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# Atrial Calpains: Mediators of Atrialmyopathies in Atrial Fibrillation

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**Abstract**

Atrial fibrillation (AF) is associated with substantial structural changes at cell and tissue level. Cellular hypertrophy, disintegration of sarcomeres, mitochondrial swelling and apoptosis have been described as typical histo-morphologic alterations in AF. Main initiators for cellular alterations in fibrillating atrial myocytes are cytosolic calcium overload and oxidative stress. Calpains are intracellular Ca2+- activated proteases and important mediators of calcium overload. Activation of calpains and down-regulation of the calpain inhibitor, calpastatin, contribute to myocardial damage in fibrillating atria. Thus, deregulations of the expression, activity, or subcellular localization of calpain within atrial myocytes have been established as important mediators of atrial myopathy during AF.

## Molecular Structure And Regulation Of Calpains And Calpastatin

Calpains are intracellular Ca2+- activated proteases and important mediators of the actions of calcium. The regulated cleavage by calpain is critical in a variety of calcium-regulated cellular processes such as muscle contraction, neuronal excitibility, secretion, signal transduction, cell proliferation, differentiation, cell cycle progression, and apoptosis. Deregulation of calpain caused by a disruption of calcium homeostasis during cardiac pathologies such as atrial fibrillation, heart failure, hypertrophy, or ischemia reperfusion, critically contributes to myocardial damage.[1-5]

The term "calpain" was originally used for classical μ- and m-calpains that are heterodimers composed of a large 80 kDa catalytic subunit encoded by either CAPN1 for μ -calpains (calpain 1), or by CAPN2 for m-calpains (calpain 2), and a common 30 kDa regulatory subunit encoded by CAPNS1 (calpain 4). Whereas the small subunit is identical for both enzymes, the large subunits share 55-65% sequence homology.[5-6] The μ- and m-calpains differ in their requirements of intracellular Ca2+, which need to be micromolar and millimolar in concentration, respectively. Based on sequence homology, 14 human genes have been identified as members of the calpain large catalytic 80 kDa family, and 2 human genes for the small regulatory 30 kDa family.[7-8]

**Key Words:**
Atrial Fibrillation, Calpain, Calcium, Protease, Pathophysiology.

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The typical calpains are composed of four domains originally found in μ- and m-calpains (Figure 1).[7-8] The N-terminal region of domain I of the large subunit comprises a single α-helix responsible for stabilizing of the domain arrangement. Domain II contains the catalytic site (Cys, His, and Asn residues) and is composed of the subdomains IIa and IIb. Domain III binds phospholipids in a Ca2+-dependent manner and is supposed to mediate Ca2+-dependent membrane translocation of calpains. Domain IV, at the C-terminal end of the large subunit, is a Ca2+-binding domain containing five EF-hand motifs. The large catalytic subunit interacts with the small regulatory subunit through the fifth EF-hand motifs in domain IV and VI to form a heterodimeric calpain. Atypical calpains lack EF-hand motifs and, therefore, cannot form a dimer with the small regulatory subunit.

Most calpains are ubiquitously expressed, but the expression of some calapins, including calpains 3, 6, 8, 9, and 12, is rather tissue-specific.[6-8] Although calpains are cytoplasmic enzymes, several studies have shown that μ-calpain, m-calpain, and calpain 10 are present also in mitochondria.[9-10] Calpains exists in the cytosol as inactive enzymes, which translocate to membranes in response to increased intracellular calcium levels. As mentioned before, at the membrane, calpains become activated in the presence of calcium and phospholipids.[11-12]

Based on data from both in vitro and in vivo studies, calpain can

play a regulatory role in important processes including remodeling of cytoskeletal proteins, modulation of signal transduction pathways, degradation of cell cycle-regulating enzymes, regulation of gene expression, and initiation of apoptotic pathways. The proteolytic activity of calpains is not specific for certain amino acid residues or motifs, but recognizes the overall three-dimensional structure of its substrates.[7-8] Many proteins have been identified as potential substrates of calpain. These include a large number of cytoskeletal and myofibrillar proteins (myosin, troponin, tropomyosin, titin),[13-15] membrane-associated proteins (receptors, ion channels),[16-24] metabolic enzymes,[25-26] signaling-modulated kinases and phosphatases (PKC, calcineurin),[27-31] transcription factors (NF-αB),[32-33] and proteins involved in apoptotic signaling.[10,34-39]

Under physiological conditions, the activity of calpain is thightly regulated. Regulatory mechanisms include the existance of a specific endogenous inhibitor, calpastatin. The binding of calpastatin to calpain is calcium-dependent and reversible.[7] Calpastatin contains four equivalent inhibitory domains, each bearing three conserved regions (A, B, and C). The regions A and C bind to domain IV of the large subunit and to domain VI of the small subunit of calpain, respectively.[4] The region B shows inhibitory activity itself.[7,40] Both μ- and m-calpains exhibit similar sensitivity to calpastatin. The presence of the two calmodulin-like domains IV and VI are necessary for effective inhibition of calpastatin. Thus, calpastatin can inhibit only dimeric calpain like μ-, m-calpain, and calpain.[9] The atypical calpains are resistant to the inhibitory actions of calpastatin.[40] The cloning and molecular characterization of calpastatin revealed the existence of potential phosphorylation sites for protein kinase C, cAMP- and cGMP-dependent protein kinase, and casein kinase II.[41] Thus, these motifs are supposed to play an important role in molecular regulation of the calpain/calpastatin system.

An additional mechanism for regulation of calpain activity is its phosphorylation by protein kinase A (PKA)[42] or by extracellular-signal regulated kinase (ERK).[43-44] Glading et al.[44] provided evidence that m-calpain can be activated after direct phosphorylation at Ser-50 by ERK in the absence of cytosolic calcium influx. However, more recent studies indicated that the phosphorylation of calpain by ERK and PKA regulates the enzyme indirectly by controlling its cellular redistribution. The phosphorylation by ERK promotes the translocation of m-calpain to the membrane and the binding to phospolipids which in turn facilitates the activation of calpain. In contrast, PKA–dependent phosphorylation of calpain at Ser-369 prevents domain movement and the formation of the calpain active site.[41,43]

Most recently, it was shown that intracellular acidosis attenuates the activation of calpain.[45] The study by Inserte demonstrated that the prolongation of intracellular acidosis during reperfusion by either post-conditioning or by acidic perfusion of isolated rat hearts has cardioprotective effects through inhibition of calpain activation. Further investigations are necessary to demonstrate a relation between delayed acidosis and the putative involvement of kinases in the cardioprotective mechanisms.

Finally, results of many in vitro and in vivo studies support the potential value of cardioprotective strategies based on interfering with calpain activation. The first generations of synthetic calpain inhibitors inactivate calpains by forming a covalent bond within the active site of the calpain catalytic domain (E-64, leupeptin, ALLN, ALLM),[46-49] or by interacting with the Ca2+-binding domain of the calpain large subunit (PD150606).[50] Persisting problems resulting from low membrane permeability and target selectivity led to the continuing development of calpain inhibitors. Several studies have used screening approches to identify new calpain inhibitor templates, and these have produced a wide range of peptidic, peptidmimetic, and non-peptide inhibitors.[51]They target the active site of calpain and exhibit an improved pharmacokinetic profile.

Amongst the synthetic calpain inhibitors developed so far, calpastatin peptide analogues and their truncated versions stand out due to their unique selectivity and affinity profile.[59-60] Calpastatin is the only known inhibitor with absolute specificity for both μ- and m-calpains, but its large molecular mass (110 kDa), making it membrane-impermeable, limits its therapeutic use.

## Regulation Of Calpains In Fibrillating Atria

Pathophysiologically, atrial fibrillation (AF) goes along with profound changes of the electrophysiologic and structural appearance of atrial tissue.[4,53-59] Previous studies have shown that AF is characterized by an initial intracellular calcium overload, which causes atrial electrical remodeling.[4,57] In addition, abnormal calcium handling during AF contributes to mechanical atrial dysfunction after successful cardioversion.[1,60-62] The histopathology of fibrillating atria is similar to that of chronically ischemic ventricular myocardium.[53,63] Ischemia/reperfusion injury is mediated by increased levels of cytosolic calcium, which causes activation of the calcium-dependent proteases μ- and m-calpain.[4,64-65]

## Contribution Of Calpains To The Contractile Dysfunction During AF

Recent data imply that activation of μ-calpain leads to the destruction of contractile filaments in fibrillating atria.[66] In samples from 32 patients (16 with chronic AF, 16 with sinus rhythm; SR) the atrial expression of μ- and m-calpain, calpastatin, troponins T (TnT), and C (TnC) were determined. Expression of μ-calpain was increased during AF (461±201% vs 100±34%; $p<0.05$). Amounts of m-calpain and calpastatin remained unchanged. Total calpain enzymatic activity was more than doubled during AF (35.2±17.7U vs 12.4±9.2U; $p<0.05$). In contrast to TnC, TnT levels were reduced in fibrillating atria by 26% ($p<0.05$) corresponding to a myofilament disintegration as observed by electron microscopy. In accordance with previous studies, μ-calpain had no effect on TnC levels.[67] In contrast to previous ischemia/reperfusion models, small fragments of TnT were not detected.

Loss of a regular sarcomere structure would help to explain the prolonged mechanical dysfunction of the atria after successful cardioversion of AF. Mechanical atrial dysfunction can last for several weeks after cardioversion.[4,68-70] Schotten et al. have demonstrated that there is a strong correlation between the maximum force of contraction and sarcomere content in atrial muscle preparations.[62]

Recently, down-regulation of L-type calcium-channels and altered intracellular calcium handling have been implicated in the pathogenesis of contractile dysfunction during AF.[61-62,71-73] An ex vivo study on HL-1 atrial myocytes showed that 24h electrical field stimulation at 5 Hz reduced plasmalemmal levels of L-type Ca2+ channelα1C subunit by -72% compared to controls, whereas the amounts of the potassium channel subunits Kv4.3 and Kv1.5 were not changed.[48] Although changes in number and/or function of L-type calcium-channels seem to contribute predominantly to the reduced contractility during AF, the time-course of resolution

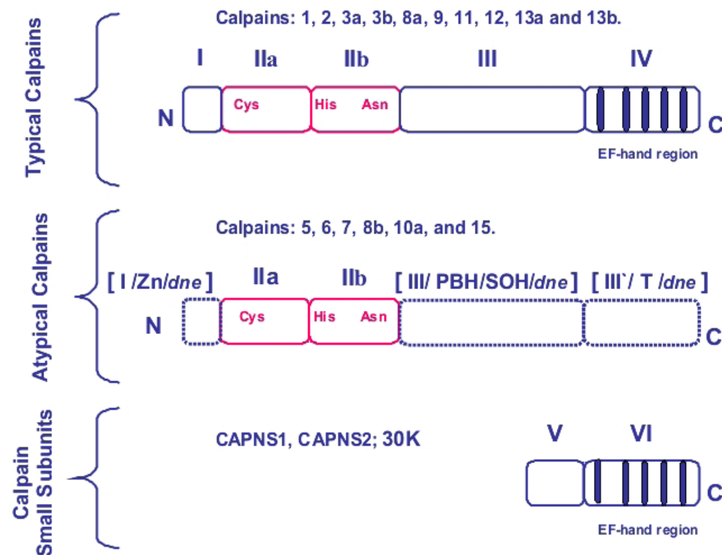


**Figure 1: Domain structure of calpains.**

The large subunit of the typical calpains comprises four domains, while the small subunit has two domains. The atypical calpains varies from the domain organisation originally found in µ- and m-calpains. All atypical calpains lack EF-hand motifs and therefore cannot form a dimer with the small subunit. Alternatives for domain I: Zn, or does not exist (dne); for domain III: SOH, PBH, or does not exist; Alternatives for domain IV: III`, T, or does not exist.

of these functional changes after restoration of sinus rhythm has not been studied in detail. A recent study implies that structural abnormalities persist significantly longer after cardioversion than functional changes of L-type calcium channels.[68] Thus, the described structural abnormalities of contractile proteins may help to explain especially the dyscontractility of the atria after cardioversion.

In conclusion, activation of µ-calpain (calpain 1) may well contribute to structural remodeling and contractile dysfunction of fibrillating atria. However, in addition to these intracellular changes, interstitial collagen accumulation as well as atrial dilatation may further contribute to sustained alterations of the atrial contractile performance.[4,70,74-76]

## Calpains Trigger Pro-Apoptotic Processes During AF

Rapid pacing induced marked structural changes; myolysis and nuclear condensation, paralleled by a 14-fold increase in calpain activity. Interestingly, inhibition of calpain prevented myofibril degradation and nuclear condensation.[4,48] Li et al. show that the inhibitor of µ-calpain, ALLM, diminishes the extent of apoptotic cell death in an in vivo canine model of AF.[51] They showed that inhibition of µ-calpain is able to reduce the rate of apoptosis (apoptosis index) and caspase expression as otherwise significantly increased after 3 weeks of atrial pacing. Anti-apoptotic proteins like bcl2 recovered in response to ALLM therapy. This is indicative of calpain activity being upstream of mitochondrial apoptotic pathways. Thus, the present paper using an in vivo model provides first evidence that inhibition of µ-calpain prevents pacing induced apoptosis. This finding has important implication since increased rate of apoptosis might affect the electrical and mechanical properties of fibrillating atrial tissue. Nevertheless, it still needs to be verified that the described molecular effects of ALLM directly influence the occurrence/persistence of AF or the mechanical atrial function.

The µ-calpain cleaves a variety of proteins to promote apoptosis with caspase-7 being just one recent example.[35] However, there is apparent crosstalk between apoptotic and necrotic pathways and activation of calpain promotes apoptosis even during caspase inhibition. In the heart, caspase-independent induction of apoptosis has been recently shown to occur via the release of apoptosis-inducing factor (AIF) from mitochondria.[4,77] This process was shown in other tissues to be mediated by elevated intracellular Ca2+-levels[78] or µ-calpain.[67] Interestingly, results of the present study are confirmed by data from previous studies obtained ex vivo.[48] The pacing-induced reduction of L-type Ca2+-channel protein was fully prevented by treatment with verapamil, the active stereoisomer of methoxyveramil D600, the calpain inhibitors PD150606 and E64d, and LaCl.[3] Interestingly, PD150606, E64d, but not verapamil, prevented structural changes such myofibril degradation, or nuclear condensation. Swelling and disruption of mitochondria are typical morphologic findings in fibrillating atrial tissue.[48] Mitochondrial dysfunction and concomitant opening of the permeability transition pore are strongly correlated with cell death,[79] and interventions to prevent pore opening have been shown to protect the myocardium.[80] AF causes severe damage of mitochondrial structure and provokes mitochondrial dysfunction, which is in part due to the frequency-dependent intracellular Ca2+-overload.[4,81-82] Accordingly, verapamil has been shown to preserve mitochondrial structure and function.[81-82] The critical requirement for mitochondrial participation in the death process is underscored by the protective effect of the mitochondrial ATP-sensitive potassium channel in preconditioning. This channel has been suggested to preserve calcium homeostasis and would, therefore, limit calpain activation.[83] Recent studies revealed the presence of m-calpain,[84-85] µ-calpain,[86] and calpain 10[87] within mitochondria. Calpain 10 has been linked to Ca2+-induced mitochondrial dysfunction and its capability to cleave complex I proteins may contribute to β-cell death in diabetes.[87-88] Intracellular Ca2+-overload as observed in AF activates mitochondrial calpains and promotes apoptotic cell death[89] via a maltidute of actions excellently reviewed by Kar et al.[10]

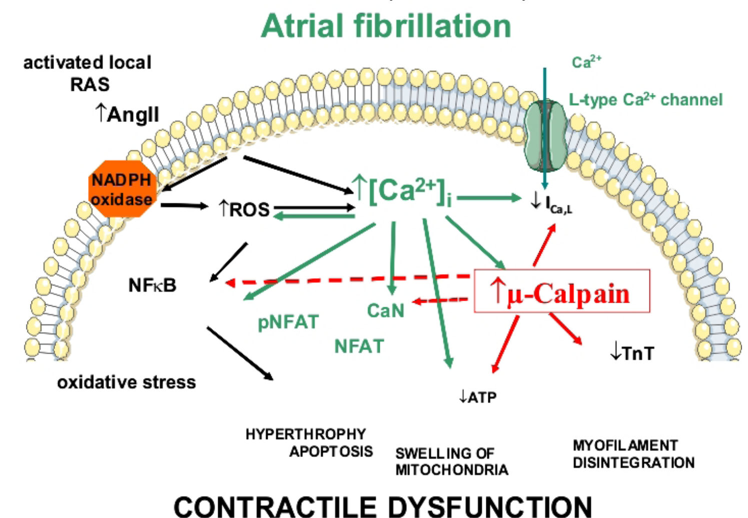


**Figure 2: Schematic representation of accompanying pathways involved in µ-calpain activation during atrial fibrillation (AF).**

Intracellular calcium overload and subsequent sequestration into mitochondria results in alteration in mitochondrial morphology and function. Concomitantly, AF causes an increased expression and activity of µ-calpain. These changes are accompanied by degradation of myofilaments and reduced amounts of troponin T (TnT). Furthermore, persistent calcium overload leads to the reduction of L-type Ca2+ channel proteins. The activation of atrial renin-angiotensin-system and altered intracellular calcium homeostasis are important factors contributing to the oxidative stress in AF. Elevated in [Ca2+]i levels and reactive oxygen species (ROS) are major activators of the immediate early response transcription factor NF-κB, which is a redox sensor in atrial tissue. Rapid pacing causes an upregulation of calcineurin (CaN), a phosphatase responsible for the translocation of transcription factor NFAT to the nucleus where it induces the expression of pro-hypertrophic genes.

**Interaction With Other Calcium-Dependent Signalpathways During AF**

Altered intracellular calcium homeostasis and activation of atrial renin-angiotensin-system have been identified as important factors contributing to the oxidative stress in the endocardium of fibrillating atrial tissue. Elevated levels of intracellular Ca2+ and reactive oxygen species (ROS) are major activators of the immediate early response transcription factor NF-αB, which is a redox sensor in atrial tissue. Typical target genes of NF-αB are pro-inflammatory cytokines such as interleukin-8 and tumor necrosis factor α (TNF-α), but also the endothelial adhesion molecules.[82,84] Interestingly, regulation of NF-kappaB signaling is linked to antioxidant levels, oxidative stress, and enhanced cell death. Concomitantly, calpain becomes activated, and treatment with calpain inhibitors, therefore, is able to restore NF-kappaB-p65 levels and to increase cell viability. Thus, calpastatin and an altered NF-kappaB-p65 signaling are crucial factors involved in oxidative stress and cell death.[90]

Calcium-dependent phosphatase, calcineurin (CnA), is also activated in atrial tissue during AF[73,91-92] Rapid pacing causes an upregulation of CnA activity that is associated with increased dephosphorylation of the transcription factor NFATc3, allowing dephosphorylated NFATc3 to enter the cell nucleus. Thereby, NFATc3 increased the transcription of genes responsible for the atrial hypertrophic cellular response, as demonstrated by an increased expression of ANP and β-myosin heavy chain.[4,91] In accordance with this finding, a study by Wang et al. indicated that calpain 1 is responsible for the formation of constitutively active calcineurin in the ventricular myocardium from patients with heart failure.[92]

Finnaly, there is apparent crossinteraction between activated calpain and calcium-dependent singalling pathways during atrial fibrillation (Figure 2). The calpain-dependent apoptosis of atrial cardiomycytes and degradation of contractile proteins contribute to the occurrence of contractile dysfunction. Moreover, activation of calcium-dependent singalling pathways (NF-αB, Calcineurin) influence the extracellular matrix and support the progress of structural remodeling in atrial tissue. These all processes collectively change the architectur of atrial tissue and may further contribute to sustained alterations in the atrial contractile performance.

## Conclusions:

Calpains are important modulators of the normal signal transduction, gene expression and muscle contraction. Deregulation of their activity is associated with cardiovascular pathologies such as heart failure, myocardial infarction, and atrial fibrillation. The increased expression and activity of calpains during atrial fibrillation may result from the deregulation in calcium homeostasis and contributes to the occurrence of contractile dysfunction due to degradation of contractile proteins. Moreover, activation of calpains initiates apoptosis processes and mediates damage of atrial cardiomyocytes. Results of in vitro and in vivo studies have shown the beneficial effects of calpain inhibitors in different models of atrial fibrillation. Therefore, this protease has attracted a special attention in the medical science as a potential drug target. Nevertheless, calpain inhibitors have not been tested in clinical trials, yet, to validate the role of calpain inhibition in the clinical setting. Persisting problems resulting from the lower target selectivity, membrane permeability, and pharmacokinetic properties hinder the introduction of calpain inhibitors into clinical trails. Therefore, efforts should be dedicated to development of compounds resolving these problems.

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