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Ca^{2+}/calmodulin-dependent protein kinase II equally induces sarcoplasmic reticulum Ca^{2+} leak in human ischaemic and dilated cardiomyopathy

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Aims The sarcoplasmic reticulum (SR) Ca^{2+} leak is an important pathomechanism in heart failure (HF). It has been suggested that Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is only relevant for the induction of the SR Ca^{2+} leak in non-ischaemic but not in ischaemic HF. Therefore, we investigated CaMKII and its targets as well as the functional effects of CaMKII inhibition in human ischaemic cardiomyopathy (ICM, $n = 37$) and dilated cardiomyopathy (DCM, $n = 40$).

Methods and results Western blots showed a significantly increased expression (by $54 \pm 9\%$) and autophosphorylation at Thr286 (by $129 \pm 29\%$, $P < 0.05$ each) of CaMKII in HF compared with healthy myocardium. However, no significant difference could be detected in ICM compared with DCM as to the expression and autophosphorylation of CaMKII nor the phosphorylation of the target sites ryanodine receptor 2 (RyR2)-S2809, RyR2-S2815, and phospholamban-Thr17. Isolated human cardiomyocytes (CMs) of patients with DCM and ICM showed a similar frequency of diastolic Ca^{2+} sparks (confocal microscopy) as well as of major arrhythmic events (Ca^{2+} waves, spontaneous Ca^{2+} transients). Despite a slightly smaller size of Ca^{2+} sparks in DCM ($P < 0.01$), the calculated SR Ca^{2+} leak [Ca^{2+} spark frequecy (CaSpF) × amplitude × width × duration] did not differ between CMs of ICM vs. DCM. Importantly, CaMKII inhibition by autocamide-2-related inhibitory peptide (AIP, 1 μmol/L) reduced the SR Ca^{2+} leak by ∼80% in both aetiologies ($P < 0.05$ each) and effectively decreased the ratio of arrhythmic cells ($P < 0.05$).

Conclusion Functional and molecular measures of the SR Ca^{2+} leak are comparable in human ICM and DCM. CaMKII is equally responsible for the induction of the 'RyR2 leakiness' in both pathologies. Thus, CaMKII inhibition as a therapeutic measure may not be restricted to patients suffering from DCM but rather may be beneficial for the majority of HF patients.

Keywords CaMKII • SR Ca^{2+} leak • Heart failure • Arrhythmias

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Introduction

It has been shown in the past that the expression of the δ-isoform of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is increased in human failing myocardium compared with non-failing control.[1,2] The resulting increase of CaMKII activity was proposed to account for the disturbed Ca^{2+} cycling properties present in failing myocardium.[2−4] Indeed, CaMKII-dependent phosphorylation of ryanodine receptor 2 (RyR2) at S2815 increases the likelihood of spontaneous diastolic openings of RyR2 clusters, leading to locally restricted Ca^{2+} release events (Ca^{2+} sparks).[5−8] The resulting diastolic Ca^{2+} loss [sarcoplasmic reticulum (SR) Ca^{2+} leak] compromises SR Ca^{2+} storage[5] and thus contractility, and can further trigger cardiac arrhythmias,[9−11] as the extrusion of spontaneously released Ca^{2+} via the Na^+/Ca^{2+} exchanger (NCX) in exchange for three Na^+ ions results in a net inward current and delayed afterdepolarizations.[12] It has been shown conclusively that an inhibition of CaMKII can improve contractility in human failing myocardium.[2] Thus, an inhibition of CaMKII could possibly integrate antiarrhythmic and inotropic therapy in heart failure (HF) and constitutes a promising approach for future therapies. However, most of the currently available data are derived from non-ischaemic animal HF models,[9,13−16] from a mixed population of human HF[2] or studies limited to human idiopathic dilated cardiomyopathy.[1,3] A recent study proposed that CaMKII-dependent SR Ca^{2+} leak may only be relevant in non-ischaemic but not in ischaemic HF[17] as mice harbouring a mutated CaMKII-dependent phosphorylation site at RyR2 (S2814A) were protected against HF resulting from trans-aortic constriction (TAC) but not myocardial infarction.[17] This would have a huge clinical impact as the therapeutic potential of CaMKII inhibition would be limited to patients with non-ischaemic HF and skip patients with underlying cardiac ischaemia. Of note, the prevalence of CAD in patients with HF was estimated to be up to 68%.[18] Therefore, the aim of the present study was to investigate a possible differential role for CaMKII-dependent RyR2 phosphorylation and its functional consequences in human ischaemic cardiomyopathy (ICM) vs. dilated cardiomyopathy (DCM).

Methods

Human myocardial tissue

All procedures were conducted in compliance with the local ethics committee, and written informed consent was received from all participants prior to inclusion.

Left ventricular myocardial tissue was taken from explanted hearts of 77 patients (ICM, $n = 37$; DCM, $n = 40$) with end-stage HF (NYHA HF classification IV) undergoing heart transplantation. The explanted hearts were acquired directly in the operating room during surgical procedures and immediately placed in pre-cooled cardioplegic solution (in mmol/L: NaCl 110, KCl 16, $MgCl_2$ 16, $NaHCO_3$ 16, $CaCl_2$ 1.2, glucose 11). Myocardial samples for western blot analysis were frozen in liquid nitrogen and stored at −80 °C immediately after excision. The remaining heart tissue was stored for cell isolation in cooled cardioprotective solution containing (in mmol/L): Na^+ 156, K^+ 3.6, Cl^- 135, HCO_3^- 25, Mg^{2+} 0.6, $H_2PO_4^-$ 1.3, SO_4^{2-} 0.6, Ca^{2+} 2.5, glucose 11.2, and 2,3-butanedionmonoxime (BDM) 10, aerated with 95% O_2 and 5% CO_2. Healthy myocardium ($n = 8$) from healthy organ donors was used as a control in western blots.

Western blots

Ventricular tissue samples were homogenized in Tris buffer containing (mmol/L): 20 Tris−HCl, 200 NaCl, 20 NaF, 1 Na_3VO_4, 1 dithiothreitol (DTT), 1% Triton X-100 (pH 7.4), and complete protease inhibitor cocktail (Roche Diagnostics). Protein concentration was determined by BCA (bicinchoninic acid) assay. The samples were denaturated at 37 and 95 °C. The homogenates were subjected to electrophoresis on 5% acrylamide gels for RyR2, 8% acrylamide gels for CaMKII, and 13% acrylamide gels for phospholamban (PLB). Membranes were probed with anti-RyR2 (1:10 000; Sigma-Aldrich, rabbit), anti-phospho-S2814-RyR2 (1:5000; Badrilla, rabbit), anti-phospho-S2808-RyR2 (1:5000; Badrilla, rabbit), anti-CaMKII (1:12 000; gift from D.M. Bers, University of California, Davis, CA, USA, rabbit), anti-phospho-CaMKII (1:1000; Thermo Scientific, mouse), anti-PLB (1:10 000, Millipore, mouse), anti-phospho-Thr17-PLB (1:10 000, Badrilla, rabbit), and anti-glyceraldehyde phosphate dehydrogenase (GAPDH; 1:50 000; Biotrend, mouse) antibodies overnight at 4 °C. Phospho-CaMKII antibody was stripped from the membrane, followed by incubation with the primary antibody against CaMKII. The membrane stripping was performed using washing steps with Tris-buffered saline and 200 mmol/L NaOH followed by incubation with 5% non-fat dry milk for 1 h at room temperature. Membranes were then incubated with secondary antimouse and antirabbit antibodies (GE Healthcare). Chemiluminescent detection was done with Immobilion Western (Millipore).

Myocyte isolation

Human myocardium was rinsed, cut into small pieces, and incubated at 37 °C in a spinner flask filled with Joklik-MEM solution (JMEM; AppliChem, Darmstadt, Germany) containing 1.0 mg/mL collagenase (Worthington type 1, 185 U/mg) and trypsin (2.5 g/L, Life Technologies) as described before.[19] After 45 min, the supernatant was discarded and fresh JMEM solution containing only collagenase was added. The solution was incubated for 10−20 min until myocytes were disaggregated using a Pasteur pipette. The supernatant containing disaggregated cells was removed and centrifuged (600 r.p.m., 3 min). Fresh JMEM with collagenase was added to the remaining tissue. This procedure was repeated 4−5 times. After every step, the centrifuged cells were resuspended in KB medium containing (mmol/L): taurine 10, glutamic acid 70, KCl 25, KH_2PO_4 10, dextrose 22, EGTA 0.5, bovine calf serum 10% (pH 7.4, KOH, room temperature). Only cell solutions containing elongated, non-granulated cardiomyocytes (CMs) with cross-striations were selected for experiments, plated on laminin-coated recording chambers, and left to settle for 1 h. Representative light microscopic pictures of isolated human CMs as well as confocal two-dimensional scans of CMs stained with Fluo-3 are shown in Supplementary material online, *Figure S1*.

Intracellular Ca^{2+} imaging

Confocal microscopy (measurement of sarcoplasmic reticulum Ca^{2+} sparks)

Cell staining and the detection of diastolic Ca^{2+} sparks were performed as described before.[19] Isolated CMs were incubated at room

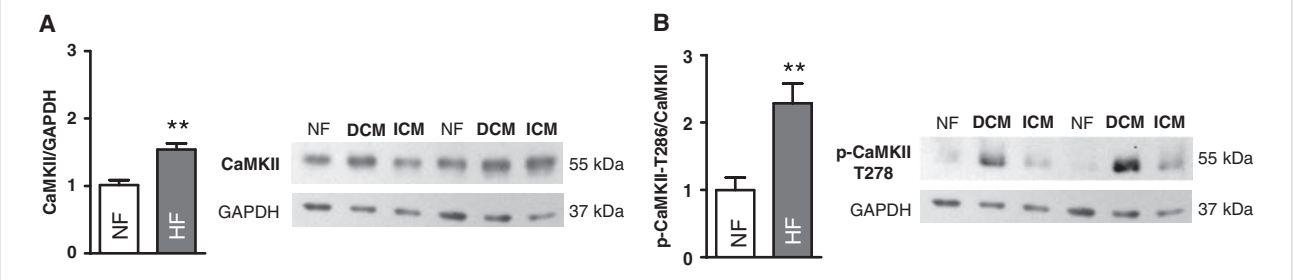

Figure 1 Expression and autophosphorylation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) in healthy [non-failing (NF), $n = 8$] and end-stage failing human myocardium [heart failure (HF), $n = 8$ dilated cardiomyopathy (DCM) + 8 ischaemic cardiomyopathy (ICM). (A) Representative western blots (right) and the respective quantification (left) show an increased expression of CaMKII in HF compared with healthy control ($P < 0.01$), which is accompanied by (B) an increased autophosphorylation of CaMKII at Thr286 (normalized to CaMKII expression, $P < 0.01$). GAPDH, glyceraldehyde phosphate dehydrogenase.

temperature for 30 min with a Fluo-3 AM loading buffer (10 μmol/L, Molecular Probes) which also contained the CaMKII inhibitor autocamide-2-related inhibitory peptide (AIP, 1 μmol/L, Alexis Corp, Switzerland) or no active agent in the control group. AIP was used in its myristoylated form to ensure cell permeability. Experimental solution contained (mmol/L): NaCl 136, KCl 4, NaH_2PO_4 0.33, $NaHCO_3$ 4, $CaCl_2$ 2, $MgCl_2$ 1.6, HEPES 10, glucose 10 (pH 7.4, NaOH, room temperature) and the respective active agent. Cells were continuously superfused during experiments. To wash out the loading buffer and remove any extracellular dye as well as to allow enough time for complete deesterification of Fluo-3 AM, cells were superfused with experimental solution for 5 min before experiments were started. Ca^{2+} spark measurements were performed with a laser scanning confocal microscope (LSM 5 Pascal, Zeiss) using a × 40 oil-immersion objective. Fluo-3 was excited by an argon ion laser (488 nm) and emitted fluorescence was collected through a 505 nm long-pass emission filter. Fluorescence images were recorded in the line-scan mode with 512 pixels per line (width of each scanline: 38.4 μm) and a pixel time of 0.64 μs. One image consists of 10 000 unidirectional line scans, equating to a measurement period of 7.68 s. Experiments were conducted under resting conditions after loading the SR with Ca^{2+} by repetitive field stimulation (10 beats at 1 Hz, 20 V). Ca^{2+} sparks were analysed with the program SparkMaster for ImageJ.[20] The mean spark frequency of the respective cell (CaSpF) resulted from the number of sparks normalized to cell width and scan rate (100 μm/s). Spark size (CaSpS) was calculated as the product of spark amplitude (F/F0), duration, and width. From this, we inferred the average leak per cell by multiplying CaSpS by CaSpF.

Cardiomyocytes showing major arrhythmic events (Ca^{2+} waves, spontaneous Ca^{2+} transients, spark clouds) were excluded from the quantification of the SR Ca^{2+} leak and classified as arrhythmic cells. The portion of arrhythmic cells out of all measured cells, the frequency of arrhythmic events, and the duration between the last stimulated Ca^{2+} transient and the occurrence of the first arrhythmic event (latency) were quantified and compared between groups.

Statistics

All data are presented as the mean ± standard error of the mean (SEM). Student's *t*-test was used for statistical analysis of all data sets except those of *Figure 3H*, for which Fisher's exact test was used. Values of $P < 0.05$ were considered as statistically significant.

Results

Expression and activity of Ca^{2+}/calmodulin-dependent protein kinase II in human healthy myocardium and heart failure

As we sought to analyse CaMKII-dependent effects on the disruption of Ca^{2+} homeostasis in human HF, we first evaluated the expression and activating autophosphorylation (Thr286) of CaMKII in human failing (HF, $n = 8$ ICM + 8 DCM) and healthy myocardium (NF, $n = 8$). Indeed, we found a robustly increased expression of CaMKII in human end-stage HF compared with NF (*Figure 1A*, increase by 54 ± 9%, $n = 16$ vs. 8, $P < 0.01$), which was accompanied by an even more pronounced increase of CaMKII autophosphorylation in HF (*Figure 1B*, increase by 129 ± 29%, $n = 16$ vs. 8, $P < 0.01$).

Ca^{2+}/calmodulin-dependent protein kinase II expression, activity, and respective target phosphorylation in human ischaemic and dilated cardiomyopathy

To detect differences in CaMKII-dependent target phosphorylation in different aetiologies of HF, we performed western blots from human myocardium of ICM and DCM patients. The characteristics of the two groups of patients as well as their medication are annotated in *Table 1*. Surprisingly, neither the expression of CaMKII (*Figure 2A*, normalized to GAPDH, $n = 20$ vs. 21, $P = 0.92$) nor its autophosphorylation at Thr286 (*Figure 2B*, p-CaMKII/CaMKII, $n = 20$ vs. 21, $P = 0.22$) were significantly varied in ICM vs. DCM. Conclusively, we did not detect any significant differences in the phosphorylation of RyR2 at the CaMKII-dependent site Ser2815 (*Figure 2D*, $n = 10$ vs. 19, $P = 0.58$) nor at Ser2809 (*Figure 2E*, $n = 10$ vs. 19, $P = 0.56$), which is known to be mainly phosphorylated by protein kinase A (PKA). The expression of RyR2 was also not different in both aetiologies (*Figure 2C*, $n = 10$ vs. 19, $P = 0.64$). Furthermore, the expression of PLB (*Figure 2F*, $n = 18$ vs. 20,

Table 1 Patient characteristics and the respective medication in the group of human ischaemic cardiomyopathy and dilated cardiomyopathy

	ICM ($n = 37$)	DCM ($n = 40$)
Male sex (%)	100.0	90.0
Age (mean ± SEM, years)	56.9 ± 0.96	53.5 ± 0.96
EF (mean ± SEM, %)	23.4 ± 1.4	22.8 ± 1.31
Ischaemic heart disease (%)	100.0	0.0
LVAD (%)	54.1	47.5
Diabetes (%)	20.6	13.2
ACE inhibitors (%)	72.7	63.2
Beta-blockers (%)	93.9	84.2
Diuretics (%)	100.0	89.5
Digoxin (%)	21.2	34.2
Catecholamines (%)	11.8	13.2
Amiodarone (%)	39.4	50.0
AT_1 receptor antagonists (%)	15.2	10.5
Aldosterone antagonists (%)	63.6	73.7
PDE inhibitors (%)	36.4	42.1
Ca^{2+} channel blockers (%)	0.0	2.6

Values are mean ± SEM or percentages.
Of note, patient characteristics of non-failing heart donors are commonly not available due to ethical reasons.
AT_1, angiotensin II receptor type 1; DCM, dilated cardiomyopathy; ICM, ischaemic cardiomyopathy; LVAD, left ventricular assist device; PDE, phosphodiesterase; SEM, standard error of the mean.

$P = 0.62$) as well as its phosphorylation at the CaMKII-dependent site Thr17 were shown to be equal in ICM and DCM (*Figure 2G*, $n = 18$ vs. 20, $P = 0.84$).

Diastolic sarcoplasmic reticulum Ca^{2+} leak in ischaemic and non-ischaemic human cardiomyopathy

To extend our western blot analysis that showed an equal phosphorylation status of key Ca^{2+} handling proteins in human ICM and DCM, we additionally performed functional experiments. We freshly isolated human CMs from both groups of patients and scanned for diastolic SR Ca^{2+} sparks (confocal microscopy, Fluo 3 AM). We found a weak trend towards higher CaSpF in DCM vs. ICM, which was not statistically significant (*Figure 3B*, CaSpF $1.01 \pm 0.13/s/10^{-4}$ m vs. $0.78 \pm 0.15/s/10^{-4}$ m, *n* cells/patients = 150/8 vs. 106/8, $P = 0.24$). However, the size of Ca^{2+} sparks was lower in CMs from DCM compared with ICM as detected sparks had a shorter duration (*Figure 3E*, DCM 23.7 ± 0.7 ms vs. ICM 27.2 ± 1.1 ms, *n* sparks/patients = 375/8 vs. 188/8, $P < 0.01$) and were narrower (*Figure 3D*, width DCM $2.0 \pm 0.05 \times 10^{-4}$ m vs. ICM $2.3 \pm 0.09 \times 10^{-4}$ m, $n = 375/8$ vs. 188/8, $P < 0.01$). Furthermore, the amplitude of detected sparks was slightly smaller in DCM compared with ICM (*Figure 3C*, $n = 375/8$ vs. 188/8, $P < 0.01$). This translated into a total calculated SR Ca^{2+} leak (=CaSpF × amplitude × width × duration) that was equal in both aetiologies of human HF (*Figure 3F*, *n* cells/patients = 106/8 vs. 150/8, $P = 0.91$).

We additionally quantified the occurrence of arrhythmic events (Ca^{2+} waves, spontaneous Ca^{2+} transients, spark clouds, see *Figure 3G*) to evaluate the arrhythmic burden in both aetiologies. As shown in *Figure 3H*, the fraction of CMs showing arrhythmic events did not significantly differ in both groups (24 out of 130 in ICM vs. 52 out of 202 in DCM, $P = 0.14$). Accordingly, the frequency of arrhythmic events was similar in ICM and DCM (*Figure 3I*, frequency = $0.65 \pm 0.09/s/10^{-4}$ m vs. $0.50 \pm 0.11/s/10^{-4}$ m, *n* cells/patients = 130/8 vs. 202/8, $P = 0.30$). As the time point of the onset of arrhythmic events also serves as a measure for the disposition towards arrhythmias, we evaluated the latency between the last stimulated Ca^{2+} transient and the occurrence of the first arrhythmic event. As shown in *Figure 3K*, no difference could be detected between both groups (latency = 0.77 ± 0.14 s vs. 0.77 ± 0.33 s, *n* cells/patients = 24/8 vs. 52/8, $P = 0.99$).

Effects of Ca^{2+}/calmodulin-dependent protein kinase II inhibition on diastolic sarcoplasmic reticulum Ca^{2+} leak in human dilated cardiomyopathy

To evaluate the contribution of CaMKII to the disruption of diastolic RyR2 closure in human DCM, we used the CaMKII inhibitor AIP (1 μmol/L). CMs treated with AIP showed a reduced CaSpF (*Figure 4B*, CaSpF AIP $0.42 \pm 0.11/s/10^{-4}$ m vs. control $0.84 \pm 0.15/s/10^{-4}$ m, *n* cells/patients = 54/4 vs. 84/3, $P < 0.05$) compared with untreated control. Furthermore, the width (*Figure 4D*, AIP $1.58 \pm 0.09 \times 10^{-4}$ m vs. control $2.00 \pm 0.08 \times 10^{-4}$ m, *n* sparks/patients = 61/3 vs. 181/3, $P < 0.01$) as well as spark duration (*Figure 4E*, AIP 16 ± 1.0 ms vs. control 23 ± 0.8 ms, $n = 61/3$ vs. 181/3, $P < 0.001$) were decreased. The amplitude of Ca^{2+} sparks was not affected by CaMKII inhibition (*Figure 4C*, $n = 61/3$ vs. 181/3, $P = 0.74$). In sum, CaMKII inhibition yielded a robust reduction of the calculated SR Ca^{2+} leak in human DCM by $85 \pm 6\%$ (*Figure 4F*, *n* cells/patients = 54/3 vs. 84/3, $P < 0.05$). Conclusively, the frequency of arrhythmic events (Ca^{2+} waves/spontaneous contractions) could also be reduced by CaMKII inhibition with AIP (*Figure 4G*, *n* cells/patients = 66/3 vs. 108/3, reduction by $56 \pm 12\%$, $P < 0.05$).

Effects of Ca^{2+}/calmodulin-dependent protein kinase II inhibition on diastolic sarcoplasmic reticulum Ca^{2+} leak in human ischaemic cardiomyopathy

Similar observations could be made for human ICM. CaMKII inhibition with AIP resulted in a tendency towards a lower CaSpF (*Figure 5B*, AIP $0.31 \pm 0.07/s/10^{-4}$ m vs. control $0.55 \pm 0.12/s/10^{-4}$ m, *n* cells/patients = 75/3 vs. 85/3, $P = 0.10$) and significantly decreased spark amplitude (*Figure 5C*, *n* sparks/patients = 57/3 vs. 106/3, $P < 0.001$), width (*Figure 5D*, AIP $1.5 \pm 0.09 \times 10^{-4}$ m vs. $2.3 \pm 0.11 \times 10^{-4}$, $n = 57/3$ vs. 106/3,

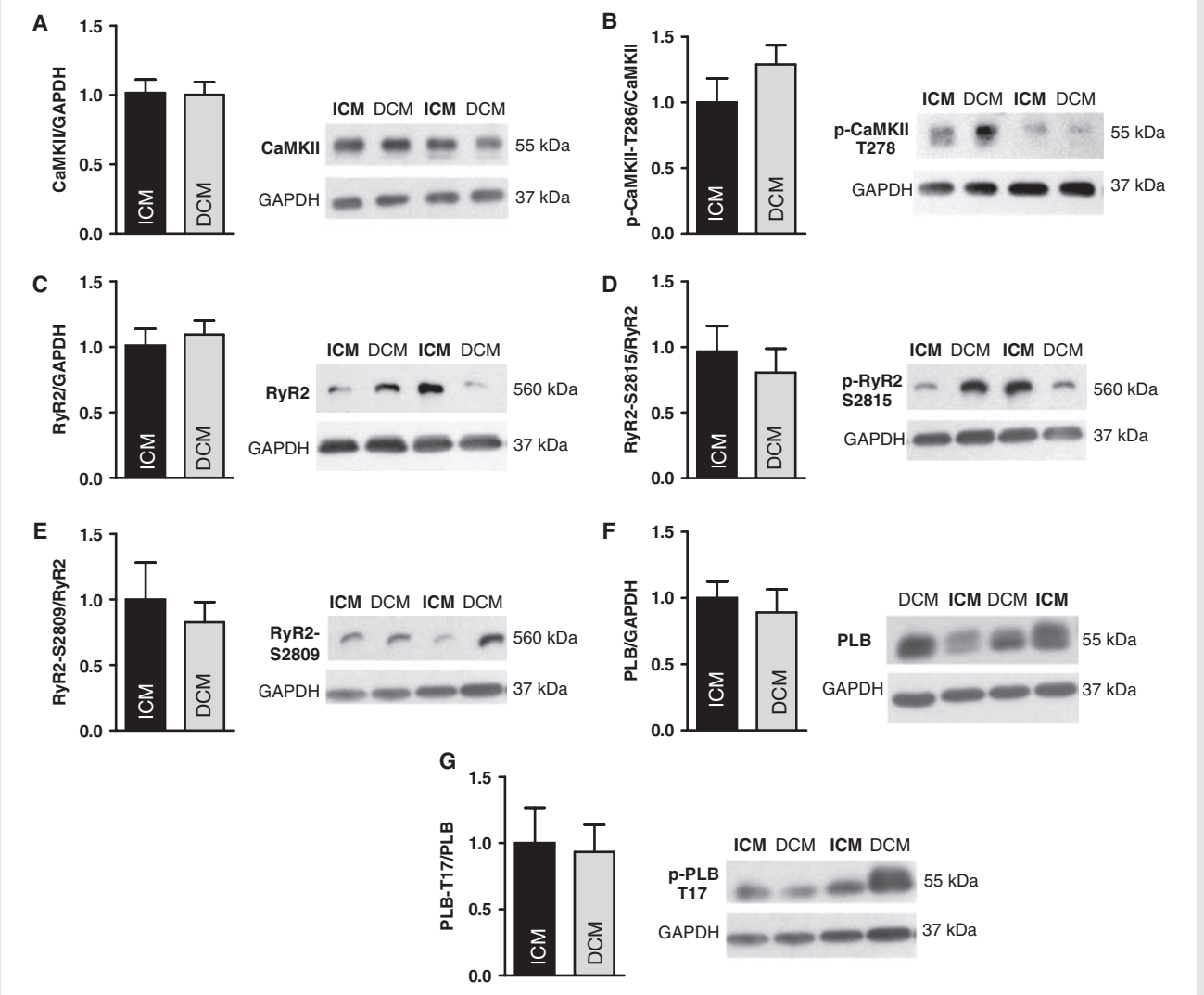

Figure 2 Comparison of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) expression and target phosphorylation between human ischaemic (ICM) and dilated (DCM) cardiomyopathy. Each figure shows representative western blots on the right and the respective quantification on the left. Neither CaMKII expression (A, $n = 20/21$) nor its autophosphorylation (B, $n = 20/21$) were significantly different in ICM vs. DCM. Additionally, ryanodine receptor 2 (RyR2) was found to be equally expressed (C, $n = 10/19$) and equally phosphorylated at Ser2815 (D, normalized to RyR2 expression, $n = 10/19$) and Ser2809 (E, normalized to CaMKII expression, $n = 10/19$) in both groups. We also did not detect a different expression of phospholamban (PLB, F) or a different phosphorylation at Thr17 (*G*, normalized to PLB expression, $n = 18/20$). GAPDH, glyceraldehyde phosphate dehydrogenase

$P < 0.001$) and duration (*Figure 5D*, AIP 18.0 ± 1.2 ms vs. control 26.6 ± 1.4 ms, $n = 57/3$ vs. 106/3, $P < 0.001$). Most importantly, this translated into a reduction of the total SR Ca^{2+} leak by $80 \pm 5\%$ (n cells/patients = 75/3 vs. 85/3, $P < 0.05$) and into a robust reduction of arrhythmic events by $82 \pm 10\%$ in human ICM (n cells/patients = 79/3 vs. 95/3, $P < 0.05$).

Discussion

The current study reveals that (i) CaMKII expression and autophosphorylation are increased in human HF compared to healthy myocardium, but (ii) are similar in human ICM and DCM. (iii) This results in an equal phosphorylation pattern of the target proteins RyR2 (S2809, S2815) and PLB (T17) in ICM and DCM. Conclusively, (iv) the diastolic SR Ca^{2+} leak and the frequency of arrhythmic events are equal in both groups of human HF and (v) can be significantly attenuated by CaMKII inhibition with AIP. Thus, CaMKII inhibition as a therapeutic approach to treat contractile dysfunction and arrhythmias would have the same potential benefit in HF patients of both subgroups.

Our findings confirm several studies showing that CaMKIIδ expression[1] and its activity[3] are increased in human HF and that the resulting hyperphosphorylation of RyR2 at S2815 is an important pathomechanism in the development of an increased SR Ca^{2+}

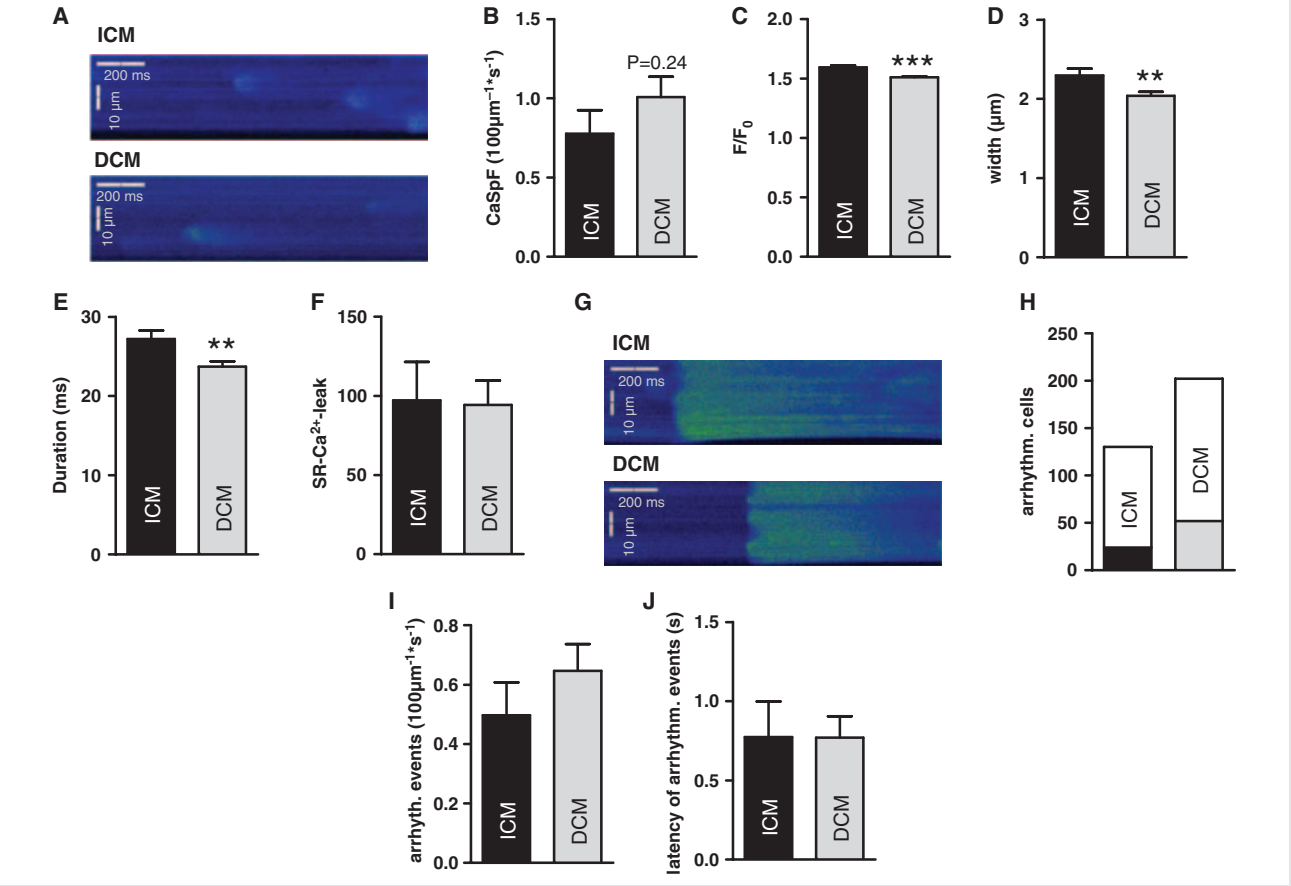

Figure 3 Quantification of the diastolic sarcoplasmic reticulum (SR) Ca^{2+} leak and the occurrence of arrhythmias in human dilated cardiomyopathy (DCM) and ischaemic cardiomyopathy (ICM). (*A*) Representative confocal line scans of cardiomyocytes (CMs) from both groups. (*B*) CMs from human DCM had a similar spark frequency (CaSpF) compared with ICM (*n* cells/patients = 150/8 vs. 106/8, $P = 0.24$). (*C*) The amplitude of Ca^{2+} sparks was slightly smaller in DCM (*n* sparks/patients = 375/8 vs. 188/8, $P < 0.01$). Furthermore, CMs from DCM had a smaller spark width (*D*, $n = 375/8$ vs 188/8, $P < 0.01$) and a significantly shorter spark duration (*E*, $n = 375/8$ vs. 188/8, $P < 0.01$). In sum, the total calculated SR Ca^{2+} leak was equal in both groups (*F*, CaSpF × amplitude × width × duration, $n = 150/8$ vs. 106/8, $P = 0.91$). (*G*) Representative confocal line scans of Ca^{2+} waves/spontaneous contractions in ICM and DCM. The ratio of arrhythmic CMs out of all measured cells (*H*, 24/130 vs. 52/202, $P = 0.14$) as well as the frequency of arrhythmic events (*I*, *n* cells/patients = 130/8 vs. 202/8, $P = 0.30$) was similar in both aetiologies. Furthermore, there was no difference as to the latency of the first arrhythmic event after stopping stimulation (*J*, *n* cells/patients = 24/8 vs. 52/8, $P = 0.99$).

leak.[2,5–8] Recently we could demonstrate that CaMKII inhibition can in fact improve contractility in human failing myocardium.[2] On the basis of these studies, CaMKII inhibition has been more and more perceived as a promising approach for future treatments of HF that would potentially integrate antiarrhythmic and inotropic properties. However, this concept has been questioned by a recent study suggesting that CaMKII-dependent dysregulation of RyR2 properties might only occur in non-ischaemic but not in ischaemic HF,[17] which would deny the majority of HF patients a possible benefit from CaMKII inhibition. Knock-in mice harbouring RyR2 with a mutated CaMKII-dependent phosphorylation site (S2814A) showed relative protection against HF development induced by TAC (increased afterload) but did not seem to be better off after ligating the LAD triggering ischaemic HF.[17] Our study shows that this conclusion that was mostly based on an elegant mouse model does not translate into human myocardium. The fact that the deletion of the main CaMKII-dependent phosphorylation site at RyR2 exerts protective effects against afterload-induced HF is in line with a previous study showing that CaMKII is required in the transition from pressure overload-induced hypertrophy to HF in mice.[16] Indeed, the crucial involvement of CaMKII-dependent SR Ca^{2+} leak induction in the development of HF and arrhythmias has mostly been demonstrated in non-ischaemic animal models[9,14,16] due to their better feasibility. The involvement of CaMKII in the development of HF due to ischaemia is far less obvious. However, a recent study performed in a mouse model of ischaemia–reperfusion (I/R) showed that autonomous CaMKIIδ activitiy increases shortly after I/R damage and leads to a hyperphosphorylation of target sites at RyR2 and PLB additionally to changes in gene transcription mediated by

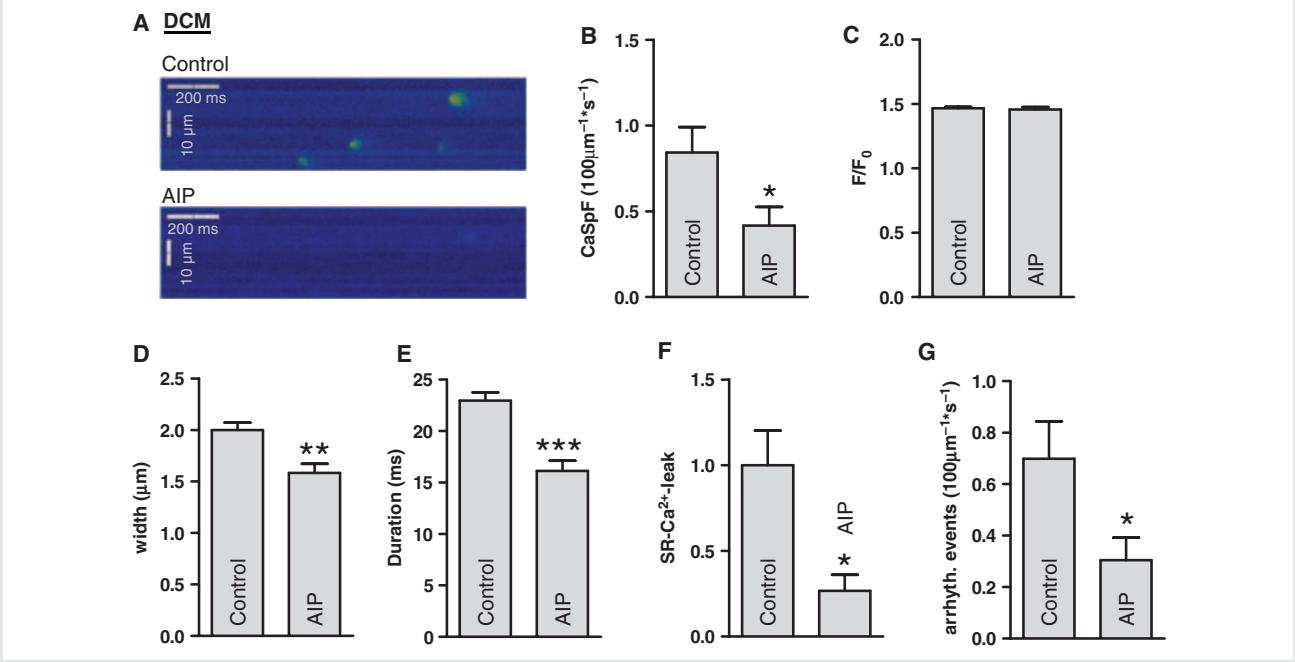

Figure 4 Effects of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) inhibition [autocamide-2-related inhibitory peptide (AIP)] on SR Ca^{2+} leak in human dilated cardiomyopathy (DCM). (*A*) Representative confocal line scans of untreated control cardiomyocytes (CMs; upper lane) and CMs treated with AIP (lower lane). (B) CaMKII inhibition significantly reduced diastolic Ca^{2+} spark frequency (CaSpF) compared with control (*n* cells/patients = 54/8 vs. 84/8, $P < 0.05$). The amplitude of Ca^{2+} sparks was not significantly altered (*C*, *n* sparks/cells = 61/3 vs. 181/3). Furthermore, spark width (*D*, $P < 0.01$) and duration (*E*, $P < 0.001$, $n = 61/3$ vs. 181/3 each) were decreased upon CaMKII inhibition. Thus, CaMKII inhibition yielded a robust reduction of the total calculated sarcoplasmic reticulum (SR) Ca^{2+} leak (*F*, CaSpF × amplitude × width × duration, *n* cells/patients = 54/3 vs. 84/3, $P < 0.05$) and conclusively also reduced the frequency of arrhythmic events (*G*, *n* cells/patients = 66/3 vs. 108/3, $P < 0.05$).

nuclear factor-κB (NF-κB).[21] Conclusively, CaMKII knockout mice showed protection against I/R-induced heart damage.[21] This study thus disagrees with an earlier study proposing that PKA and not CaMKII may be a critical mediator of HF progression after cardiac ischaemia,[22] as mice with a mutation of the PKA-dependent phosphorylation site at RyR2 (S2808A) were relatively protected against the development of HF after myocardial infarction. However, these results could not be confirmed by a later study using mouse lines with the same mutation (S2808A).[23] An inherent downside of animal models of cardiac ischaemia is that currently applied techniques may not validly mimic ischaemic heart disease found in patients with CAD. These patients develop sclerosis of coronary arteries over decades and often go through several myocardial infarctions until the ischaemic heart disease leads to overt HF. Furthermore, microvessels are equally affected and cause borderline blood supply to various regions in the heart. This situation differs significantly from animal models in which previously healthy hearts are infarcted by sudden ligation of the LAD. The fact that the genetic deletion of CaMKII-dependent RyR2 phosphorylation did not show any benefit in this context[17] cannot easily be transferred to human CAD. Furthermore, the myocardial infarctions induced by LAD ligation ranged from 30% to 60% of the total myocardium, which may in general be difficult to compensate for. Thus, comprehensive data from human HF myocardium subdivided into aetiology were needed to resolve this issue. To the best of our knowledge, our study is the first to combine western blot experiments (CaMKII expression, phosphorylation status of target proteins) with functional measurements (diastolic SR Ca^{2+} leak, effects of CaMKII inhibition) in different aetiologies of human HF. Previously available data from human HF were all derived from mixed populations of human failing myocardium[2,19] or from idiopathic dilated cardiomyopathy.[1,3] Our western blots of human myocardium show an increased expression and autophosphorylation of CaMKII in HF (ICM + DCM) compared with healthy myocardium but no significant differences between ICM and DCM as to the expression of CaMKII and the phosphorylation of its targets RyR2-S2809, RyR2-S2815, and PLB-T17. We intentionally included myocardial samples of a large number of patients to increase the validity of this approach. A previous study detected a significant hyperphosphorylation of RyR2 at S2815 compared with healthy myocardium only in DCM but not in ICM.[17] Of note, this study included 11 ICM and 6 control samples, and a tendency towards higher phosphorylation in ICM compared with non-failing samples could already be guessed, although this was not significant. Our functional measurements show equal ranges of the SR Ca^{2+} leak and a similar frequency of arrhythmic events in ICM vs. DCM, which corroborates our western blot data. Furthermore, the inhibition of CaMKII by AIP, which is widely used for this purpose, yielded a significant

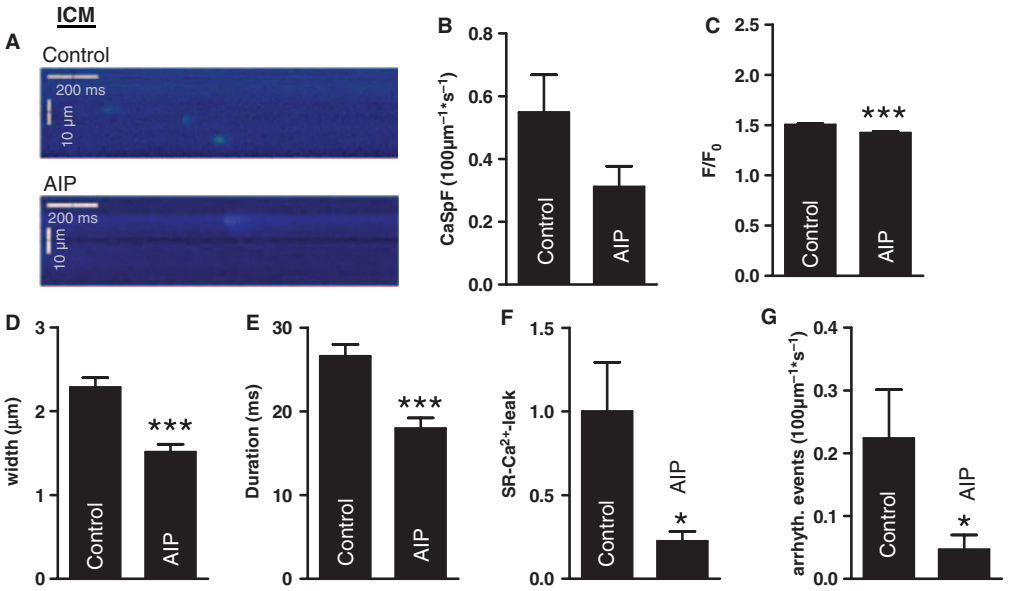

Figure 5 Effects of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) inhibition [autocamide-2-related inhibitory peptide (AIP)] on sarcoplasmic reticulum SR Ca^{2+} leak in human ischaemic cardiomyopathy (ICM). (*A*) Representative confocal line scans of untreated control cardiomyocytes (CMs; upper lane) and CMs treated with AIP (lower lane). CaMKII inhibition with AIP led to a tendency towards a lower Ca^{2+} spark frequency (CaSpF) compared with untreated control (*B*, *n* cells/patients = 75/3 vs. 85/3, $P = 0.10$) and significantly decreased spark amplitude (*C*), width (*D*), and duration (*E*, *n* sparks/patients = 57/3 vs. 106/3 and $P < 0.001$ each). This translated into a significantly decreased SR Ca^{2+} leak (*F*, CaSpF × amplitude × width × duration, n cells/patients = 75/3 vs. 87/3, $P < 0.05$) and a reduction of the frequency of arrhythmic events (*G*, *n* cells/patients = 79/3 vs. 95/3, $P < 0.05$).

reduction of the SR Ca^{2+} leak and effectively suppressed arrhythmias in both groups.

Our study thus demonstrates that the therapeutic value of CaMKII inhibition may not be restricted to non-ischaemic aetiologies of HF. This is especially important as many HF patients suffering from CAD and subsequent myocardial infarctions could also possibly benefit from CaMKII inhibition. However, as most of the patients included were male, further studies are needed to investigate possible gender-dependent differences in the development of the SR Ca^{2+} leak. Furthermore, the total number of patients in this experimental study is still relatively low. Clinical trials with large patient cohorts are needed to corroborate the positive effects of CaMKII inhibition on the inclination towards arrhythmias and the development of heart failure.

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Supplementary Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. (*A*) Representative bright field pictures (light microscopy, Zeiss, ×40) of isolated failing human ventricular cardiomyocytes. (*B*) Exemplary confocal 2D scans of human ventricular cardiomyocytes loaded with Fluo-3.

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