

Calpain system and its involvement in myocardial ischemia and reperfusion injury

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Abstract

Calpains are ubiquitous non-lysosomal Ca^{2+} -dependent cysteine proteases also present in myocardial cytosol and mitochondria. Numerous experimental studies reveal an essential role of the calpain system in myocardial injury during ischemia, reperfusion and post-ischemic structural remodelling. The increasing Ca^{2+} -content and Ca^{2+} -overload in myocardial cytosol and mitochondria during ischemia and reperfusion causes an activation of calpains. Upon activation they are able to injure the contractile apparatus and impair the energy production by cleaving structural and functional proteins of myocytes and mitochondria. Besides their causal involvement in acute myocardial dysfunction they are also involved in structural remodelling after myocardial infarction by the generation and release of proapoptotic factors from mitochondria. Calpain inhibition can prevent or attenuate myocardial injury during ischemia, reperfusion, and in later stages of myocardial infarction.

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Key words: Calpain; Calpain inhibition; Calcium overload; Myocardial injury; Ischemia; Reperfusion; Myocardial infarction; Remodelling

Core tip: Calpains, calcium-dependant cytosolic cysteine proteases, are essentially involved in the pathophysiology of myocardial infarction. Their inhibition has shown in animal experiments an enhanced tolerance towards ischemia, a reduction of myocardial infarction and reperfusion injury, and an improvement of the process of remodelling. The availability of specific calpain inhibitors offers new prophylactic and therapeutic possibilities for patients with myocardial infarction, revascularisation and coronary surgery.

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INTRODUCTION

Calpains are calcium-dependent, cytosolic cysteine proteases and are expressed as two “ubiquitous” isoenzymes (μ - and m -calpains) and several “tissue specific” isoforms (n -calpains). Their primary structure contains as well calmodulin-like calcium-binding proteins as well as papain protease-like components, reflected by the term calpain^[1]. A non-lysosomal Ca^{2+} -activated cysteine protease was isolated for the first time by Guroff^[2] 1964 from rat brain. Calpains are meanwhile found in all cells of vertebrates that have been examined^[2-5], in cells of invertebrates^[6,7] and fungi^[8], but not in bacteria and plants.

Besides their physiological functions they are also implicated in pathophysiological processes^[4,9-12], especially with disturbed calcium homeostasis^[4,13,14]. Thus, calpains were found to be involved in myocardial tissue damage resulting from ischemia and reperfusion^[15,16]. Calpain inhibition on the other hand ameliorates, respectively, prevents these lesions in animal experiments with potential prophylactic and therapeutic implications even in clinical

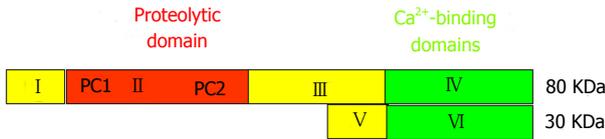


Figure 1 Domain structure of the catalytic 80-kDa and the regulatory 30-kDa subunits of the μ - and m-calpain dimers.

situations.

The following review will give an overview of the physiological and pathophysiological basis of the calpain system and finally focus on its role in myocardial ischemia, infarction and reperfusion and the effectiveness of calpain inhibition based on experimental studies.

BASICS OF THE CALPAIN SYSTEM

Nomenclature

The terms μ -calpain and m-calpain were first used by Cong *et al*^[17] in 1989. They indicate the micromolar (μ -calpain) respectively millimolar (m-calpain) Ca^{2+} -concentrations required for their activation. Thus, μ -calpain is activated in the presence of 3-50 $\mu\text{mol/L}$ Ca^{2+} and m-calpain in the presence of 400-800 $\mu\text{mol/L}$ Ca^{2+} ^[17,18]. Meanwhile, more than 25 proteins with structural similarities were identified as calpains or calpain-like molecules. The genes assigned to 15 of these proteins are numerically named as CAPN1 up to CAPN15 and their coded molecules are named as calpain1 up to calpain15, correspondingly. Calpain1 as well as Calpain2 are biologically active as proteases not as monomers but only as dimers with an identical 30-kDa subunit each. Both biologically active calpains are usually called μ -calpain (calpain 1 + 30-kDa subunit) and m-calpain (calpain 2 + 30-kDa subunit), respectively^[4,12].

According to Suzuki *et al*^[19] calpains are subdivided into two main categories: (1) “typical” calpains with a calmodulin-like domain IV at their COOH-terminus; and (2) “atypical” calpains without this component. Typical calpains are μ -calpain, m-calpain and the calpains 5, 7, 10, 13 and 15 which are also named “ubiquitous” calpains as they are present in almost all cells of vertebrates. In contrast to the “ubiquitous” calpains the “tissue-specific” calpains are exclusively expressed in special cells and tissues, such as calpain 3 in skeletal muscle^[20], calpain 6 in placenta and embryonic muscles^[21], calpain 8 and 9 in the gastrointestinal tract^[22], calpain 11 in the testis^[23], and calpain 12 in hair follicles^[24].

Domain structure of μ - and m-calpain

Both proteases μ - and m-calpain exist as dimers with two subunits of 80-kDa and 30-kDa each (Figure 1)^[25,26]. The larger 80-kDa catalytic subunits of μ -calpain and m-calpain are coded in humans by different genes on chromosome 11 respectively chromosome 1^[27]. On the base of their amino acid sequences they are composed of four regions/domains: (1) a N-terminal domain; (2) a catalytic CysPc protease domain consisting of two protease core regions PC1 and PC2; and (3) a C2-like Ca^{2+} -regulated

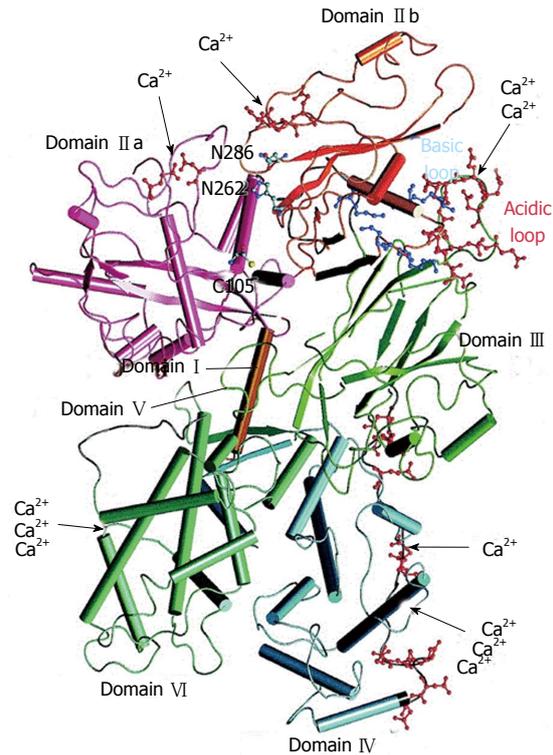


Figure 2 Crystallographic structure of human m-calpain by Suzuki *et al*^[33].

phospholipid-binding domain, and IV a Ca^{2+} -binding penta-EF-hand domain^[28-31].

Domain I contains an amphipathic alpha-helix in the N-terminus of μ -calpain which was shown to be important in targeting and migrating of μ -calpain into the intermembrane space of mitochondria. Domain I of m-calpain, however, does not contain a similar N-terminal component^[32].

Domain II represents the catalytic CysPc protease domain. It consists of two separate protease core domains PC1 with a cysteine (Cys) residue and PC2 with a histidine (His) residue and an asparagine (Asn) residue. These residues form a catalytic triade as known from cysteine proteases such as papain or cathepsin (Figure 2). Both core domains PC1 and PC2 have Ca^{2+} -binding sites for a single Ca^{2+} by each^[33,34].

Domain III is structurally related to C2 domains and can bind phospholipids in a Ca^{2+} -dependent manner. It links the Ca^{2+} -binding domains with the catalytic domain II and is supposed to be involved in the adjustment of the calpain activity *via* electrostatic interactions^[35].

Domain IV shows a slight sequence homology to calmodulin (51%-54%) and has five Ca^{2+} -binding COOH-terminal EF-hand motifs. The fifth motif binds to the corresponding EF-hand sequences of domain VI of the smaller 30 kDa subunit and, thus, contributes to the dimer formation of both calpain subunits^[4,31,33,36].

The smaller regulatory 30 kDa subunit, responsible for the stability of the larger catalytic subunit, consists of the N-terminal Gly-rich domain V and the Ca^{2+} -binding calmodulin-like penta-EF-hand domain VI. The long stretches of Gly residues and an unordered structure of

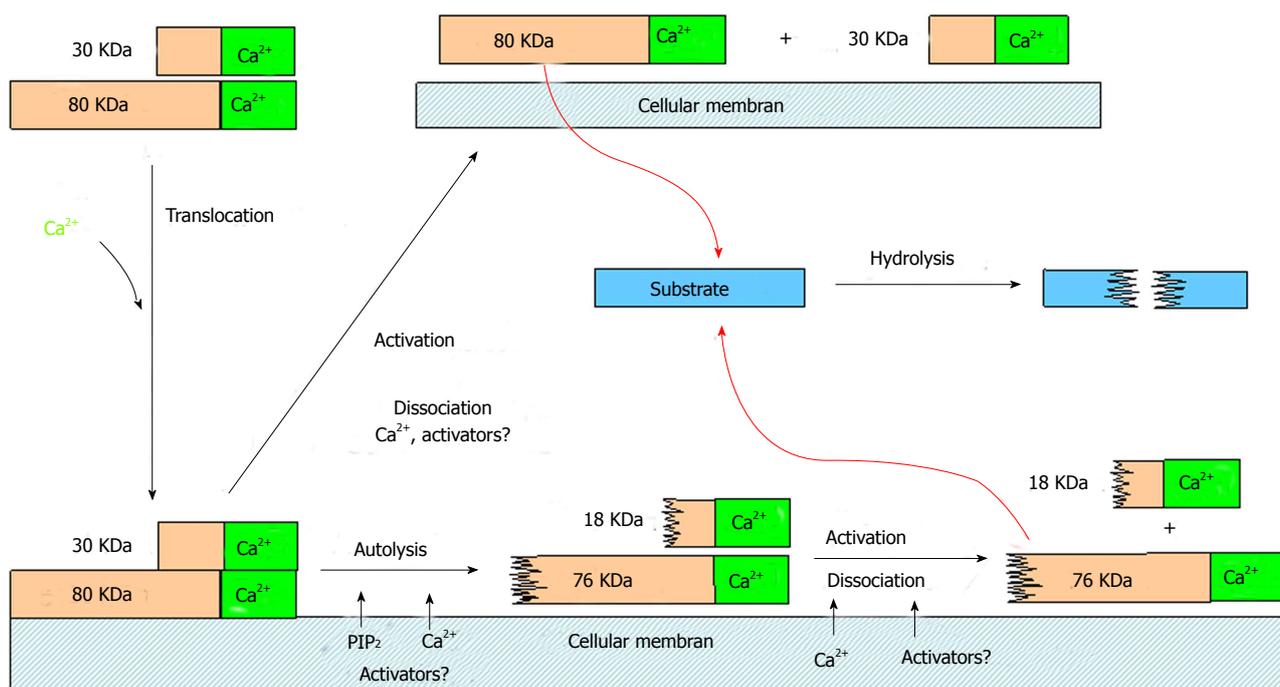


Figure 3 Mechanisms and consequences of calpain activation at biological membranes. Modified from Suzuki *et al*^[40].

the amino acid sequence in domain V are supposed to bind to other molecules and structures.

The “calmodulin-like” domain VI is involved in Ca^{2+} -binding and dimerization by their penta-EF-hand motifs, as also known from domain V of the 80-KDa subunit^[4,31,37,38].

Activation of μ - and m-calpain

Increase of the intracellular Ca^{2+} -concentration is the decisive trigger for calpain activation. The Ca^{2+} -binding core domains PC1 and PC2 of domain II and the terminal EF-hand motifs of domain V and VI cause electrostatic conformational changes in these domains. By this electrostatic switch mechanism the PC1 and PC2 core domains approaches each other. Thus the distance of the Cys-residue from the α His- and Asn-residues of the initially inactive catalytic triade shrinks from 10 to approximately 3.7 Å to form the proteolytic active centre^[50,39]. Simultaneously, the change of conformation intensifies the affinity of calpain to membrane phospholipids and thus induces its translocation to the cell membranes (Figure 3)^[40,41].

Immediately with the binding of Ca^{2+} the autolysis of both subunits of the calpain dimers happens by splitting off the NH2-terminal amino acids. The 80-kDa subunits of μ - and m-calpain are reduced by this process to active fragments of 76-kDa and 78-kDa, respectively, and both 30-kDa subunits are reduced to fragments of 18-kDa each^[42-44]. The autolysis facilitates the dissociation and re-association of the calpain dimers, but is not necessary for their activation, as the dissociated 80-kDa subunits are enzymatically full active^[45].

Confusion still exists with regard to the Ca^{2+} -concentration required for calpain activation. The *in vitro* con-

centrations for μ -calpain (3-50 μ mol/L) and m-calpain (200-1000 μ mol/L) to cause a half-maximal calpain activity are far above the physiological concentrations of 100-300 nmol/L necessary in living cells^[46-48]. Additional mechanisms and factors are therefore supposed to contribute to the activation and activity in a physiological environment. Autolysis is known to increase the Ca^{2+} -sensitivity of μ - and m-calpain for activation^[19,49], however, the problem remains, that far higher Ca^{2+} -concentrations are required to initiate autolysis as they occur in a physiological environment^[50]. Autolysis normally happens in contact with biological membranes in presence of phospholipids such as PIP_2 which considerably reduces the Ca^{2+} -concentration necessary for autolysis^[10,51]. Thus, in presence of PIP_2 autolysis of μ -calpain already happens with 10^{-5} - 10^{-7} mol Ca^{2+} .

In addition, activator proteins from rat brain lower the Ca^{2+} -concentrations necessary for autolysis of μ -calpain to a tenth^[52] and from rat skeletal muscle for autolysis of m-calpain from 400 μ mol/L to 15 μ mol/L^[53]. Both activators are Ca^{2+} -binding proteins combining with calpains and becoming effective upon contact with cell membranes. Further activator proteins are known which increase the catalytic activity of calpains against particular substrates twice^[54], ten times^[55] or twenty-five times^[56] without influencing the required Ca^{2+} -concentration.

Regulation of calpain activity

Calpastatin is the only known specific endogenous inhibitor and regulator of μ - and m-calpain. In addition also H-kininogen and α 2-macroglobulin are inhibiting calpain besides other proteases^[57]. Human calpastatin is encoded by a single gene on chromosome 5^[58] and expressed in several isoforms from 17.5 to 107 kDa^[59-61].

It consists of four inhibitory domains I, II, III and IV, and one N-terminal domain L without inhibitory capability^[62, 63]. Each inhibitory unit inhibits one calpain molecule competitively by blocking the substrate access to the catalytic centre^[64, 65]. Calpastatin inhibits exclusively calpain and not other proteases^[57]. Binding of calpastatin to calpain and its inhibition is Ca²⁺-dependent. The Ca²⁺-concentrations for this are lower as needed for the half-maximal proteolytic activity of μ - and m-calpain^[66]. Calpains and calpastatin are found in physical proximity within the cells^[67, 68]. Therefore, mechanisms are necessary to enable calpain to perform its biological purpose, since calpastatin already binds to calpain with increasing Ca²⁺-concentrations. Thus, the translocation of calpain to the membranes could cause a spatial distance to calpastatin. Furthermore, special mechanisms/factors could lower the threshold for Ca²⁺ to activate calpain without influencing the binding of calpastatin^[3]. With regard to activation and deactivation of calpain many questions are still open concerning a regulating, respectively, modifying role of substrate phosphorylation.

Localization of μ - and m-calpain in cell and tissue

In all examined cells of vertebrates μ -calpain, m-calpain and calpastatin are found at least as the only constituents of the calpain system or they exist in various combinations with great varying patterns of distribution. Thus, human erythrocytes and platelets only contain μ -calpain, and smooth muscles of vessels and stomach predominantly contain m-calpain, whereas, in skeletal muscles and kidneys of the most representatives of vertebrates nearly equal amounts of μ - and m-calpain are found^[67, 69, 70]. Both calpains as well as calpastatin are exclusively localized intracellular and apparently associated with subcellular structures. Thus, 93% of the μ -calpain are found in human red blood cells within the cytosol and 7% membrane associated^[71]. Most of the μ -calpain, m-calpain and calpastatin is localized close to the Z-disc in the myofibrils of skeletal and cardiac muscle, smaller amounts are found in the I- and A-bands. In mitochondria and nuclei only a tenth, respectively, a fifth of calpains and calpastatin was identified compared to their concentration in the Z-disc region^[67, 72, 73]. Calpain and calpastatin are normally localized with a close spatial proximity.

Substrates for calpain

Normally, calpains have only access to intracellular substrates, whereby their cleavage decisively depends on the local activity of calpain and its inhibitor calpastatin. Many proteins are cleaved by calpains *in vitro*, but there is no conclusive evidence that they cannot also be splitted by calpain *in vivo*.

Calpain cleaves the cytoskeleton and membrane-associated proteins: adducin^[74], ankyrin^[75], caldesmon^[9], cadherin^[76, 77], C-protein^[78], desmin^[79], dystrophin^[80], the filamin/actin-binding proteins MAP1 and MAP2^[81], myosin^[82], the neurofilament-proteins NFH, NFM and NFL^[83], NR2-subunit^[84], the anchoring protein PSD-95

of NMDA-receptors^[85], α II-spectrin^[16], β -spectrin^[86], talin^[87, 88], titin^[89], tropomyosin and troponin I^[78], troponin T^[90], vimentin^[79, 91], and vinculin^[92].

Furthermore, kinases, phosphatases and transcription factors are cleaved, such as: EGF-rezeptor-kinase^[93], myosin light-chain kinase^[94], protein-kinase C^[95], calcineurin^[96], inositol-polyphosphat-4-phosphatase^[97], protein-tyrosin-phosphatase-1B^[98], the transcription factors c-Jun, c-Fos^[99, 100], and p53^[101, 102].

PHYSIOLOGICAL FUNCTIONS AND PATHOPHYSIOLOGICAL IMPLICATIONS OF THE CALPAIN SYSTEM

Physiological function of μ - and m-calpain

Calpains are not seen to play an essential role in the intracellular protein digestion. In contrast to lysosomal proteases and the proteasome calpains split proteins by a limited proteolysis into large fragments with potential regulatory and signalling functions^[4]. Many studies including experiments with transgenic mice indicate, that calpains are involved in the embryonic development and cell function^[103-105], cytoskeletal/membrane attachments/cell motility^[79, 81, 86-88, 106], intracellular signal transduction^[95, 107-109], cell cycle^[110, 111], regulation of gene expression^[99, 101], apoptosis^[112-115], and in the long-term potentiation of synaptic transmission^[84, 85, 116].

Involvement of calpains in inherited and acquired diseases

A lacking synthesis of calpains or the dysregulation of the calpain activity disturbing the proteolysis of structural and regulatory proteins is found in a series of genetic and acquired diseases, such as: limb girdle muscular dystrophy (LGMD2A)^[117, 118], muscular dystrophy (type Duchenne and Becker)^[119], diabetes mellitus (type 2)^[120], gastric cancer^[121], Alzheimer's disease^[122-125], multiple sclerosis^[126, 127], and cataract formation^[127].

THE KEY ROLE OF CALCIUM HOMEOSTASIS WITHIN THE CALPAIN SYSTEM

Regulation of Ca²⁺-homeostasis

Many vital cell functions are regulated by the concentration of intracellular available Ca²⁺, such as muscle contraction, neurotransmitter release, glandular secretion, and intercellular communication^[128, 129]. And last but not least, calpains are Ca²⁺-activated proteases. Because of its key role, normally the Ca²⁺ concentration is controlled at different cellular levels *via* mitochondria, plasmalemma/sarcolemma and endoplasmatic reticulum. The transmembrane transport of ions is regulated actively, selectively and directionally-oriented by voltage gated ion channels, by ATP-consuming ion pumps (Na⁺-K⁺-ATPases, Ca²⁺-ATPases, proton-ATPases) and by the concentration gradient due to carrier proteins (Na⁺/H⁺-exchanger,

$\text{Na}^+/\text{HCO}_3^-$ -symporter, $\text{Na}^+/\text{Ca}^{2+}$ -exchanger)^[130-133]. Failing of this control mechanisms may result in an excessive intracellular accumulation of Ca^{2+} (Ca^{2+} -overload) with severe cellular dysfunction up to cell death^[14,134,135].

Events with increasing myocardial Ca^{2+} concentration

Studies with isolated perfused mammalian hearts have shown an increasing cytosolic Ca^{2+} concentration during hypoxia in hearts of rabbits^[136] and ferrets^[137], during ischemia in hearts of rabbits^[138] and rats^[139], and during post-ischemic reperfusion in hearts of rats^[140] and ferrets^[141]. Severe burn trauma also augments the Ca^{2+} content in myocytes^[142,143] and mitochondria^[144] of rat hearts. The same effect can be observed upon exposure of isolated perfused rabbit hearts^[145] and isolated rat cardiomyocytes^[146,147] to hydroxyl free radicals. In analogy to the heart, a Ca^{2+} -overload was also observed in rat brains^[148,149] during hypoxia/ischemia and in the spinal cord^[150] after traumatization.

Disturbance of Ca^{2+} homeostasis in the heart: Pathomechanisms and consequences

The underlying mechanisms and consequences of an imbalance in Ca^{2+} homeostasis are documented the most extensively in heart during hypoxia, ischemia and post-ischemic reperfusion. They are initiated by the decreasing ATP generation and developing acidosis resulting from oxygen deficiency. The activation of the Na^+/H^+ -exchanger (NHE-1)^[152,151,152], which causes the influx of Na^+ into the cell for exchange with H^+ in order to regulate pH, and the simultaneous inhibition of the Na^+/K^+ -ATPase^[153], due to lack of ATP, plays a key role in the intracellular Ca^{2+} -overload. Thus, Na^+ accumulates intracellular and lowers the transmembranous Na^+ gradient, which is the driving force behind the $\text{Na}^+/\text{Ca}^{2+}$ -exchanger by transporting Ca^{2+} out off the cell, resulting in Ca^{2+} -accumulation. The $\text{Na}^+/\text{Ca}^{2+}$ -exchanger which represents a bidirectional transport system is also able to transport Ca^{2+} in exchange with Na^+ in a reverse mode into the cell^[152,154,155]. Driving forces for this are the increasing intracellular Na^+ concentration and depolarisation of the sarcolemma.

Today, disturbance of Ca^{2+} -homeostasis is seen as the main triggering factor of cardiac dysfunction and myocardial injury during ischemia and reperfusion, such as the myocardial stunning, a long-lasting reversible reduction of heart contraction after ischemia^[156-158], or like the Ca^{2+} -overload induced hypercontracture during reperfusion/reoxygenation^[14,159-161], or the incidence of arrhythmias during reperfusion^[162]. Other factors, such as reactive oxygen species or inflammation seem to play a minor role in these situations^[163].

Many studies demonstrate as a consequence of an increasing intracellular Ca^{2+} -concentration the activation of calpains, which cleave numerous functional and structural proteins, and thereby decisively contribute to ischemic and postischemic injury. Thus, the activation of the calpain system during hypoxia or ischemia is well documented in the myocardium of rats^[164-167] and humans^[168],

as well as in the brain of rats^[169-171]. In rat renal proximal tubules hypoxia induces the increase of μ -calpain activity^[172], whereas calpain inhibition reduces the renal functional and structural damage following ischemia and reperfusion^[173]. Hypoxia was also found to up-regulate the activity and gene expression of calpains in endothelial cells of the pulmonary artery^[174].

ROLE OF CALPAINS IN MYOCARDIAL ISCHEMIA/REPERFUSION INJURY

Global ischemia

Most studies on the implication of calpains for myocardial dysfunction and failure are based on experiments in isolated perfused mammalian hearts, in which the duration of perfusion stop (global ischemia) is restricted to enable at least a recovery with reperfusion.

Global ischemia in isolated perfused rat hearts was found to induce a time-dependent translocation of m-calpain to the membrane initially not associated with calpain activation which occurred only during reperfusion and intracellular pH normalization^[175]. Under comparable conditions, a loss of myofibrillar desmin, α -actinin, and spectrin was observed in guinea pig hearts, which was reduced by calpain inhibitor I^[176]. Immunohistochemical studies revealed the proteolysis of caldesmon and α -fodrin at the intercalated discs and the sarcolemma after post-ischemic reperfusion in rat hearts. Degradation of both proteins could be suppressed and myocardial function improved by calpain inhibitor I^[16,177]. The inhibition of α -fodrin degradation associated with the attenuation of myocardial dysfunction could also be observed after cardioplegic cardiac arrest in rat hearts in the presence of calpain inhibitor SNJ-1945^[178]. As a result of calpain activation, the essential Ca^{2+} -handling proteins Ca^{2+} -ATPase (SERCA2a) and the SERCA regulatory protein PLB were degraded upon global ischemia and reperfusion in a working rat heart preparation. Their degradation, the depression of cardiac performance and the release of lactate dehydrogenase, indicating the myocardial damage, could be significantly attenuated by calpain inhibition with calpain inhibitor III (MDL28170)^[179]. As an indicator of myocardial tissue damage creatine phosphokinase and lactate dehydrogenase are released from myocytes into the perfusion fluid during reperfusion in concentrations dependent on the duration of ischemia (Figure 4). Calpains seem to be responsible or to contribute to these effects, as calpain inhibition with A-705239 significantly reduces the enzyme release^[180].

Cardiac muscle contraction is initiated by Ca^{2+} via troponin/tropomyosin which are known as substrates of calpain. Therefore, their cleavage is supposed to be jointly responsible for myocardial dysfunction in ischemia/reperfusion injury. With regard to this, degradation of troponin T (TnT) was observed during ischemia/reperfusion of isolated perfused rat hearts and was reduced by calpain inhibition with PD150606 and PD151746^[181]. In addition, "overexpression of calpastatin by gene trans-

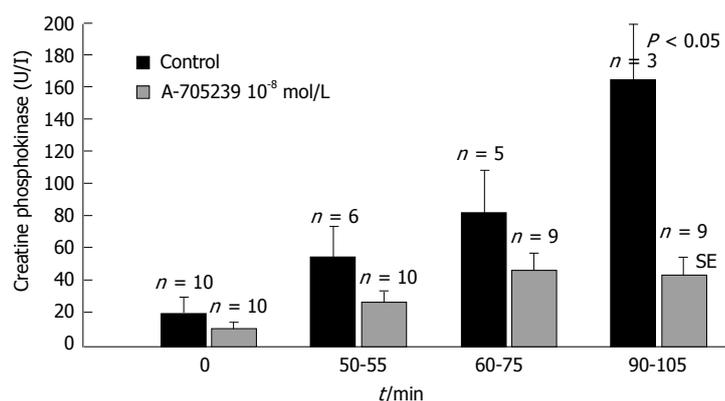


Figure 4 Release of creatine phosphokinase into the perfusion fluid of isolated rabbit hearts subjected to ischemia and reperfusion^[180]. Control experiments without inhibitor are represented by black-coloured columns and inhibitor (A-705239 10⁻⁸ mol/L) treated hearts by grey-coloured columns. Data are expressed as means \pm SE of $n = 10$ experiments each. Both groups differ significantly ($P < 0.05$) at the end of reperfusion.

fer prevents troponin I (TnI) degradation and ameliorates contractile dysfunction in rat hearts subjected to global ischemia followed by reperfusion^[182].

Mitochondrial function impairment

Damage of mitochondria plays a central role in the pathophysiology of reperfusion injury *via* the impairment of oxidative metabolism, respectively, energy production and the generation and accumulation of metabolic products toxic to the myocytes. Cardiac mitochondria are located subsarcolemmal beneath the plasma membrane and interfibrillar between the myofibrils^[183-185]. In animal and human hearts μ -calpain, m-calpain and calpain 10 are present in cytosol and in the intermembrane space of mitochondria^[67,186-189]. Cytosolic calcium content is found to increase in hearts of rats and rabbits during myocardial ischemia and reperfusion and is made responsible for the subsequent activation of calpains^[190,191]. The damage of Ca²⁺-handling proteins by direct cleaving or detaching the Na⁺/K⁺-ATPase and the Na⁺/Ca²⁺-exchanger from their binding ankyrin^[174,192], and by proteolysis of sarcoplasmic reticulum Ca²⁺-ATPase (SERCA)^[179,193] and Ryanodine receptor RyR^[194], sustains Ca²⁺-influx and calpain activation and aggravates myocardial injury. Thus, SERCA2a and the SERCA regulatory protein PLB were found to be degraded upon global ischemia and reperfusion in a working rat heart preparation. Their degradation, the depression of cardiac performance and the release of lactate dehydrogenase, indicating the myocardial damage, could be significantly attenuated by calpain inhibition with calpain inhibitor III (MDL28170)^[179].

One of the most serious consequences of mitochondrial damage by calpains is the impairment of oxidative phosphorylation with loss of ATP generation. Damage to mitochondrial oxidative metabolism can be caused on various levels of the electron transport chain (ETC). In isolated renal cortical mitochondria from rats and rabbits calpain 10 was shown to cleave complex I subunits of the ETC, which could be prevented by pretreatment with calpeptin^[195]. The impairment of mitochondrial respiration is documented in isolated perfused rabbit hearts^[180,196]. State 3 respiration decreased significantly during 45 min of global ischemia and further decreased during 60 min of reperfusion, and this reaction could be

significantly attenuated by addition of calpain inhibitor A-705239 to the perfusion fluid (Table 1).

Reduced state 3 respiration reflects the impairment of the electron transport chain (ETC), above all complex I, which is an early target of myocardial ischemia^[197].

Calpain inhibitor A-705239 administered before ischemia and reperfusion also attenuated the increase in permeability of the inner mitochondrial membrane (mitochondrial permeability transition), as reflected by the reduced state 4 respiration and leak-respiration^[180].

Besides their deleterious effect on mitochondrial oxidative metabolism, calpains are also recognized to cause the generation and release of substances toxic to myocytes.

During reperfusion, mitochondria generate reactive oxygen species that lead to additional mitochondrial and myocyte injury^[197-200].

Dependent on the degree of oxidative damage in concert with mitochondrial calcium overload and calpain activation, mitochondrial permeability transition can occur by formation of inner membrane pores^[201,202]. Mitochondrial permeability transition can result in disruption of the outer mitochondrial membrane and the release of cytochrome c, a key step inducing apoptosis^[203]. Cytochrome c is detectable in the cytosol of rabbit myocardium at 30 min of ischemia^[204], whereas cytochrome c content decreases in subsarcolemmal mitochondria^[205]. Mitochondrial calpain plays an important role in programmed cell death by generation or release of apoptotic factors in mitochondria during ischemia and reperfusion. Thus, the cleavage of Bid, a pro-apoptotic BH3-only Bcl-2 family member, is documented in isolated perfused adult rabbit hearts during ischemia/reperfusion, and in secondary *in vitro* studies recombinant Bid was cleaved by calpain to an active fragment that was able to mediate cytochrome c release^[206]. It was also shown, that activated mitochondrial μ -calpain, mostly located in the intermembrane space, cleaves and releases apoptosis inducing factor (AIF) from isolated mouse heart mitochondria. Besides, mitochondrial μ -calpain activity increased in buffer perfused mouse hearts during ischemia/reperfusion whereas the mitochondrial AIF content decreased. Inhibition of mitochondrial μ -calpain using MDL-28170 preserved the AIF content within the mitochondria and

Table 1 Effect of calpain inhibitor A-705239 on impairment of mitochondrial function following myocardial ischemia and reperfusion^[180]

	<i>n</i>	State 3 respiration (nmol O ₂ /min per milligram)	State 4 respiration (nmol O ₂ /min per milligram)	RCI (state3 rate): (state 4 rate)	Leak respiration(nmol O ₂ /min per milligram)	Stimulation by cytochrome c %
Control						
Before ischemia	4	6.4 ± 1.1	0.5 ± 0.1	12.5 ± 2.7	0.15 ± 0.07	6.0 ± 10.0
Ischemia 45 min	8	3.5 ± 1.4 ^{bc}	0.9 ± 0.3 ^a	4.4 ± 2.5 ^a	0.32 ± 0.14 ^a	10.0 ± 6.0
Reperfusion 60 min	4	2.6 ± 1.3 ^{bc}	0.9 ± 0.3 ^a	3.2 ± 2.1 ^a	0.43 ± 0.29	28.0 ± 16.0
A-705239 treated hearts						
Before ischemia	4	6.8 ± 1.3	0.6 ± 0.1	12.4 ± 1.1	0.12 ± 0.06	16.0 ± 9.0
Ischemia 45 min	9	5.0 ± 0.8 ^{bc}	0.6 ± 0.2	8.2 ± 2.3 ^{bc}	0.20 ± 0.14 ^a	15.0 ± 13.0
Reperfusion 60 min	5	4.2 ± 1.2 ^{bc}	0.7 ± 0.2	6.4 ± 2.7 ^a	0.26 ± 0.24	

Data are presented as means of 4 to 9 experiments mean ± SD measured as duplicates or triplicates. A significant difference from baseline before ischemia is represented by ^a*P* < 0.05, and between both groups by ^c*P* < 0.05.

reduced cardiac injury^[186].

Partial ischemia and myocardial infarction

In contrast to models of global ischemia, in the experimental setting of partial ischemia by temporary occlusion of coronary arteries the duration of ischemia can be extended in time to enable irreversible myocardial damage to a restricted area with myocardial infarction without the risk of early global heart failure with reperfusion. In isolated perfused rat hearts it was shown, that during a 30 min occlusion of the left anterior descending coronary artery calpain translocates to the cell membranes without being activated initially. Calpain activation, as indicated by the hydrolysis of α -fodrin, only started with the onset of reperfusion and could be prevented by calpain inhibition with MDL-28170, just as the infarct size could be reduced by 32%^[175].

Inhibition of α -fodrin degradation and improvement of left ventricular function by calpain inhibitor SNJ-1945, administered 30 min before a gradual and partial coronary occlusion, was also found after mild ischemic-reperfusion in another study in rat hearts^[207]. Protecting effects of calpain inhibition on myocardial injury could also be demonstrated by own experiments with inhibitor administration both before and during reperfusion. "Two novel calpain inhibitors (A-705239 and A-705253) were studied in isolated perfused rabbit hearts subjected to a 60 min occlusion of the ramus interventricularis of the left coronary artery (below the origin of the first diagonal branch), followed by 120 min of reperfusion^[208,209]. The inhibitors were added to the perfusion fluid in various final concentrations from the beginning of the experiments before the coronary artery was blocked. The infarct size was significantly reduced in presence of both calpain inhibitors. The best effect was achieved with 10⁻⁸ mol/L A-705253 which reduced the infarcted area by 61.8 % (Figure 5A). In a second study in isolated perfused rabbit hearts subjected to a 60 min occlusion of the ramus interventricularis of the left coronary artery followed by 120 min of reperfusion calpain inhibitor A-705253 and/or the Na⁺/H⁺-exchange inhibitor cariporide[®] were added to the perfusion fluid at the beginning of reperfusion solely or in combination^[210]. The infarct size was signifi-

cantly reduced dose-dependently in presence of both inhibitors (Figure 5B). The best effect was achieved with 10⁻⁶ mol/L A-705253, which reduced the infarcted area by 33.6%. Cariporide[®] (10⁻⁶ mol/L) reduced the infarct size in the same extent. The combination of both inhibitors, however, didn't further improve cardioprotection. Thus, the protective effect can be attributed exclusively to its influence on the calpain system, since the combination of both inhibitors didn't augment the protective effect of sole calpain inhibition. The calpain inhibitor A-705253 is known to directly block the catalytic centre of activated calpains, whereas the Na⁺/H⁺-exchange inhibitor cariporide[®] prevents or reduces the ischemic intracellular Ca²⁺-overload and thus prevents or reduces the following calpain activation". This is shown in postischemic perfused rat and rabbit hearts where reduced calpain activation^[211] and calcium overload^[212] were observed upon inhibition of Na⁺/H⁺-exchange. Even in patients undergoing coronary bypass surgery pretreatment with cariporide[®] reduced mortality and the risk of myocardial infarction^[213], however, cerebrovascular events increased^[214]. In accordance with the findings in rabbit hearts, also in pigs undergoing occlusion of the left anterior descending coronary artery for 45 min followed by 6 h of reperfusion infarct size was reduced by 35% and hemodynamic alterations attenuated using calpain inhibitor A-705253^[215]. In experiments with isolated mouse hearts undergoing ischemia and reperfusion infarct size was decreased and ventricular function improved in calpain-1 knockout mice, whereas myocardial injury was greatly increased in transgenic mice hearts with calpain-1 overexpression^[216].

No sufficient information is available to what extent polymorphonuclear leukocytes (PMN) contribute to ischemic/reperfusion injury. In one study in isolated rat hearts perfused with PMNs, exposed to 20 min of ischemia and followed by 45 min of reperfusion, calpain inhibition with Z-Leu-Leu-CHO reduced the adherence of PMNs to the vascular endothelium and improved ventricular function, however, controls without PMNs are missing^[217]. Thus, with regard to the numerous experiments discussed in this review, which were all performed without PMNs in the perfusion fluid, polymorphonuclear leukocytes appear not to be essential for reperfusion

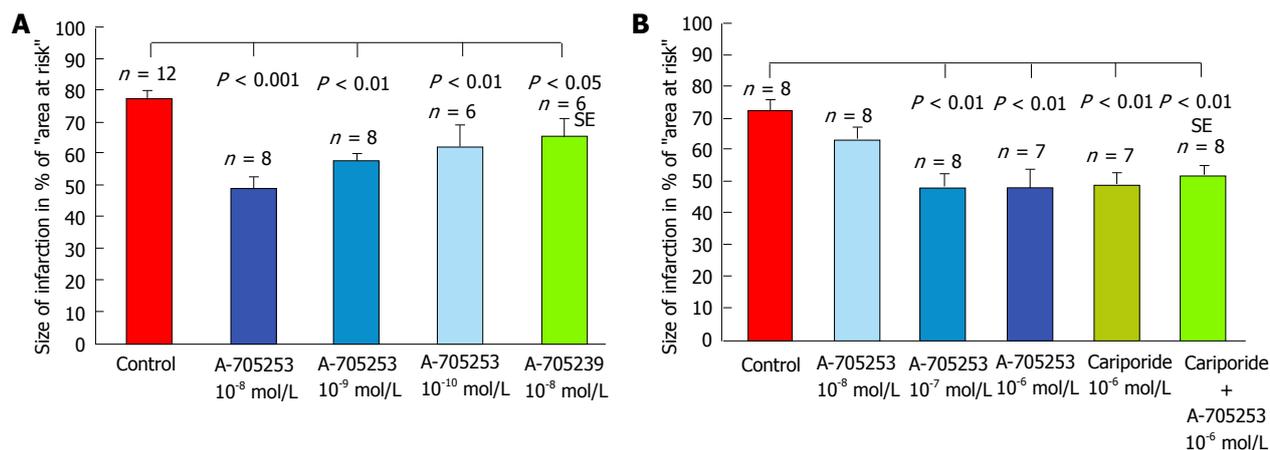


Figure 5 Development of myocardial infarction in isolated perfused rabbit hearts after occlusion of ramus interventricularis of left coronary artery for 60 min, followed by 120 min of reperfusion^[209]. A: The inhibitors were added to the perfusion fluid before ischemia; B: With reperfusion. Infarct size is expressed in percentage of the area at risk (the transiently not perfused myocardium). Control experiments without inhibitor are represented by a red-coloured column and inhibitor treated hearts by blue-coloured columns. Data are presented as means \pm SE. Infarct size is significantly reduced by calpain inhibition in all treated hearts compared to untreated controls.

injury.

Remodelling after myocardial infarction

Myocardial infarction is followed by a progressive structural remodelling of the heart, replacing and reconstructing the irreversibly damaged myocardium^[186,205,206]. After the early phase of ischemia-induced myocyte necrosis a longer lasting myocyte death by apoptosis can be observed. Proapoptotic factors are generated and released from myocardial mitochondria already during ischemia and reperfusion which are considered to be essentially involved in remodelling after myocardial infarction^[186,205,206]. Characteristics of apoptosis, DNA fragmentation and chromatin condensation, could be detected in isolated perfused rabbit hearts subjected to 30 min ischemia and 4 h reperfusion^[220]. In ischemic/reperfused rat hearts undergoing 30 min coronary occlusion followed by 6 h reperfusion the administration of calpain inhibitor I (CAI) 10 min before reperfusion significantly reduced DNA fragmentation and infarct size^[221]. Comparable results were achieved in mouse hearts with persistent coronary artery ligation for 4 d. Calpain inhibition with calpeptin was started 15 min before artery occlusion and continued during the observation time. Calpeptin administration reduced apoptotic cell death, as detected by TUNEL staining, and reduced infarct size and myocardial dysfunction^[222]. The important contribution of calpains to the process of myocardial remodelling is also documented by a transgenic mouse model with cardiomyocyte-specific deletion of gene *Capn4* (*Capn4-ko*) which is indispensable for μ - and m-calpain stability and activity. Mice were subjected to persistent left coronary artery ligation and followed up for 30 d. Deletion of *Capn4* reduced infarct expansion, apoptosis, myocardial remodelling and dysfunction^[223].

CONCLUSION

Numerous studies have shown an essential contribution

of calpains in myocardial injury following ischemia and reperfusion. Proven prevention or attenuation of post-ischemic heart damage by calpain inhibition with various tested inhibitors could offer a novel prophylactic or therapeutic approach for patients with myocardial infarction, revascularisation and coronary surgery.

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