) and the densities of bands were measured using the public domain NIH image program. For all RNA samples, the density of an individual mRNA band was divided by that of the GAPDH mRNA band to correct for differences in RNA loading and/or transfer.

2.3. Measurement of protein levels using immunohistology

Hearts of sham-operated and infarcted animals were fixed by infusion of 4% phosphate-buffered formaldehyde, rapidly excised and stored in the same formaldehyde solution. To arrest the hearts in diastole, the fixation was performed in a Ca^{2+}-free solution. Following removal of the atria and large vessels, the ventricles were cut in a standardized fashion into five transversal slices as described previously (Sandmann et al., 2000). All slices were weighted to obtain the total heart weight and embedded in paraffin. For immunohistological analysis, three serial 4-μm sections of all slices were cut from the apical side, deparaffinated and digested for 10 min at 37 °C with 0.1% protease (Sigma, Deisenhofen, Germany) in basis buffer (10 mM Tris base, 40 mM Tris–HCl, 150 mM NaCl, pH 7,4) to unmask antigens. The three sections were separately incubated for 30 min at 37 °C with a 1:200 dilution of primary antibodies for calpain I, calpain II and calpastatin (Chemicon, Hofheim, Germany). After washing three times in basis buffer, the sections were incubated for 1 h at 37 °C with a alkaline phosphatase-conjugated monoclonal secondary antibody (APAAP kit, DAKO, Hamburg, Germany). Using this method, calpain I-, calpain II- and calpastatin-labeled cardiac tissue regions appeared in a red color, whereas the remaining tissue showed a yellow color. All sections were counterstained with Mayer's hematoxylin solution (Sigma) and coverslipped with Kaiser's glycerol

gelatin (MERCK, Darmstadt, Germany). For determination of protein levels of the three calpains, the sections were placed in a universal microscope (Leica DMR, Leica Microscopy and Systems, Wetzlar, Germany), scanned systematically at fivefold lens magnification and computerized using a surface determination method which employed onscreen visualization of the cardiac sections in direct light (Leica Imaging Systems, Cambridge, UK). Following calibration, the software digitized red-stained tissue areas as a blue color and expressed the protein content of the calpains as percentage of the corresponding tissue region (right ventricle, interventricular septum, left ventricular free wall).

2.4. Cardiac morphology

From hearts excised 14 days post infarction, additional 4-μm sections were cut from slice 3, which has been shown to have the largest left ventricular circumference (Passier et al., 1996), and routinely stained with the collagen-specific stain picrosirius red (Sirius Red, C.I. 3570, Polysciences, Warrington PA, USA, in aqueous picric acid). Using this method, collagen I and III content was clearly detectable within the collagen accumulating tissue showing a red color and the remaining tissue a yellow color. The histology of picrosirius red-stained sections showed a transmural infarction. Infarct size, left ventricular dilation, septal thickness and interstitial collagen volume fraction were estimated using the computerized surface determination method as described above. Briefly, the sections were scanned at 25-fold lens magnification and the borderline between the infarcted area and the remaining myocardial muscle was marked exactly with a pointer. Infarct size was calculated by the computer program as the percentage of the left ventricular circumference. Left ventricular dilation and septal thickness were determined according to the method as published previously (Sandmann et al., 1998). Interstitial collagen volume fraction of the noninfarcted myocardium was evaluated by the software basing on density of the picrosirius red-stained collagen fibers of tissue areas in direct light and was calculated as the sum of all connective tissue areas divided by the sum of all connective tissue and muscle areas of the noninfarcted myocardium. All immunohistological and morphometric measurements were performed in a blinded fashion by a single observer.

2.5. Measurement of enzyme activity

Calpains I and II activity was measured fluorimetrically by the release of 7-amino-4-methyl-coumarin (AMC) from the synthetic fluorogenic substrate for calpain, Suc-Leu-Tyr-AMC (Calbiochem, Darmstadt, Germany, and Affinity Research, Mamhead, UK) (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). In these experiments, right ventricle, interventricular septum and left ventricular free wall of hearts of sham-operated and infarcted animals were homogenized (approximately 0.6–1.2 g) in five volumes of buffer (25 mM imidazole/HCl containing 5 mM cysteine and 1 mM EDTA, pH 7.5) for 1 min using an IKA Ultra-Turrax (1000 rpm, T25, Janke and Kunkel, Germany). The homogenates were centrifuged at 100,000*g* for 1 h (Beckman Ti 70.1 rotor, 37,000 rpm), 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 precooled buffers and centrifugations. 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. 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). To measure Ca^{2+}-independent activity, $CaCl_2$ was replaced by 20 μl EDTA at 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). The fluorescence was measured in 220 μl of the stopped reaction mixture using a spectrofluorometer (Fluorolite 1000, Japan, lamp voltage 6 V). An AMC standard curve was included in each experiment. 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.

2.6. Statistical analysis

Statistical evaluation was performed with SYSTAT software using one-way analysis of variance (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 means ± standard error of the mean (S.E.M.). Further details of statistical analysis are given in the legends to the figures.

3. Results

3.1. Effects of Ca^{2+} channel antagonists on transcriptional regulation of calpain I, calpain II and calpastatin in the infarcted myocardium

Reverse transcription followed by polymerase chain reaction amplification of total RNA resulted in single bands

of the predicted size for calpain I (152 bp), calpain II (117 bp) and calpastatin (110 bp) as well as for the house-keeping gene, GAPDH (916 bp). The GAPDH signals did not change in the rat myocardium throughout the time course following infarction compared to the noninfarcted myocardium and, thus, were used as internal control (Fig. 1, fourth lane). Therefore, the expression levels of the three cardiac calpains were related to myocardial GAPDH signals and expressed by folds of increase from those of sham-operated rats. Calpain I mRNA abundance increased steadily in the interventricluar septum of infarcted rat hearts at the four time points studied, was significantly increased 3 days post infarction (twofold) and reached maximal expression 14 days post infarction (threefold) compared to noninfarcted rat hearts (Fig. 1, first lane). Calpain I mRNA expression was unchanged in the right ventricle and the left ventricular free wall at any time point measured following infarction (data not shown). The post infarction increase of calpain I mRNA expression was abolished by pretreatment for 1 week followed by treatment up to 14 days post coronary occlusion with the L-type Ca^{2+} channel antagonist, amlodipine, whereas chronic treatment with the L-/T-type Ca^{2+} channel antagonist, mibefradil, reduced calpain I up-regulation on days 7 and 14 post infarction compared to placebo treatment (Fig. 2A). Calpain I mRNA up-regulation in the interventricular septum 14 days post infarction was significantly decreased in chronic amlodipine-treated infarcted animals compared to chronic mibefradil-treated infarcted animals (Fig. 2A). Calpain I mRNA expression was not affected by amlodipine and mibefradil in sham-operated animals.

In contrast, calpain II mRNA expression of placebo-treated infarcted animals was increased in the left ventricular free wall on day 1 post infarction (twofold), reached maximal expression on day 3 post infarction (threefold) and remained elevated for 7 and 14 days post infarction compared to sham-operated animals (Fig. 1, second lane). No significant differences in the transcription of calpain II were observed in the right ventricle and interventricular septum following infarction (data not shown). As illustrated in Fig. 2B, chronic amlodipine treatment decreased calpain II mRNA up-regulation in the left ventricular free wall on day 3 post infarction. In contrast, chronic treatment of infarcted animals with mibefradil totally prevented calpain II mRNA up-regulation in the left ventricular free wall at all four time points post infarction studied (Fig. 2B). Calpain II mRNA up-regulation in the left ventricular free wall of mibefradil-treated infarcted animals was significantly reduced 1 and 3 days post infarction compared to amlodipine-treated infarcted animals (Fig. 2B). In sham-operated animals, amlodipine and mibefradil had no effect on mRNA expression of calpain II.

The mRNA levels of calpastatin in the infarcted rat heart were unchanged in the three tissue samples, right ventricle, interventricular septum and left ventricular free wall, throughout the 14 days post infarction period when compared to hearts of sham-operated rats and were unaffected by long-term treatment with the Ca^{2+} channel antagonists, amlodipine and mibefradil (Fig. 1, third lane).

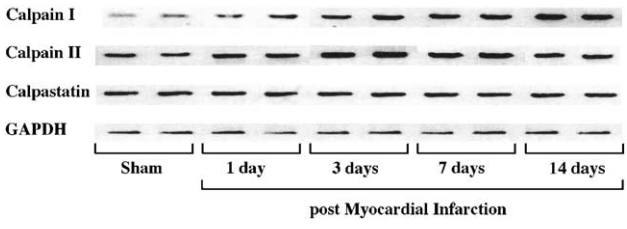

Fig. 1. Representative experiments using reverse transcription-polymerase chain reaction amplification showing mRNA expression of calpain I in the interventricular septum (IS), calpain II in left ventricular free wall (LVFW), calpastatin and GAPDH of placebo-treated infarcted animals on days 1, 3, 7 and 14 post infarction compared to sham-operated animals ($n=7$).

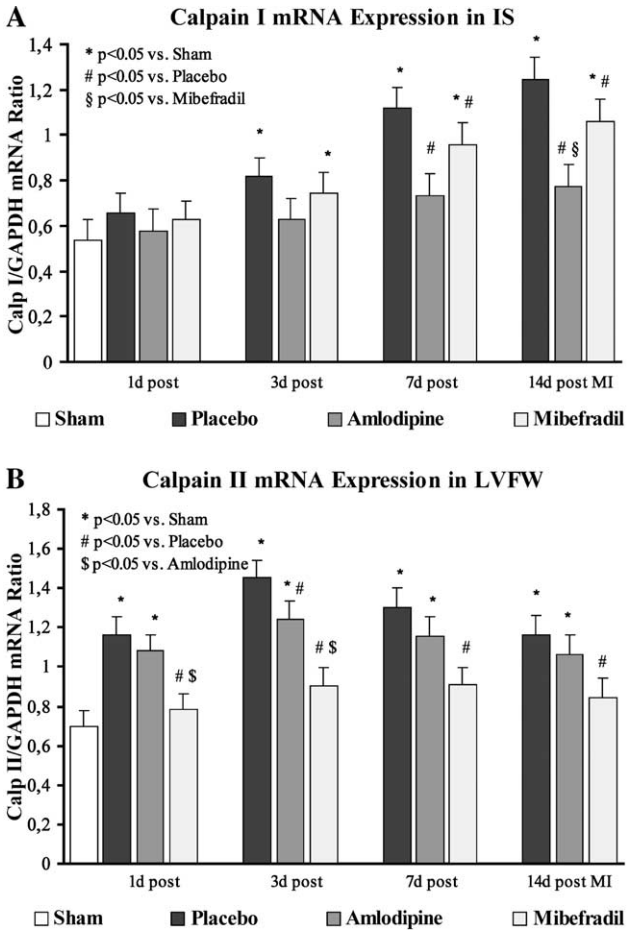

Fig. 2. Relative changes of calpain I mRNA expression in the interventricular septum (IS) (A) and of calpain II mRNA expression in the left ventricular free wall (LVFW) (B) determined by densitometric analysis and normalized to GAPDH signals of sham-operated and infarcted animals treated either with placebo (0.9% saline), amlodipine (4 mg/kg/day) or mibefradil (10 mg/kg/day) on days 1, 3, 7 and 14 post infarction. $^{*}P<0.05$ compared to sham, $^{\#}P<0.05$ compared to placebo, $^{\S}P<0.05$ compared to mibefradil, $^{\$}P<0.05$ compared to amlodipine. Data represent means ± S.E.M. ($n=7$).

3.2. Effects of Ca^{2+} channel antagonists on protein levels of the cardiac calpains post infarction

Immunohistological studies were performed to measure the protein content of calpain I, calpain II and calpastatin in the left ventricular free wall and the interventricular septum of the infarcted and noninfarcted rat myocardium. As shown in Fig. 3A, calpain I-stained red areas were most prominent 14 days post infarction within the interventricular septum of infarcted rat hearts. To a minor extent, calpain I was also found in the infarcted region and in the right ventricle showing the basal level of this enzyme in the rat myocardium. In contrast, calpain II-stained regions were abundantly present within the left ventricular free wall of the infarcted myocardium with highest detected levels on day 3 post infarction and was not detectable within the interventricular septum and right ventricle of the noninfarcted myocardium at any time point post infarction measured (Fig. 3B). Fig. 3C and D show computerized images where calpains I and II, respectively, were digitized as a blue color. Calpastatin was evenly distributed in the infarcted rat myocardium (right ventricle, interventricular septum and left ventricular free wall) throughout the time period studied (1, 3, 7 and 14 days post infarction).

As demonstrated for calpain I mRNA expression, pretreatment of infarcted animals with the L-type Ca^{2+} channel antagonist, amlodipine, totally prevented the post infarction up-regulation of the calpain I protein in the interventricular septum 14 days post infarction compared to placebo-treated infarcted animals (Fig. 4A). The L-/T-type Ca^{2+} channel antagonist, mibefradil, also decreased calpain I protein up-regulation in the interventricular septum of infarcted animals compared to placebo but was significantly increased compared to sham-operated and amlodipine-treated infarcted animals (Fig. 4A). In contrast, the infarction-induced increase in calpain II protein levels in the left ventricular

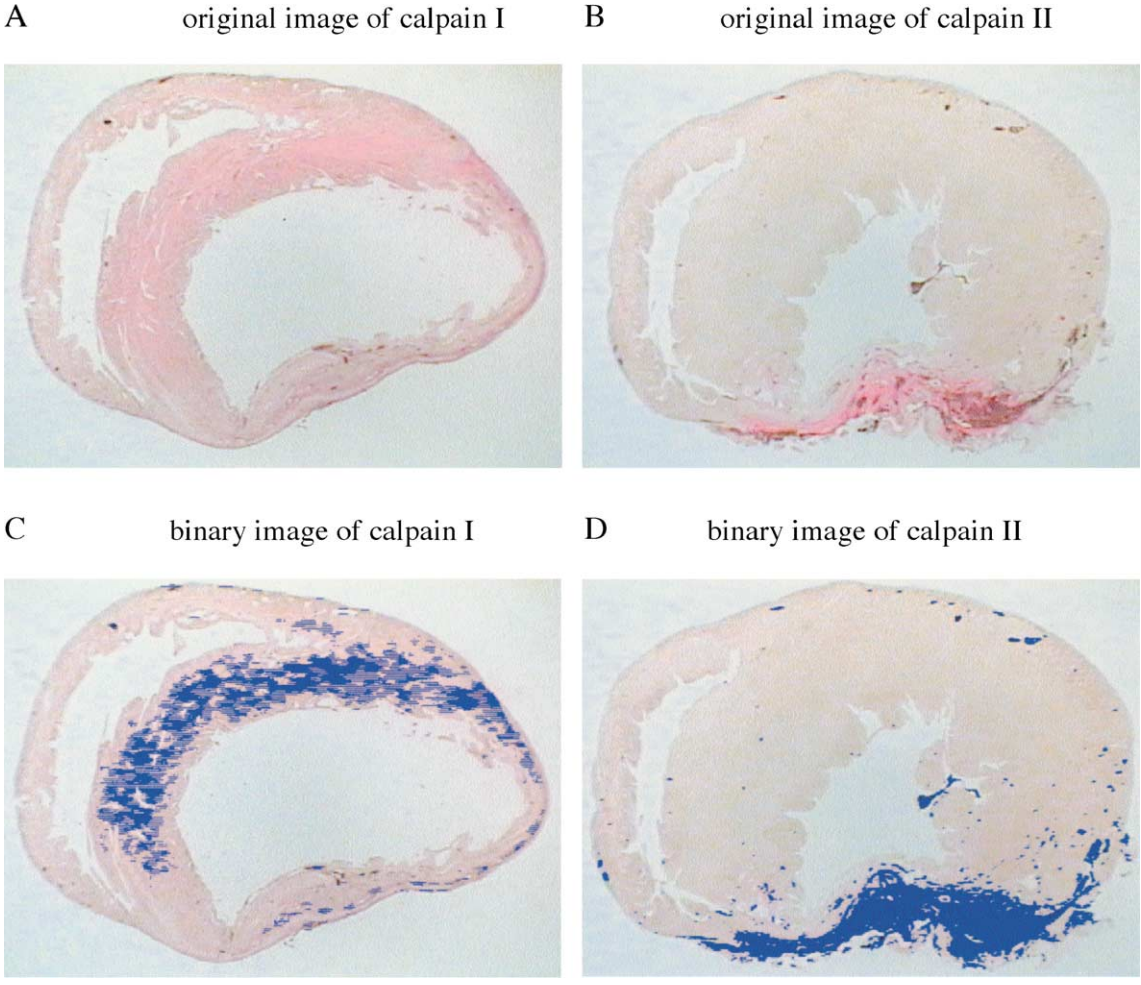

Fig. 3. Photomicrographs show immunhistological stainings of calpain I and II within the left ventricular free wall (LVFW) and the interventricular septum (IS) untreated myocardium in direct light. Red-stained areas of immunostainings show in the original images at fivefold lens magnification the existence of calpain I predominantly in the interventricular septum (A), whereas calpain II-stained areas occurred predominantly in the left ventricular free wall (B) of the infarcted rat heart. (C) and (D) show the computerized binary images where calpain-stained red areas are digitized and displayed as a blue color. For measurement of protein levels of calpain I and II, the detected blue areas were normalized to the whole tissue area of the left ventricular free wall and the interventricular septum.

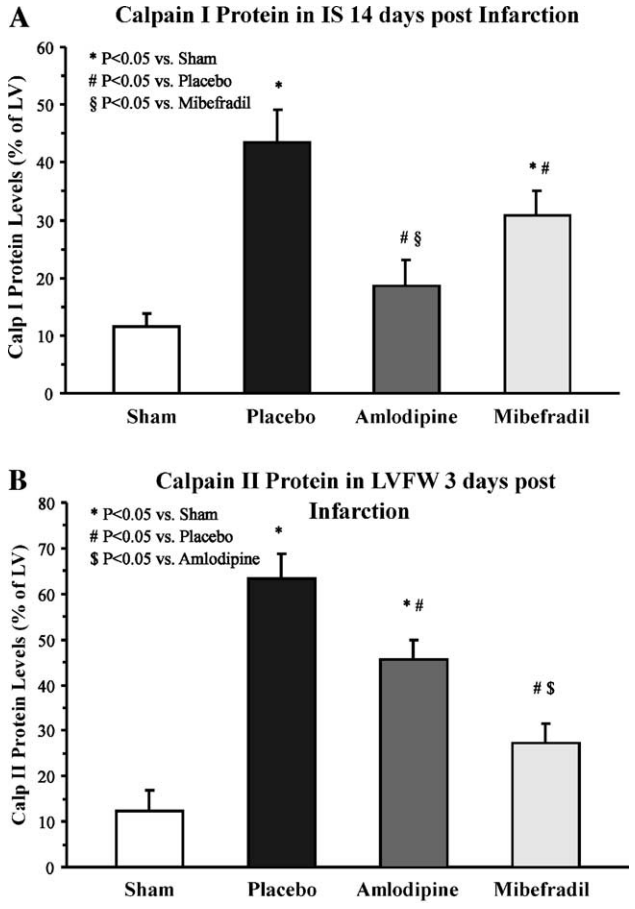

Fig. 4. Effects of chronic treatment with amlodipine (4 mg/kg/day) and mibefradil (10 mg/kg/day) on calpain I protein levels in the interventricular septum (IS) 14 days post infarction (A) and on calpain II protein levels in the left ventricular free wall (LVFW) 3 days post infarction (B) compared to sham-operated and placebo-treated infarcted animals. $^{*}P<0.05$ compared to sham, $^{\#}P<0.05$ compared to placebo, $^{\S}P<0.05$ compared to mibefradil, $^{\$}P<0.05$ compared to amlodipine. Data represent means ± S.E.M. ($n=9$).

free wall of infarcted rat hearts 3 days post infarction was prevented by chronic mibefradil treatment, whereas amlodipine treatment had a much smaller effect to decrease calpain II protein in the left ventricular free wall following infarction as shown by the significant difference compared to mibefradil treatment (Fig. 3B).

Myocardial calpastatin protein concentration was unaffected by chronic treatment with the Ca^{2+} channel antagonists, amlodipine and mibefradil (data not shown).

3.3. Cardiac morphometry

Fourteen days post myocardial infarction, the cardiac index (ratio of total heart weight to body weight) was increase in placebo-treated infarcted animals compared to sham-operated animals. Cardiac index of Ca^{2+} channel antagonist-treated infarcted animals tended to decrease compared to placebo-treated infarcted animals but was not statistically significant (Table 1). The infarct size was 49.1 ± 2.8% in placebo-treated infarcted animals. Pretreatment of infarcted animals with both Ca^{2+} channel antagonists, amlodipine and mibefradil, reduced infarct size compared to placebo treatment (Table 1). In these experiments, the L-/T-type Ca^{2+} channel antagonist, mibefradil, showed a greater tendency to limit the infarct size (not significantly different) when compared to the L-type Ca^{2+} channel antagonist, amlodipine. Left ventricular dilation was increased in all infarcted animals compared to sham-operated animals and was significantly reduced by amlodipine and mibefradil pretreatment as compared to placebo treatment (Table 1). However, mibefradil showed a greater tendency to prevent left ventricular dilation compared to amlodipine as the values were not different from those of sham-operated animals, whereas left ventricular dilation of amlodipine-treated animals were significantly increased compared to sham-operated animals. Septal thickness was increased in placebo-treated infarcted animals compared to sham-operated animals (Table 1). Amlodipine abolished and mibefradil reduced septal thickening in infarcted animals. Fourteen days post infarction, interstitial collagen volume fraction of the noninfarcted myocardium was markedly increased in all infarcted animals when compared to sham-operated animals. Amlodipine and mibefradil significantly reduced interstitial collagen volume fraction compared to placebo treatment with a greater efficacy of amlodipine than mibefradil (Table 1). Thus, amlodipine predominantly reduced the cardiac remodeling process in the noninfarcted myocardium, whereas mibefradil more

Table 1
Morphometric data of rat hearts 14 days after induction of myocardial infarction or sham surgery

	Sham	Placebo	Amlodipine	Mibefradil
Cardiac index (total heart weight to body weight) (g)	0.25 ± 0.01	0.30 ± 0.01[a]	0.28 ± 0.01	0.28 ± 0.01
Infarct size (%)		49.1 ± 2.8	42.8 ± 1.5[b]	39.4 ± 2.1[b]
Left ventricular dilation (mm)	5.26 ± 0.07	7.12 ± 0.06[a]	6.35 ± 0.04[a,b]	6.01 ± 0.04[b]
Septal thickness (mm)	1.71 ± 0.08	2.16 ± 0.09[a]	1.82 ± 0.06[b]	1.87 ± 0.05[a,b]
Interstitial collagen volume fraction (noninfarcted myocardium) (%)	2.91 ± 0.68	9.83 ± 1.75[a]	5.82 ± 0.89[a,b,c]	7.13 ± 1.07[a,b]

Indicated are animals that underwent sham surgery (sham), placebo-treated MI rats and rats treated with amlodipine (4 mg/kg/day) or mibefradil (10 mg/kg/day) beginning 7 days before induction of MI. Data are presented as means ± S.E.M ($n=9$).

[a] Significant versus sham ($P<0.05$).

[b] Significant versus placebo ($P<0.05$).

[c] Significant versus mibefradil ($P<0.05$).

effectively limited infarct size and prevented left ventricular dilation.

3.4. Proteolytic activity of calpains I and II in the infarcted myocardium

To investigate the contribution of calpains I and II in the cardiac remodeling process of the myocardium post infarction, the proteolytic activity of both enzymes was measured. Following chromatographic separation using a linear NaCl gradient (0–500 mM), calpain I eluted in the fractions 9–12 (180–240 mM NaCl), whereas calpain II occurred in the fractions 13–16 (260–320 mM NaCl) according to findings by Spalla et al. (1985). The calpain I activity in the interventricular septum of placebo-treated infarcted animals increased from days 1 to 14 post infarction compared to sham-operated animals. At this time point, a threefold increase in the proteolytic activity of this enzyme was measured (Fig. 5A). Chronic L-type Ca^{2+} channel blockade with amlodipine prevented and chronic L-/T-type Ca^{2+} channel blockade with mibefradil reduced the infarction-induced increase in the activity of calpain I when compared to placebo treatment. Calpain I activity in the interventricular septum 14 days post infarction was significantly decreased in amlodipine-treated infarcted animals compared to mibefradil-treated infarcted animals (Fig. 5A). In contrast, calpain II activity was maximally increased (threefold) in the left ventricular free wall 3 days post infarction compared to sham-operated animals (Fig. 5B). Treatment with amlodipine reduced and with mibefradil abolished the increase in calpain II activity (Fig. 5B). Calpain II activity in the left ventricular free wall 3 days post infarction was significantly decreased in mibefradil-treated infarcted animals compared to amlodipine-treated infarcted animals (Fig. 5B). Thus, amlodipine inhibited more effectively the enzymatic activity of calpain I in the noninfarcted myocardium, whereas the calpain II activity was more inhibited by mibefradil in the infarcted myocardium.

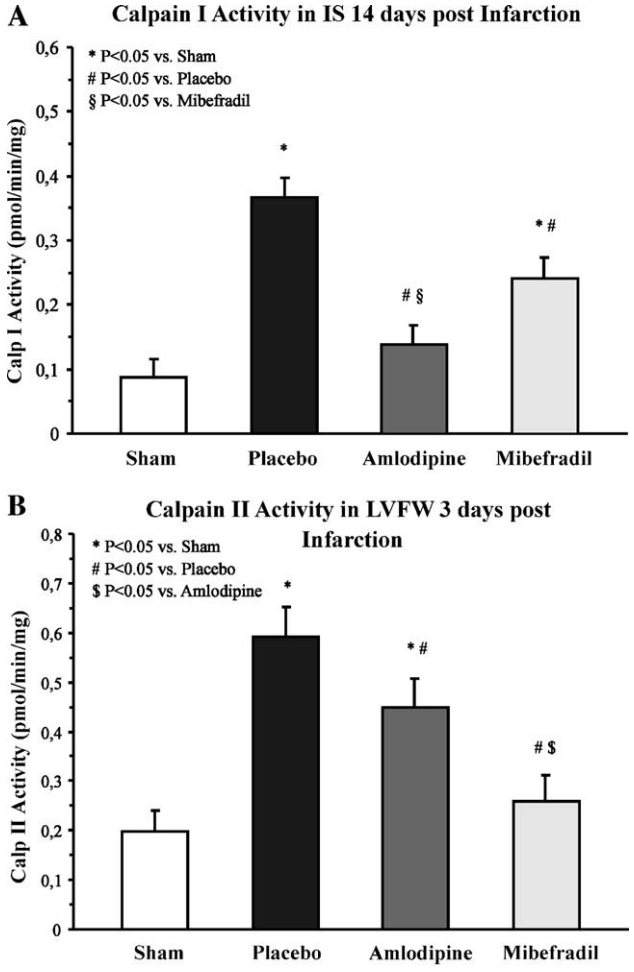

Fig. 5. Effects of chronic treatment with amlodipine (4 mg/kg/day) and mibefradil (10 mg/kg/day) on proteolytic activity (pmol AMC/min/mg protein) of calpain I in the interventricular septum (IS) 14 days post infarction (A) and of calpain II protein levels in the left ventricular free wall (LVFW) 3 days post infarction (B) compared to sham-operated and placebo-treated infarcted animals. $^{*}P<0.05$ compared to sham, $^{\#}P<0.05$ compared to placebo, $^{\S}P<0.05$ compared to mibefradil, $^{\$}P<0.05$ compared to amlodipine. Data represent means ± S.E.M. ($n=7–8$).

4. Discussion

The present study demonstrates that myocardial infarction induces an up-regulation of gene expression, protein levels and enzyme activity of calpain I in the noninfarcted myocardium in the later state of infarction (14 days) and of calpain II in the infarcted myocardium in the earlier state of infarction (3 days). These results indicate a differential regional and temporal up-regulation of calpains I and II in the infarcted rat heart. Fourteen days after permanent ligation of the left coronary artery, the hearts of placebo-treated infarcted animals featured an infarct size of 49% and a left ventricular dilation accompanied by an increase in septal thickness and an elevation in interstitial collagen volume fraction of the noninfarcted myocardium. These findings indicate that myocardial infarction had induced a dilatative hypertrophy and myocardial fibrosis accounting in part for the progressive deterioration in the mechanical behavior of the myocardium (Gay, 1990; Sandmann et al., 1999). Whereas both Ca^{2+} channel antagonists, amlodipine and mibefradil, limited infarct size, amlodipine more effectively reduced septal thickness and interstitial collagen volume fraction suggesting an antihypertrophic and antifibrotic action of this drug in infarct-induced heart failure.

4.1. Role of cardiac calpains in the infarcted myocardium

Previous studies have demonstrated that calpain I is up-regulated by sustained stimuli and contributes to the regulated protein turn-over during differentiation or hypertrophy (Pussard et al., 1993; Arthur and Belcastro, 1997). In fact, our data demonstrate that the cardiac remodeling coincided with transcriptional, translational and functional up-regula-

tion of calpain I in the noninfarcted myocardium. These findings are consistent with the idea that calpain I is involved in the structural remodeling process of the heart in the later phase of infarction. Indeed, calpain I, activated in the pressure overload and stunned myocardium, has been shown to alter the microstructure of matrix and myofilament proteins leading to cardiac hypertrophy and reduction of Ca^{2+} responsiveness of the contractile filaments (Gao et al., 1996; Contard et al., 1991). The present study further demonstrated that calpain I was not significantly up-regulated in the infarcted myocardium supporting the hypothesis that this enzyme is not involved in ischemic cell damage following infarction but contributes to adaptive mechanisms of the hypertrophying myocardium.

In contrast, mRNA, protein and activity of calpain II were increased in the infarcted myocardium during the early phase of infarction. Calpain II has been shown to be up-regulated by processes which are associated with an excess elevation of intramyocardial Ca^{2+} (Pussard et al., 1993). The ligation of the left coronary artery, as performed in the present experiments, is thought to be associated with an intracellular accumulation of Ca^{2+} within the infarcted (ischemic) myocardium (Katz and Reuter, 1979). Thus, the infarct-induced abnormal rise in intramyocardial Ca^{2+} as a result of an increased Ca^{2+} influx through Ca^{2+} channels, an elevated Ca^{2+} release by the sarcoplasmatic reticulum (Wier, 1990) and/or a stimulated Na^{+}–Ca^{2+} exchanger activity (Siffert and Akkerman, 1989) might be the initial signal to up-regulate calpain II in this cardiac region. This is in agreement with the finding that calpain II was not significantly up-regulated in the noninfarcted myocardium indicating that a pathological rise in intramyocardial Ca^{2+} is required for activation of this enzyme. Additionally, Ca^{2+} overload has been shown to act as a specific mechanism to activate calpain II (Toyo-oka and Ross, 1981; Steenbergen et al., 1987). In agreement with these findings, in our experiments, myocardial infarction increased the activity of calpain II in the infarcted myocardium which, in turn, might be responsible to accelerate tissue injury following infarction.

4.2. Contribution of cardiac Ca^{2+} channels in the regulation of calpains I and II

A central aim of our study was to compare the effects of a long-term L-type Ca^{2+} channel blockade versus a combined L-/T-type Ca^{2+} channel blockade on the regulation of cardiac calpain I, calpain II and calpastatin by using an in vivo animal model of myocardial infarction.

The present experiments showed that chronic treatment with the L-type Ca^{2+} channel antagonist, amlodipine, reduced the infarct-induced up-regulation of calpain I gene, protein and activity in the noninfarcted myocardium implicating that intramyocardial Ca^{2+} is involved in the transcriptional, translational and functional up-regulation of cardiac calpain I post infarction. Steenbergen et al. (1990) demonstrated that cardiac L-type Ca^{2+} channels are stimulated following infarction leading to an increased Ca^{2+} influx into ventricular myocytes. Pasquet et al. (1996) demonstrated that the Ca^{2+} influx through these channels activates the calpains directly and/or by stimulation of Ca^{2+} release from intracellular stores. We further showed that chronic amlodipine treatment prevented the septal thickening and reduced cardiac fibrosis suggesting that chronic L-type Ca^{2+} channel blockade by amlodipine limited the structural remodeling process in the noninfarcted myocardium via inhibition of calpain I up-regulation in this cardiac region. A prevention of the left ventricular remodeling in the noninfarcted rat myocardium by long-term treatment with amlodipine has been demonstrated earlier by Shimada et al. (1998). Since the reactive fibrosis post infarction has been shown not to be related to myocyte necrosis and independent of the size of myocardial infarct (Sawamura et al., 1990; Brilla et al., 1991), the ability of L-type Ca^{2+} channel antagonists to decrease interstitial collagen content seems to be directly related to modulation of intracellular Ca^{2+}-dependent signaling mechanisms, such as mediation by calpain I, in cardiac fibroblasts (Olsen et al., 1989). The reduction of myocardial fibrosis and cardiac hypertrophy by L-type Ca^{2+} channel antagonists post infarction (Weishaar and Bing, 1980) resulted in an improved myocardial performance evidenced by an increase in cardiac contractility and a decrease in inner left ventricular pressure (Sandmann et al., 2001a; Hoff et al., 1989). Additionally, evidence has been presented that inhibition of cardiac L-type Ca^{2+} channels by verapamil attenuated the infarct-induced increase in calpain I activity in the noninfarcted myocardium (Yoshida et al., 1993). It is, therefore, possible that the inhibition of calpain I up-regulation by amlodipine was directly related to the decrease in intramyocardial Ca^{2+} by the reduction of Ca^{2+} influx through cardiac L-type Ca^{2+} channels and, thereby, prevented structural remodeling and cardiac fibrosis post infarction.

Our study further demonstrated that chronic treatment with the L-/T-type Ca^{2+} channel antagonist, mibefradil, inhibited transcriptional, translational and functional up-regulation of calpain II in the infarcted (ischemic) myocardium suggesting that the early Ca^{2+} influx through T-type Ca^{2+} channels seem to be involved in the regulation of cardiac calpain II. This effect was associated with a reduction of infarct size and left ventricular dilation. An infarct size-limiting property of mibefradil has previously been demonstrated after permanent coronary artery ligation (Sandmann et al., 1998). In view of these findings, the results of the present study nourish the hypothesis that the reduction of infarct size by mibefradil might be related to the prevention of calpain II up-regulation in the infarcted (ischemic) myocardium. Indeed, calpain inhibitors have been found to protect the myocardium from ischemia-induced damage via reduction of calpain II activity in this cardiac region (Urthaler et al., 1997; Iwamoto et al., 1999; Tolnai and Korecky, 1986). The greater efficacy of mibefradil than

amlodipine to decrease infarct size, as also demonstrated previously (Sandmann et al., 2001a), could be partially explained by the additional blockade of cardiac T-type Ca^{2+} channels which have been found to exert an increased Ca^{2+} channel current in the myocardium post infarction (Qin et al., 1995). In contrast to healthy ventricular myocytes of adult rat hearts, in which no T-type Ca^{2+} channel current was detectable (Lory et al., 1990; Bogdanov et al., 1995), an increase in T-type Ca^{2+} channel current was measured in cells of cardiomyopathic hamsters (Sen and Smith, 1994) suggesting that this Ca^{2+} channel type participates in the regulation of intramyocardial Ca^{2+} under pathological conditions. Additionally, an abnormal Ca^{2+} channel current of T-type Ca^{2+} channels was shown in ventricular myocytes of failing hearts (Nuss and Houser, 1993). Thus, it seems reasonable to suppose that the functional importance of T-type Ca^{2+} channels is more pronounced in ischemic myocytes of the failing heart. Indeed, in earlier experiments, we found that the infarct-induced abnormal increase in systolic and diastolic intramyocardial Ca^{2+} in the ischemic myocardium was attenuated by long-term L-/T-type Ca^{2+} channel blockade by mibefradil (Sandmann et al., 1999). The inhibition of Ca^{2+} influx into ischemic myocytes could stem from the fact that mibefradil blocks Ca^{2+} entry more potently in depolarized cells because ischemic myocardial tissue is depolarized (Janse and Kleber, 1981). Thus, the greater efficacy of mibefradil compared to amlodipine in reduction of infarct size might be related to the combined L- and T-type Ca^{2+} channel blocking action of the drug (Mishra and Hermsmeyer, 1994).

Calpastatin expression, on the other hand, was not up-regulated whether in the noninfarcted nor in the infarcted myocardium at mRNA or protein levels suggesting that transcriptional and translational expression of the naturally occurring calpain inhibitor was not affected in infarct-induced heart failure. These results are in keeping with findings of Sorimachi et al. (1997), showing that cardiac calpastatin was not up-regulated in the ischemic–reperfused rat heart. Additionally, calpastatin has been shown to be a substrate for both calpain isoforms (Doumit and Koohmaraie, 1999) implying that the ischemia-induced increase in calpains I and II expression and activity seems to be responsible for abnormal calpastatin degradation in the myocardium. Thus, it appears that the calpain–calpastatin ratio was shifted towards calpain leading to abnormal protein degradation and increased myocardial damage during cardiac ischemia. Therefore, a reduction of cardiac calpains I and II gene, protein and activity up-regulation as observed under Ca^{2+} channel antagonist treatment might be cardioprotective via decreasing the amount of activable calpains I and II levels within the myocardial tissue and preserve cardiac function in infarct-induced heart failure via prevention of myocardial remodeling and limitation of infarct size.

In summary, our study demonstrates for the first time that the infarct-induced cardiac remodeling of the noninfarcted myocardium is correlated with calpain I up-regulation in this region, whereas calpain II was up-regulated earlier post infarction in the infarcted (ischemic) region suggesting a contribution of this enzyme to the myocardial tissue injury. Long-term treatment with the L-type Ca^{2+} channel antagonist, amlodipine, attenuated calpain I up-regulation and reduced myocardial fibrosis of the noninfarcted myocardium implicating that this drug effectively protected the heart against cardiac remodeling post infarction. In contrast, chronic treatment with the L-/T-type Ca^{2+} channel antagonist, mibefradil, reduced calpain II up-regulation in the infarcted myocardium and decreased infarct size. Thus, both agents seems to positively influence pathological alterations of the infarcted heart by limiting the calpain-associated structural myocardial remodeling process via different pathways.

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References

Arthur, G.D., Belcastro, A.N., 1997. A calcium stimulated cysteine protease involved in isoproterenol induced cardiac hypertrophy. Mol. Cell. Biochem. 176, 241–248.

Bogdanov, K.Y., Ziman, B.D., Spurgeon, H.A., Lakatta, E.G., 1995. L- and T-type calcium currents differ in finch and rat ventricular cardiomyocytes. J. Mol. Cell. Cardiol. 27, 2581–2593.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248–254.

Brilla, C., Janicki, J.S., Weber, K.T., 1991. Impaired diastolic function and coronary reserve in genetic hypertension: role of interstitial fibrosis and medial thickening of intramyocardial coronary arteries. Circ. Res. 69, 107–115.

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal. Biochem. 162, 156–159.

Contard, F., Koteliansky, V., Marotte, F., Dubud, I., Rappaport, L., Samuel, J.L., 1991. Specific alterations in the distribution of extracellular matrix components within rat myocardium during the development of pressure overload. Lab. Invest. 64, 65–75.

Doumit, M.E., Koohmaraie, M., 1999. Immunoblot analysis of calpastatin degradation: evidence for cleavage by calpain in post mortem muscle. J. Anim. Sci. 77 (6), 1467–1473.

Gao, W.D., Liu, Y., Mellgren, R., Marban, E., 1996. Intrinsic myofilament alterations underlying the decreased contractility of stunned myocardium. A consequence of Ca^{2+}-dependent proteolysis. Circ. Res. 78, 455–465.

Gay, R.G., 1990. Early and late effects of captopril treatment after large myocardial infarction in rats. JACC 16 (4), 967–977.

Hoff, P.T., Tamura, Y., Luchesi, B.R., 1989. Cardioprotective effects of amlodipine in the ischemic–reperfused heart. Am. J. Cardiol. 64, 101I–116I.

Hong, S.C., Goto, Y., Lanzino, G., Solea, S., Kassell, N.F., Lee, K.S., 1994. Neuroprotection with a calpain inhibitor in a model of focal cerebral ischemia. Stroke 25, 663–669.

Iwamoto, H., Miura, T., Okamura, T., Shirakawa, K., Iwatate, M., Kawamura, S., Tatsuno, H., Ikeda, Y., Matsuzaki, M., 1999. Calpain inhibitor-1 reduces infarct size and DNA fragmentation of myocardium in ischemia/reperfusion rat heart. J. Cardiovasc. Pharmacol. 33, 580–586.

Janse, M.J., Kleber, A.G., 1981. Electrophysiological changes and ventricular arrhythmias in the early phase of regional myocardial ischemia. Circ. Res. 49, 1069–1081.

Katz, A.M., Reuter, H., 1979. Cellular calcium and cardiac cell death. Am. J. Cardiol. 44 (1), 188–190.

Lampi, K.J., Kadoya, K., Azuma, M., David, L.L., Shearer, T.R., 1992. Comparison of cell-permeable calpain inhibitors and E 64 in reduction of cataract in cultured rat lenses. Toxicol. Appl. Pharmacol. 117, 53–57.

Lory, P., Rassendren, F.A., Richard, S., Tiaho, F., Nargeot, J., 1990. Characterization of voltage-dependent calcium channel expressed in *Xenopus* oocytes injected with mRNA from rat heart. J. Physiol. 429, 95–112.

Min, J.Y., Sandmann, S., Meissner, A., Unger, Th., Simon, R., 1999. Differential effects of mibefradil, verapamil, and amlodipine on myocardial function and intracellular Ca^{2+} handling in rats with chronic myocardial infarction. J. Pharmacol. Exp. Ther. 291 (3), 1038–1044.

Mishra, S.K., Hermsmeyer, K., 1994. Selective inhibition of T-type calcium channels by RO 40-5967. Circ. Res. 75, 144–148.

Nayler, W.G., Britnell, S., 1991. Calcium antagonists and tissue protection. J. Cardiovasc. Pharmacol. 18 (1), S1–S5.

Nuss, B.H., Houser, S.R., 1993. T-type calcium current is expressed in hypertrophied adult feline left ventricular myocytes. Circ. Res. 73, 777–782.

Olsen, R., Seewald, M., Powis, G., 1989. Contribution of external and internal Ca^{2+} to changes in intracellular free Ca^{2+} produced by mitogens in Swiss 3T3 fibroblasts: the role of dihydropyridine sensitive Ca^{2+} channels. Biochem. Biophys. Res. Commun. 162, 448–455.

Pasquet, J.M., Dachary-Prigent, J., Nurden, T., 1996. Calcium influx is a determining factor of calpain activation and microparticle formation in platelets. Eur. J. Biochem. 239, 647–654.

Passier, R.C.J.J., Smits, J.F.M., Verluyten, M.J.A., Daemen, M.J.A.P., 1996. Expression and localisation of renin and angiotensinogen in rat heart after myocardial infarction. Am. J. Physiol. 271, H1040–H1048.

Permutt, M.A., Bernal-Mizrachi, E., Inoue, H., 2000. Calpain 10: the first positional cloning of a gene for type 2 diabetes? J. Clin. Invest. 106, 819–821.

Pussard, S., Cottin, P., Brustis, J.J., Talmat, S., Elamrani, N., Ducastaing, A., 1993. Quantitative measurement if calpain I and II mRNAs in differentiating rat muscle cells using a competitive polymerase chain reaction method. Biochemistry 75, 885–890.

Qin, D., Jian, P., Boutjdir, M., El-Sherif, N., 1995. Expression of T-type calcium channel current in remodeled hypertrophied left ventricle from adult rat following myocardial infarction (Abstract). Circulation 92 (Suppl. I), I587.

Rechsteiner, M., Rogers, S.W., 1996. PEST sequences and regulation by proteolysis. Trends Biochem. Sci. 21 (7), 267–271.

Reddy, P.A., Anandavalli, T.E., Anandaraj, M.P.J.S., 1986. Calcium activated neutral protease (milli- and microCNP) and endogenous CANP inhibitor of muscle in Duchenne muscular dystrophy (DMD). Clin. Chim. Acta 160, 281–288.

Saito, K.I., Elce, J.S., Hamos, J.E., Nixon, R.A., 1993. Widespread activation of calcium-activated neutral protease (calpain) in the brain in Alzheimer disease: a potential molecular basis for neuronal degeneration. Proc. Natl. Acad. Sci. U. S. A. 90, 2628–2632.

Sandmann, S., Spitznagel, H., Chung, O., Xia, Q.G., Illner, S., Jänichen, G., Rossius, B., Daemen, M.J.A.P., Unger, Th., 1998. Effects of the calcium channel antagonist mibefradil on haemodynamic and morphological parameters in myocardial infarction-induced cardiac failure in rats. Cardiovasc. Res. 39, 339–350.

Sandmann, S., Min, J-Y., Meissner, A., Unger, Th., 1999. Effects of the calcium channel antagonist mibefradil on haemodynamic parameters and myocardial Ca^{2+}-handling in infarct-induced heart failure in rats. Cardiovasc. Res. 44, 67–80.

Sandmann, S., Bohle, R.M., Dreyer, Th., Unger, Th., 2000. The T-type calcium channel blocker mibefradil reduced interstitial and perivascular fibrosis and improved haemodynamic parameters in myocardial infarction-induced cardiac failure in rats. Virchows Arch. 436 (2), 147–157.

Sandmann, S., Claas, R., Cleutjens, J.P.M., Daemen, M.J.A.P., Unger, Th., 2001a. Calcium channel blockade limits cardiac remodeling and improves cardiac function in myocardial infarction-induced heart failure in rats. J. Cardiovasc. Pharmacol. 37, 64–77.

Sandmann, S., Yu, M., Unger, Th., 2001b. Transcriptional and translational regulation of calpain in the rat heart after myocardial infarction—effects of AT1 and AT2 receptor antagonists and ACE inhibitor. Br. J. Pharmacol. 132, 767–777.

Sasaki, T., Kikuchi, T., Yumoto, N., Yoshimura, N., Murachi, T., 1984. Comparative specificity and kinetic studies on porcine calpain I and calpain II with naturally occurring peptides and synthetic fluorogenic substrates. J. Biol. Chem. 259 (20), 12489–12494.

Sawamura, I., Hazama, F., Kinoshita, M., 1990. Histological and histometrical study of myocardial fibrosis in spontaneously hypertensive rats of the stroke-prone strain. Jpn. Circ. J. 54, 1274–1282.

Sen, L., Smith, T.W., 1994. T-type Ca^{2+}-channels are abnormal in genetically determined cardiomyopathic hamster hearts. Circ. Res. 75 (1), 149–155.

Shimada, T., Yoshiyama, M., Takeuchi, K., Omura, T., Takemoto, Y., Kim, S., Iwao, H., Yoshikawa, J., 1998. Long acting calcium antagonist amlodipine prevents left ventricular remodeling after myocardial infarction in rats. Cardiovasc. Res. 37, 618–626.

Siffert, W., Akkerman, J.W.N., 1989. Na^{+}/H^{+} exchange and Ca^{2+} influx. FEBS 259, 1–4.

Sorimachi, H., Saido, T.C., Suzuki, K., 1994. New era of calpain research. FEBS Lett. 343, 1–5.

Sorimachi, Y., Harada, K., Saido, T.C., Ono, T., Kawashima, S., Yoshida, K., 1997. Downregulation of calpastatin in rat heart after brief ischemia and reperfusion. J. Biochem. (Tokyo) 122 (4), 743–748.

Spalla, M., Tsang, W., Kuo, T.H., Giacomelli, F., Wiener, J., 1985. Purification and characterization of two distinct Ca^{2+}-activated proteinases from hearts of hypertensive rats. Biochem. Biophys. Acta 830, 258–266.

Steenbergen, C., Murphy, E., Levy, L., London, R.E., 1987. Elevation of cytosolic free calcium concentration early in myocardial ischemia in perfused rat heart. Circ. Res. 60, 700–707.

Steenbergen, C., Murphy, E., Watts, J.A., London, R.E., 1990. Correlation between cytosolic free calcium, contracture, ATP, and irreversible ischemic injury in perfused rat hearts. Circ. Res. 66, 135–146.

Suzuki, K., Imajoh, S., Emori, Y., Kawasaki, H., Minami, Y., Ohno, S., 1987. Calcium activated neutral protease and its endogenous inhibitor: activation at the cell membrane and biological function. FEBS Lett. 220, 271–277.

Tolnai, S., Korecky, B., 1986. Calcium-dependent proteolysis and its inhibition in the ischemic rat myocardium. Can. J. Cardiol. 2, 42–47.

Toyo-oka, T., Ross, J.R., 1981. Ca^{2+} sensitivity change and troponin loss in cardiac natural actomyosin after coronary occlusion. Am. J. Physiol. 240, H704–H708.

Urthaler, F., Wolkowicz, P.E., Digerness, S.B., Harris, K.D., Walker, A.A., 1997. MDL-28170, a membrane-permeant calpain inhibitor, attenuates stunning and PKC_{ε} proteolysis in reperfused ferret hearts. Cardiovasc. Res. 35, 60–67.

Wang, K.K.W., Villalobo, A., Roufogalis, B.D., 1989. Calmodulin-binding proteins as calpain substrates. Biochem. J. 262, 693–706.

Weishaar, R.E., Bing, R.J., 1980. The beneficial effect of a calcium channel blocker, diltiazem, on the ischemic–reperfused heart. J. Mol. Cell. Cardiol. 12, 993–1009.

Wier, W.G., 1990. Cytosolic $[Ca^{2+}]$ in mammalian ventricle: dynamic control by cellular process. Annu. Rev. Physiol. 52, 467–485.

Yoshida, K., Yamasaki, Y., Kawashima, S., 1993. Calpain activity alters in rat myocardial subfractions after ischemia or reperfusion. Biochim. Biophys. Acta 1182, 215–220.