Cell. Mol. Life Sci. 63 (2006) 333–342
1420-682X/06/030333-10
DOI 10.1007/s00018-005-5353-3
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Cellular and Molecular Life Sciences

Research Article

Activation of the calcineurin signaling pathway induces atrial hypertrophy during atrial fibrillation

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Received 5 August 2005; received after revision 15 November 2005; accepted 17 November 2005
Online First 2 January 2006

Abstract. Atrial tachyarrhythmia (AF) alters intracellular calcium homeostasis and induces cellular hypertrophy of atrial myocytes. The impact of the calcium-dependent calcineurin pathway on the development of AF-induced atrial hypertrophy has not yet been analyzed. In this study, atrial tissue samples from patients with sinus rhythm and chronic persistent atrial fibrillation (CAF) were used to determine changes in expression and activity of calcineurin A (CnA), and its relation to CnA-regulated transcription factors NFATc1–4, and hypertrophic markers ANP, troponin I, and β-MHC. CnA phosphatase activity and CnAβ protein contents were significantly upregulated in patients with CAF. Calcineurin activation led to dephosphorylation, redistribution, and subsequent accumulation of NFATc3 in nuclei during CAF, and expression of hypertrophic genes was increased. CAF-dependent changes were reproduced by ex vivo pacing (2–4 Hz) of human atrial tissue slices. FK506 abolished the hypertrophic response induced by electrical-field stimulation. Atrial tachyarrhythmia causes atrial hypertrophy by activation of the CnA signal pathway, which thereby contributes to structural remodeling of human atria.

Key words: Fibrillation; calcium; calcineurin; hypertrophy; pacing.

Atrial fibrillation (AF) is the most common arrhythmia in clinical practice and is a potential cause of thromboembolic events [1, 2]. Several studies have shown that AF induces significant changes in the electrophysiological properties and morphologic appearance of trial myocytes [3–8]. Most of these AF-induced alterations are related to a cellular calcium overload, which is due to an increased influx of calcium through L-type calcium channels [5, 9, 10]. Increased levels of intracellular calcium have been found to cause ‘atrial electrical remodeling’ [5, 9]. Furthermore, calcium-dependent proteases like calpain are activated during AF contributing to ‘structural remodeling’ of atrial myocytes [10].

The presence of hypertrophied atrial myocytes during AF is one consistent finding of several studies [5–8]. Hypertrophy of cardiomyocytes influences chamber geometry and mechanical performance. Furthermore, cellular hypertrophy increases anisotropic conduction and an increase in cell size may thereby contribute to the development of an arrhythmogenic substrate [11–14]. However,

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Table 1. Characteristics of the patients

Characteristic	SR	CAF
Age (years)	61 ± 13	67 ± 7
Male/female [n (%)]	11/2 (85/15)	8/5 (62/38)
Myocard infarction [n (%)]	6 (46)	3 (23)
Valve disease		
Mitral regurgitation [n (%)]	5 (38)	8 (61)
Mitral stenosis [n (%)]	0	3 (23)
Aortic stenosis [n (%)]	1 (8)	1 (8)
Aortic regurgitation [n (%)]	4 (31)	4 (31)
Hypertension [n (%)]	10 (77)	9 (69)
Corornary artery disease [n (%)]	11 (85)	5 (38)
NYHA functional class	2.7 ± 0.6	2.7 ± 0.5
Left ventricular ejection fraction (%)	53 ± 18	54 ± 10
Left atrial diameter (cm)	4.4 ± 0.7[a]	5.2 ± 0.5[a]
Therapy before heart surgery:		
Beta-blocker [n (%)]	6 (46)	5 (38)
Nitrates [n (%)]	9 (69)	4 (31)
Calcium channel blocker [n (%)]	2 (15)	2 (15)
ACE inhibitor/AT1 antagonists [n (%)]	12 (92)	10 (77)

Values are the mean ± SD, [a]$p < 0.05$. NYHA, New York Heart Association, ACE, angiotensin-converting enzyme; AT1, angiotensin II type I receptor blocker.

the underlying pathophysiology of atrial hypertrophy during AF has not been elucidated.

Calcineurin (CnA) is a protein phosphatase known to dephosphorylate a class of transcription factors, nuclear factors of activated T cells (NFATs), allowing their translocation into cell nuclei [15]. In this way, CnA induces a hypertrophic response in cardiomyocytes characterized by an increased expression of hypertrophic marker genes such as atrial natriuretic peptide (ANP), β-myosin heavy chain (β-MHC), and cardiac troponin I (cTnI) [16–19]. The immunosuppressive drug FK506 can inhibit CnA activity and has been shown to prevent calcineurin-induced hypertrophy of cardiomyocytes in different models [15, 20–23].

The purpose of this study was to assess the role of the calcium-dependent expression of CnA signaling and its effect on atrial hypertrophy in atrial tissue of patients with chronic persistent atrial fibrillation (CAF).

Materials and methods

Patients. Right atrial appendages were obtained from 26 patients undergoing cardiac bypass surgery or mitral/aortic valve replacement. Tissue samples were taken from 13 consecutive patients with chronic persistent AF (≥6 months; CAF) and from 13 matched patients with sinus rhythm (SR). The clinical characteristics are shown in table 1. For *ex vivo* pacing, atrial tissue samples were taken from 15 additional patients (age 69 ± 8 years) with SR. The baseline characteristics of these patients were similar to the clinical controls. The study was approved by the local ethics committee and all patients gave written informed consent to participate in the study.

RNA isolation and reverse transcription. Samples of human atrial appendages were rapidly frozen in liquid nitrogen and stored until further analysis. Total RNA was prepared from about 250 mg of atrial tissue or 100 mg of atrial tissue slices, as described recently [24] by applying the method of Chomczynski and Sacchi [25]. One microgram of total RNA was transcribed into cDNA using AMV reverse transcriptase (Promega, Mannheim, Germany) as described previously [24].

Quantitative PCR. Quantitative PCR was performed using the iCycler (BioRad, Munich, Germany). All samples were analyzed in triplicate. A 25-µl reaction mixture consisted of 1× HotStartTaq Master Mix (Qiagen, Hilden, Germamy), 0.5 µl of a 1:1000 dilution of SYBR-Green I (Molecular Probes, Eugene, Oregon, USA), 1 µl cDNA, and 0.5 µmol/l of the specific primers listed in table 2.
Initial denaturation at 95 °C for 15 min was followed by 40 cycles with denaturation at 95 °C for 30 s, annealing at the temperature indicated in table 2 for 30 s, and elongation at 72 °C for 30 s. Quantities of GAPDH mRNA were used to normalize cDNA contents. The fluorescence intensity of the double-strand specific SYBR-Green I, reflecting the amount of PCR product actually formed was read in real-time at the end of each elongation step. Then amounts of specific initial template mRNA were calculated by determining the time point at which the linear increase in sample PCR product started, relative to the corresponding points of a standard curve; these are given as arbitrary units.

Protein extraction from atrial tissue. Frozen tissue samples (250 mg) from right atrial appendages were pulverized in liquid nitrogen and subsequently homogenized in 1 ml lysis buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 100 mM NaCl, 0.5% Triton X-100, 10% glycerol, 10 mM K_2HPO_4, 0.5% NP-40), containing a protease inhibitor cocktail (Boehringer Mannheim), 1 mM sodium vanadate, 0.5% sodium deoxycholate, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 20 mM NaF, and 20 mM glycerol-2-phosphate (all from Sigma, Heidelberg, Germany). Tissue homogenates were centrifuged (15 min, 15,000 g at 4 °C) and the resulting supernatant was stored at –20 °C until further use.

Table 2: Primers used in quantitative RT-PCR analyses.

Gene	Product size (bp)	T_{anneal} (°C)	Sequence
CnA-β	370	59	US: 5′- GGAGGATCACCTGCTAATAC DS: 5′- CACATTGGTCCAAATGCAGG
NFATc1	136	58	US: 5′- CCAACACCAAAGTCCTGGAG US: 5′- GTGTTCTTCCTCCCGATGTC
NFATc2	589	62	US: 5′- CTATGAGACAGAAGGCAGCC DS: 5′- GCTGTCCATCTGTGGTCTTC
NFATc3	306	56	US: 5′- GTAGATCCACCTCCATCTAC DS: 5′- GAGATGATCTCTAGAAGGCC
NFATc4	544	59	US: 5′- GCCAGAGGATAGCTGGCTAC DS: 5′- GTACCTCGATCCTCAGCTCC
α-MHC	266	62	US: 5′- CTGGCTTCGAGATCTTCGAC DS: 5′- GCTTGGCCTTGAAGGTCATG
β-MHC	300	60	US: 5′- CCAGCTCCAGACTGAAGTGG DS: 5′- CTTCACCGACTCTGCGTTGC
ANP	377	60	US: 5′- ACTGGCATTCCAGCTCCTAG DS: 5′- CTCCAATCCTGTCCATCCTG
cTnI	312	58	US: 5′- CAACTACCGCGCTTATGCCAC DS: 5′- AGATCTGCAATCTCCGTGATG
GATA-4	261	58	US: 5′- CTGTCATCTCACTACGGGCA DS: 5′- CCAGGTCCGTGCAGGAATTT
MEF-2	432	58	US: 5′- CACTGGAGAAAAGAAGGGTC DS: 5′- GTGGAACCAAGTCTACCAGC
Erk2	218	60	US: 5′- CATTGCTGAAGCGCCATTCAAG DS: 5′- GCCTAACATCCTCATTTCACACC
GAPDH	600	60	US: 5′- TCCAAAATCAAGTGGGGCGATGCT DS: 5′- ACCACCTGGTGCTCAGTGTGACCC

Nuclear extracts were prepared as previously described with some modification [26]. In brief, about 250 mg of atrial tissue was washed with cold PBS, carefully homogenized and resuspended in 1 ml hypotonic buffer (10 mM Hepes, pH 7.9, 1 mM EDTA, 150 mM NaCl, 0.5 mM PMSF, 0.6% Nonidet P40, 10 mM NaF, protease inhibitor mixture). Homogenates were incubated for 10 min on ice and centrifuged (10 min, 800 g at 4 °C). Pellets were resuspended in 0.15 ml hypertonic buffer (20 mM Hepes, pH 7.9, 1 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT, 25% glycerol, protease inhibitor mixture) and incubated on ice for 20 min. Subsequently, samples were centrifuged (10 min, 13000 g at 4 °C) and supernatants (nuclear protein extract) were aliquoted and stored at –80 °C until use. The protein content of all samples was determined using the Bio-Rad Protein Assay.

SDS-PAGE and immunoblot analysis. Extracted proteins were separated by 7–10% PAGE, followed by transfer to nitrocellulose membranes (Schleicher and Schuell BioScience, Dassel, Germany). Membranes were blocked with 5% non-fat dried milk in TBS and then incubated with the primary antibodies against calcineurin-Aβ (PP2B-Aβ goat IgG, dilution in TBS 1:300; Santa Cruz, Calif.), NFATc3 (M-75 rabbit polyclonal IgG, dilution in TBS 1:150; Santa Cruz), or Phospho-p44/42 MAP kinase (Thr202/Tyr204) E10 monoclonal antibody (dilution in TBS 1:1000; New England Biolabs, Schwalbach, Germany). Anti-goat (dilution 1:5000 in TBS; Dianova, Hamburg, Germany), anti-rabbit or anti-mouse (both diluted 1:2000 in TBS; Cell Signaling, Frankfurt/Main, Germany) horseradish-peroxidase-conjugated antibodies were applied after washing the blots three times in TBS. For chemoluminescence detection, the SuperSignal West Dura Extended Duration substrate (Pierce, Rockford, Ill.) was used. Densitometric quantification for comparison of the different groups was performed only on blots processed equally and exposed on the same X-ray film.

CnA phosphatase activity. CnA phosphatase activity was measured using the calcineurin assay kit plus (Biomol, Plymouth Meeting, Pa.) according the manufacturer's instructions. The RII-phosphopeptide was used as a highly specific substrate for calcineurin. The detection of free

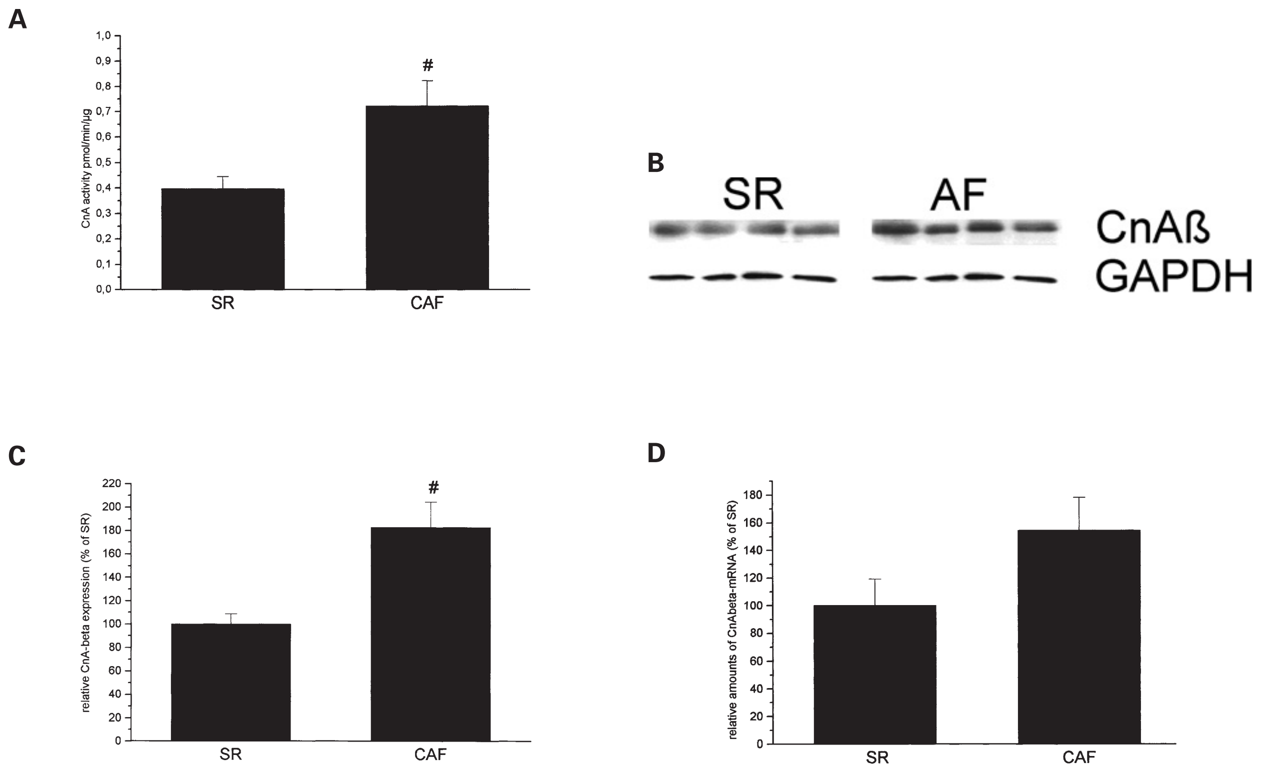


Figure 1. Expression of calcineurin in fibrillating human atria. Data represent the mean ± SE. (*A*) Quantification of calcineurin phosphatase activity in tissue from patients with CAF (n = 12) and SR (n = 9; #p < 0.05 CAF vs SR). (*B*), Representative CnAβ Western blot from whole-tissue homogenates (30 µg protein per lane). Loading control was performed with GAPDH. (*C*), Amounts of atrial CnAβ protein determined by densitometric analyses of CnAβ Western blot (n = 12, #p < 0.05 CAF vs SR). (*D*), Quantitative RT-PCR of CnAβ (n = 12, p = ns).

phosphate released from RII peptide by calcineurin was determined photometrically using the Biomol Green reagent. Calcineurin activity was calculated as total phosphatase activity minus activity in the presence of EGTA.

Culturing tissue slices and *ex vivo* pacing. Atrial tissue was obtained during open heart surgery and directly processed for culturing. The tissue was immediately sliced (350 µm) and five to nine slices were placed on top of 0.02-µm Anopore membrane tissue culture inserts (25 mm; Nunc, Wiesbaden, Germany). Four of these inserts were placed in a petri dish (8 cm diameter; Nunc), which was filled with culture medium as previously described [27]. To electrically stimulate adult human atrial tissue, a pair of custom-built carbon electrodes (12.5×6×32 mm) was submersed at the opposite ends of the petri dish. The distance of the electrodes was about 8 cm. Copper wires, which were electrically isolated with silicon rubber, were inserted into holes drilled into the carbon electrodes and connected to a stimulation unit (GRASS Stimulator). In accordance with previous *in vitro* pacing experiments, a biphasic square wave impulse (150 V) with a duration of 5 ms was used for electrical-field stimulation [27–31]. A biphasic impulse was used to minimize electrolysis at the electrodes. The minimum distance between the tissue samples and the electrodes was approximately 1.5 cm. Pacing of the tissue slices was performed within the cell culture incubator up to 24 h at 37 °C, pacing at rates of 0.6, 2.0, or 4.0 Hz. Non-paced tissue slices served as control. To determine the effect of FK506, 30 ng/ml FK506 was added to the medium throughout the pacing period.

Statistical Analysis. Differences in the amounts of mRNA, protein levels and CnA activity between the groups were evaluated using one-way ANOVA. The Pearson correlation coefficient (r) was used to determine the relationship between parameters. All values are expressed as the mean ± SE, data presented in table 1 are given as the mean ± SD. A p value <0.05 was considered to be statistically significant.

Results

The baseline patient characteristics are shown in table 1. The diameter of the left atrium was increased in patients with CAF compared to patients with SR (5.2 ± 0.5 cm vs 4.4 ± 0.7 cm in SR; p < 0.05). The severity of the underlying heart diseases and non-cardiac diseases was similar in both groups.

Calcineurin expression in fibrillating human atrial tissue. In patients with CAF, calcineurin activity was in-

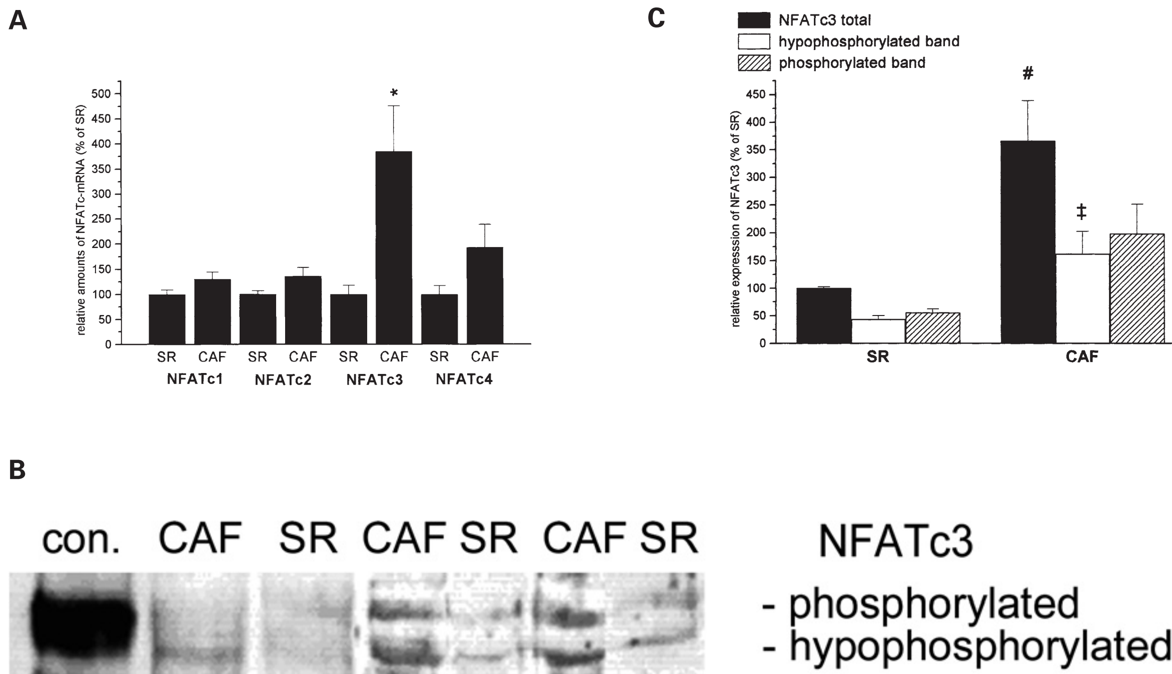


Figure 2. Expression of NFATc isoforms in human atrial myocardium. (*A*) Quantitative analysis of NFATc family members 1–4 in atrial tissue from patients with SR and CAF by real-time RT-PCR. NFATc3 mRNA amounts were significantly increased in CAF in comparison to SR (n = 13, *$p < 0.01$). (*B*) Demonstration of the nuclear accumulation (representative samples) of NFATc3 during CAF by Western blot analysis of nuclear extracts (40 µg protein per lane). The fast-moving band of NFATc3 representing the hypophosphorylated form is increased in CAF compared to SR. Whole-cell homogenates of Jurkat T cells served as positive control. (*C*) Quantification of nuclear NFATc3 protein contents by densitometric analysis of Western blots (total NFATc3: #$p < 0.05$ CAF vs SR; hypophosphorylated NFATc3: ‡$p < 0.05$ CAF vs SR; n = 6; mean ± SE) Total NFATc3 means the amounts of both phosphorylated and hypophosphorylated NFATc3.

creased 1.8-fold compared to SR (SR: 0.40 ± 0.05 pmol/min/per microgram; CAF: 0.72 ± 0.1 pmol/min/per microgram; $p < 0.05$; fig. 1A). The CnA beta isoform has been associated with a hypertrophy signal pathway [32]. Therefore, the expression of CnAβ was analyzed for both mRNA and protein levels. Atrial CnAβ protein amounts in CAF were significantly increased to 182.8 ± 21.5% (SR: 100 ± 8.3%; $p < 0.05$; fig. 1B, C). This increase in CnAβ expression was partially reflected at the mRNA level, where we observed a tendency toward increased amounts of CnAβ mRNA in atrial tissue of patients with CAF (154.5 ± 23.5%) compared to SR (100 ± 19%; fig. 1D).

Expression of NFATc1–4 in fibrillating human atrial tissue. Gene expression of the NFAT family members NFATc1, c2, c3, and c4 was quantified using RT-PCR. Amounts of atrial NFATc1 (130 ± 14% vs SR 100 ± 8.7%) and NFATc2 mRNA (136 ± 17.3% vs SR: 100 ± 6.6%) were not significantly changed in patients with CAF compared to SR (fig. 2A). However, amounts of NFATc3 mRNA were markedly increased during CAF (384.8 ± 90.6% vs SR: 100 ± 17.9%; $p < 0.01$). Notably, NFATc3 expression was significantly correlated with the diameter of the left atrium ($r = 0.46$, $p < 0.05$). There was also a tendency toward increased NFATc4 mRNA expression (CAF: 193 ± 49.8% vs SR: 100 ± 17.7%; $p = 0.056$) (fig. 2A). Furthermore, we determined the mRNA level of the transcription factors GATA-4 and MEF-2, which interact with NFATs to mediate specific gene expression. Expression levels of both transcription factors appeared unchanged during CAF [GATA-4: 105 ± 10.4% (CAF) vs 100 ± 11.0% (SR); MEF-2: 101.4 ± 9.5% (CAF) vs 100 ± 12.5% (SR); p = ns].

NFATc activation during AF. Among the NFATs analyzed in this study, an increase in NFATc3 expression was the predominant change observed in response to CAF. Therefore, the nuclear localization and the extent of phosphorylation of NFATc3 were investigated by immunoblot analyses. In full accordance with results at the mRNA level we observed an accumulation within nuclei of NFATc3 during CAF (360.6 ± 72.6% vs SR 100 ± 2.4%; n = 6, $p < 0.05$) (fig. 2B, C). Notably, the hypophosphorylated form of NFATc3 was fourfold higher within the nucleus during CAF (178.3 ± 45.8% vs SR 43.6 ± 6.6%; $p < 0.05$).

Atrial expression of hypertrophic genes. Next, we looked for changes in gene expression which could be related to development of atrial hypertrophy. RT-PCR analyses showed an increased atrial mRNA expression of the hypertrophy-associated genes ANP and cTnI in patients with CAF [ANP: 309.4 ± 59.3% (CAF) vs 100 ± 13.3% (SR); cTnI: 197.6 ± 28.7% (CAF) vs 100 ± 11.1% (SR); $p < 0.01$

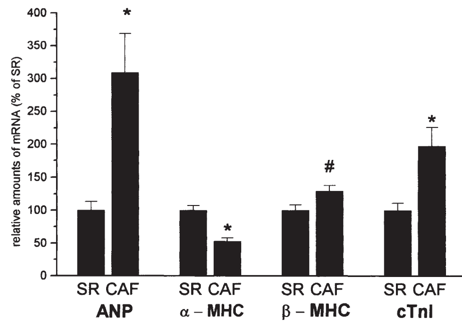


Figure 3. Atrial expression of heart hypertrophy-associated genes during CAF. ANP, β-MHC, α-MHC, and cTnI mRNA amounts in atrial tissue from patients with CAF and SR were measured by quantitative real-time RT-PCR. In CAF, amounts of ANP, β-MHC, and cTnI mRNA were significantly increased, whereas that of α-MHC was decreased (n=13, CAF vs SR: $^{*}p<0.01$, $^{\#}p<0.05$). Data represent the mean±SE.

for both). Furthermore, β-MHC mRNA amounts were increased by 29.4±8.8% during CAF (SR: 100±8.7%, $p<0.05$), whereas mRNA expression of α-MHC was decreased to 53.2±4.7% (SR: 100±7.4%, $p<0.01$) demonstrating a switch in MHC isoform expression (fig. 3).

***Ex vivo* atrial tissue slices.** To confirm the functional role of CnA signaling during CAF, additional atrial tissue slices (350 µm) obtained from patients without AF were cultured *ex vivo* and electrically paced. Staining with propidium iodide and calcein ester AM indicated a good viability of the atrial tissue slices even after prolonged (up to 8 days) culture *ex vivo* (fig. 4). In this study, analyses of expression of both mRNA and protein levels were performed after rapid-pacing for 24 h. Functional integrity of the atrial tissue slices was proven by their ability to respond to 2-Hz pacing with increased MAP kinase Erk2 mRNA expression (0 Hz: 100±34.2%; 0.6 Hz: 43±22.5%; 2.0 Hz: 202±46.7%; 0 Hz vs 2 Hz: $p<0.05$, n=15; 0 Hz vs 0.6 Hz: $p<0.05$, n=7) and activity (0 Hz: 100±5%; 2.0 Hz: 206±46%; 2 Hz vs 0 Hz: $p<0.05$, n=7).

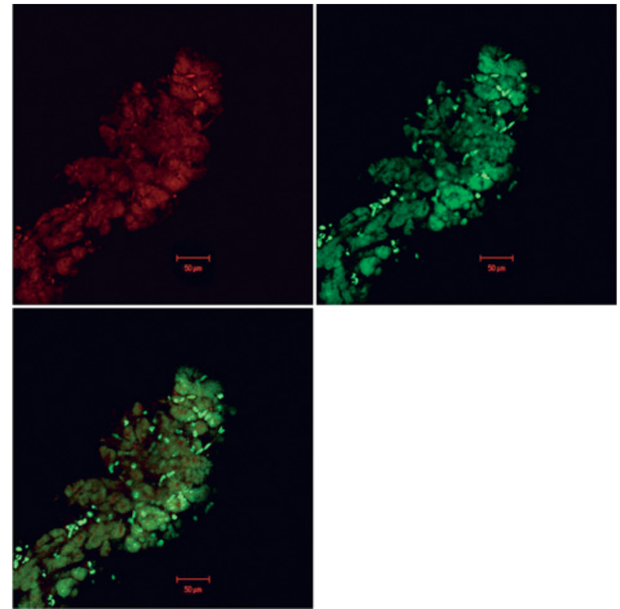
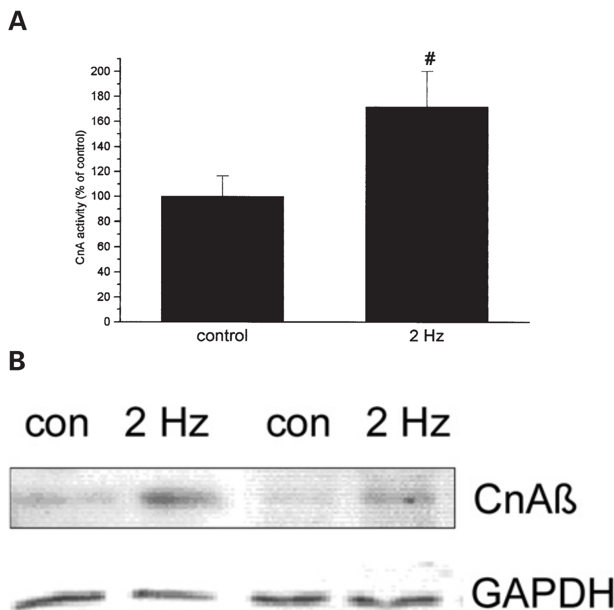


Figure 4. Viability of human atrial tissue slices cultured *ex vivo* for a period of 8 days. Propidium iodide and calcein AM (Molecular Probes) were used to identify dead (*A*) (red fluorescence) or viable (*B*) (green fluorescence) cells, respectively, as described previously [53]. (*C*) Superimposed *A* + *B*.

Pacing of *ex vivo* atrial tissue slices stimulates calcineurin expression. Rapid pacing at 2 Hz for 24 h increased calcineurin phosphatase activity by 71.8±28.3% (vs non-paced 100%; n=5, $p<0.05$) (fig. 5). This activa-



C

CnA-beta expression normalised with GAPDH

0 20 40 60 80 100 120 140 160

#

control 2 Hz

Figure 5. Effect of 2-Hz pacing (24 h) of human atrial tissue slices on the expression and activity of CnA. Data represent the mean±SE. (*A*) CnA phosphatase activity assessed in cytosolic fractions of paced and non-paced (control) tissue slices (n=5, $^{\#}p<0.05$ 2 Hz vs control). (*B*) Representative CnAβ Western blot from homogenates of paced and non-paced tissue slices (15 µg protein per lane). Loading control was performed with GAPDH. (*C*) Amounts of atrial CnAβ protein were significantly increased in response to 2-Hz pacing as determined by densitometric analyses of a CnAβ Western blot (n=5, $^{\#}p<0.05$ 2 Hz vs control).

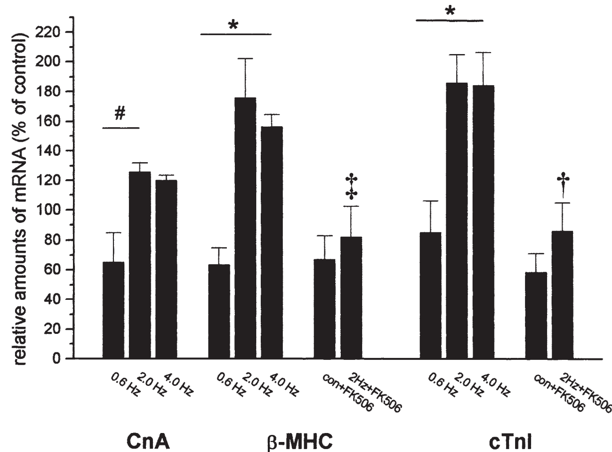


Figure 6. Induction of atrial-hypertrophy-associated genes in response to rapid pacing and its prevention by 30 ng/ml FK506. Pacing of human atrial tissue slices was performed at 0.6, 2.0, and 4.0 Hz for 24 h and then amounts of mRNA were determined by quantitative real-time RT-PCR. The pacing at 2.0 Hz (n=15) or 4.0 Hz (n=3) equally increased mRNA expression of CnAβ, cTnI, and β-MHC when compared to 0.6 Hz (n=5, *p<0.01, #p<0.05). Rapid pacing (2.0 Hz) in the presence of 30 ng/ml FK506 largely prevented this hypertrophic response (β-MHC and cTnI: †p<0.01 2 Hz vs 2 Hz + FK506; ‡p<0.05 2 Hz vs 2 Hz + FK506; n=5). Data represent the mean ± SE (100%=non-paced controls).

tion was supported by the Western blot analyses of whole homogenates from atrial tissue slices. As shown in figure 5B, C, pacing at 2 Hz elevated significantly the expression of CnAβ by 38.8 ± 13.4% (vs non- paced, n=5, p<0.05).

Pacing-induced expression of hypertrophy-associated genes. The rate-dependent expression of hypertrophy-associated genes was investigated at the mRNA level using the following pacing frequencies: 0.6 Hz (n=5), 2.0 Hz (n=15), and 4.0 Hz (n=3). Rapid pacing of atrial slices increased amounts of CnAβ mRNA about 2-fold, irrespective of whether the frequency being applied was 2.0 or 4.0 Hz, compared to the pacing at 0.6 Hz (0.6 Hz: 62.2 ± 19.6%; 2.0 Hz: 125.8 ± 5.8%; 4.0 Hz: 120.0 ± 3.5%; p<0.05) (fig. 6). Pacing at 2.0 Hz increased amounts of cTnI mRNA 2.2-fold compared to pacing at 0.6 Hz (2 Hz: 186.1 ± 18.5% vs 0.6 Hz: 85.4 ± 20.8%, p<0.01). Pacing at 4.0 Hz did not further elevate the expression of cTnI (4 Hz: 184.3 ± 22.0%). Similarly, the expression of β-MHC was raised 2.5-fold by pacing at 2.0 Hz compared to pacing at 0.6 Hz (2 Hz: 175.7 ± 26.1%, 0.6 Hz: 63.4 ± 11.3, p<0.05). Again, the rise in the pacing frequency from 2 to 4 Hz did not change the expression of β-MHC mRNA (fig. 6). However, the changes in gene expression of cTnI and β-MHC observed after *ex vivo* pacing of atrial tissue slices for 24 h closely resembled those observed in patients with CAF (fig. 3).

Effect of FK506 during *ex vivo* pacing. Next, we investigated the effects of the calcineurin inhibitor, FK506, on the mRNA expression of cTnI and β-MHC. As described above, 2- or 4-Hz pacing affected gene expression to the same degree. Therefore, a pacing frequency of 2.0 Hz was chosen to analyze effects of pharmacological interventions. The mRNA level of β-MHC (82.0 ± 20.4%; p<0.05), and cTnI (86.4 ± 18.4%; p<0.01) remained at baseline levels when pacing was performed in the presence of 30 ng/ml FK506 (fig. 6). Thus, the administration of FK506 prevented the hypertrophic response to pacing at 2.0 Hz.

Discussion

We observed that CAF causes a significant activation of atrial CnA. This upregulation of CnA activity was associated with increased dephosphorylation of the transcription factor NFATc3, allowing dephosphorylated NFATc3 to enter the cell nucleus, where it increased the transcription of genes responsible for the atrial hypertrophic cellular response, characterized by an increased expression of cTnI, ANP, and β-MHC. Importantly, we were able to reproduce the observed findings in fibrillating atria using an *ex vivo* culture model of human atrial tissue slices. In this tissue model, treatment with FK506 prevented the tachycardia-induced hypertrophic response.

Calcineurin-NFATc signaling and myocardial hypertrophy. Calcineurin (protein phosphatase 2B, PP2B) is a calcium-activated Ser-Thr phosphatase composed of a catalytic A subunit (59–63 kDa) and a regulatory B subunit (19 kDa) [15]. Three catalytic genes (A subunit) have been identified, of which CnAα and CnAβ are present in the heart [32]. The induction of cardiac hypertrophy has been associated with an increase in CnAβ (but not in CnAα) expression [32, 33]. An elevation in the intracellular calcium concentration leads to calmodulin saturation and the subsequent activation of calcineurin [34]. Activated calcineurin binds directly to NFAT transcription factors located in the cytoplasm and dephosphorylates NFAT allowing NFAT nuclear import [15, 35]. Five NFATs have been identified, of which NFATc1–4 are regulated by calcineurin-mediated dephosphorylation. Previous data imply, however, that NFATc3 has a pivotal role in regulating the hypertrophic program [36]. For the transactivation of hypertrophic marker genes e.g. ANP [17] and β-MHC [16, 18, 19, 22], NFATs are known to cooperate with other transcription factors such a GATA-4 [36, 37] and MEF-2 [38]. Recent data showed that NFAT binds to and regulates transcription of the cTnI gene, and thereby plays a critical role in the structural architecture of developing myocardium. Interestingly, inhibition of NFAT transcriptional activity during cardiac de-

velopment causes thinning of the atrial myocardium [39]. In patients with CAF we observed an increase in the mRNA expression of NFATc3 (fig. 2). In addition, an increase in nuclear NFATc3 protein is evident for both phosphorylated and hypophosphorylated NFATc3. As a consequence, there is a shift in the balance between nuclear and cytosolic NFATc3, which leads to higher levels of NFATc3 within the nucleus. As NFATc3 is rephosphorylated in the nucleus and then exported into the cytoplasm, there is also more phosphorylated NFATc3 in the nucleus in patients with CAF. A theoretical model of this scenario has been recently outlined [40].

In addition to different models of cardiac hypertrophy [for reviews see refs. 15, 41, 42], the calcineurin-NFAT signaling pathway has been associated with pacing-induced hypertrophy [43–45]. First studies that used the electrical stimulation of neonatal rat ventricular myocytes have demonstrated dramatic increases in cellular size and a five- to tenfold increase in ANP after 3 days pacing [31]. At that time it was suggested that the cellular changes are associated with increased calcium influx and calmodulin activity. Recently, Xia et al. [22] provided evidence that NFATc4-GATA4-MEF2 signaling is involved in the pacing-induced stimulation of β-MHC and adenylosuccinate synthetase (Adss1) gene expression in neonatal ventricular cardiomyocytes. This effect could be prevented by cyclosporine A, an inhibitor of calcineurin, suggesting that the activation of calcineurin is involved in the signaling cascade.

These previous results are in full accordance with the data of the present study. To the best of our knowledge, our study is the first to analyze the calcineurin signaling cascade in fibrillating human atrial tissue. Remarkably, *ex vivo* pacing of human atrial slices resulted in significant activation of calcineurin signaling and induction of β-MHC and cTnI genes. There has been one study which investigated the CnA-NFAT pathway in atrial tissue in a porcine model of AF [46]. In that study, increased cNA activity with subsequent NFATc3 and NFATc4 translocation into nuclei were demonstrated after 4 weeks of rapid atrial pacing. However, the functional consequences of NFAT translocation, especially with regard to hypertrophic changes, were not assessed.

AF-induced myocardial hypertrophy. AF is known to induce a cellular calcium overload due to an increased influx of calcium through L-type calcium channels [5, 9]. Increased levels of intracellular calcium during AF have been found to activate calcium-dependent proteases, such as calpain, which appears to cleave several structural cellular proteins [10]. In addition to these structural cellular changes, morphologic studies in animal and human fibrillating atrial tissue have uniformly revealed the presence of hypertrophied atrial myocytes [6–9]. The pathophysiologic mechanisms responsible for the AF-induced cellular hypertrophy have not been elucidated so far. Therefore, the present study is the first to analyze the link between the AF-induced cellular calcium overload and atrial hypertrophy. The presented regulation of the calcium-dependent CnA signaling closes an important gap in our knowledge about the molecular mechanisms involved in structural remodeling of atrial myocytes in patients with atrial tachyarrhythmias. Nevertheless, activation of other, primarily Ca-independent, hypertrophic pathways may also contribute to the development of atrial hypertrophy. This is supported by the observed increase in MAP-kinase Erk2 activation in this study. MAP kinase-dependent cellular effects are induced by several signaling cascades. For example, peptide hormones like angiotensin II or endothelin-1 may modulate the tachycardia-dependent hypertrophic response *in vivo*, in particular in patients with AF and left ventricular dysfunction [9, 15, 24].

The impact of cellular hypertrophy on arrhythmogenesis is supported by a recent study demonstrating that an increase in cell size is of extraordinary importance for anisotropic conduction properties in cardiac tissue. Spach et al. [11] have clearly shown that cell size may be even more important than gap junction distribution for explaining the different transverse and longitudinal propagation V_{max}. Therefore, the increase in cell size of atrial myocytes may contribute to the development of an arrhythmogenic substrate, which allows AF to persist. In addition, CnA-dependent alterations in tissue architecture and chamber geometry, which are supported by the significant correlation between NFATc3 expression and left atrial diameter, may further contribute to an impaired mechanical function of fibrillating atria, and such cellular changes might thereby increase the risk of thromboembolic events in these patients [12–14, 24, 47].

The antihypertrophic effects of FK506 in the present study suggest a potential therapeutic approach in patients with CAF, although the clinical potential is limited due to the immunosuppressive action of FK506 [15]. Of note, first reports suggest that the combination of free radical scavenger drugs (EGb 761) and low-dose FK506 has antiarrhythmic effects [48]. Thus, studies are warranted to analyze the effect of long-term FK506 or cyclosporine A (CsA) therapy on the incidence of AF in organ-transplanted patients, and to analyze the effect of low-dose FK506/CsA treatment regimens to prevent AF-induced atrial alterations.

Culturing tissue slices. Our knowledge about functional cellular changes during atrial arrhythmia is limited because repetitive atrial biopsies are not feasible *in vivo*. Henry McIlwain and coworkers have introduced the *ex vivo* culture technique of tissue slices [49, 50]. Initially, brain tissue was cultured, which is also very sensitive to hypoxia. However, the culture technique is meanwhile ac-

cepted to keep tissue slices viable up to 10 days [27, 51, 52]. Similarly, murine ventricular tissue slices (300 µm thick) can be cultured for hours, showing stable oxygen consumption [28]. Furthermore, electrical pacing has been used to study electromechanical coupling in such slices, demonstrating reproducible and consistent electrical capture of tissue slices *in vitro* [28]. In the present study, adult human atrial tissue slices (mean patient age was 69 years) could be cultured for several days. Tissue viability was confirmed using established vital staining techniques.

In addition to the previously reported effects of electrical pacing *in vitro* [27–31], we were able to reproduce the previous pacing experiments using human atrial tissue slices. The pacing technology used in this study is in accordance with these previous studies. High pacing rates have been found to induce poisoning of the culture medium due to the generation of free radicals and polarization effects around the electrodes. This is a limitation of the *ex vivo* pacing model; high atrial rates observed during AF *in vivo* cannot be reproduced 1:1 *ex vivo*. However, pacing at 2 Hz reproduced the molecular alterations found in atrial tissue from patients with CAF and tissue viability could be proven. In addition, pacing at higher frequency (4.0 Hz) did not lead to stronger or even conflicting effects. Thus, the presented culture model is the first to set up *ex vivo* experiments to address the functional role of different signaling pathways in human atrial tissue during induced tachycardia. This is especially true as our findings indicate that cellular and molecular responses *ex vivo* largely mirror the pathophysiologic alterations observed *in vivo*, and therefore, tissue culture models allowing the maintenance of the physiological interaction of different cell types may have substantial advantages compared with cultures of isolated cardiomyocytes.

Acknowledgements. We thank Katja Mook for excellent technical assistance. This work was supported by grants from the 'Kultusministerium des Landes Sachsen-Anhalt, Germany' (3517 A/0603M) and by the 'Bundesministerium für Bildung und Forschung, Germany' (Kompetenznetz Vorhofflimmern, grant 01GI0204).

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