CaMKII-Dependent Diastolic SR Ca$^{2+}$ Leak and Elevated Diastolic Ca$^{2+}$ Levels in Right Atrial Myocardium of Patients With Atrial Fibrillation

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Rationale: Although research suggests that diastolic Ca$^{2+}$ levels might be increased in atrial fibrillation (AF), this hypothesis has never been tested. Diastolic Ca$^{2+}$ leak from the sarcoplasmic reticulum (SR) might increase diastolic Ca$^{2+}$ levels and play a role in triggering or maintaining AF by transient inward currents through Na$^+$/Ca$^{2+}$ exchange. In ventricular myocardium, ryanodine receptor type 2 (RyR2) phosphorylation by Ca$^{2+}$/calmodulin-dependent protein kinase (CaMK)II is emerging as an important mechanism for SR Ca$^{2+}$ leak.

Objective: We tested the hypothesis that CaMKII-dependent diastolic SR Ca$^{2+}$ leak and elevated diastolic Ca$^{2+}$ levels occur in atrial myocardium of patients with AF.

Methods and Results: We used isolated human right atrial myocytes from patients with AF versus sinus rhythm and found CaMKII expression to be increased by 40±14% (P<0.05), as well as CaMKII phosphorylation by 33±12% (P<0.05). This was accompanied by a significantly increased RyR2 phosphorylation at the CaMKII site (Ser2814) by 110% and 83% (P<0.05). This was accompanied by a significantly increased RyR2 phosphorylation at the CaMKII site (Ser2814) by 110% and 83% (P<0.05). Most likely, this resulted from an increased SR Ca$^{2+}$ leak in AF (P<0.05), which was not attributable to higher SR Ca$^{2+}$ load. Tetracaine experiments confirmed that SR Ca$^{2+}$ leak through RyR2 leads to the elevated diastolic Ca$^{2+}$ level. CaMKII inhibition normalized SR Ca$^{2+}$ leak and cytosolic Ca$^{2+}$ levels without changes in L-type Ca$^{2+}$ current.

Conclusion: Increased CaMKII-dependent phosphorylation of RyR2 leads to increased SR Ca$^{2+}$ leak in human AF, causing elevated cytosolic Ca$^{2+}$ levels, thereby providing a potential arrhythmogenic substrate that could trigger or maintain AF. (*Circ. Res. 2010;106:1134-1144.)

Key Words: Ca$^{2+}$/calmodulin-dependent protein kinase II ■ sarcoplasmic reticulum Ca$^{2+}$ leak ■ atrial fibrillation ■ Ca$^{2+}$ sparks ■ ryanodine receptor

Atrial fibrillation (AF) is the most frequent sustained arrhythmia in clinical practice.1 In AF, both structural and electrophysiological remodeling of the atrial myocardium occurs, leading to increased susceptibility to arrhythmias, thus perpetuating the condition (“AF begets AF”). The most prominent alteration is a shortening in action potential duration (APD),2 which is believed to favor reentry of excitation being largely attributable to a reduction of L-type Ca$^{2+}$ current ($I_{Ca,L}$).3,4 Although this reduces the Ca$^{2+}$ influx per excitation, the rate of excitation is increased, ie, the atrial heart rate during AF. There is evidence suggesting that cellular Ca$^{2+}$ overload may occur in AF and might play a role in its pathogenesis.5-9 Interestingly, acute rapid pacing induces increased cellular Ca$^{2+}$ loading in atrial myocytes from sinus rhythm dogs.10 Elevation of diastolic [Ca$^{2+}$] through increased sarcoplasmic reticulum (SR) Ca$^{2+}$ spark frequency is regarded as an arrhythmogenic mechanism in failing ventricular myocardium: Ca$^{2+}$ sparks are believed to participate as crucial events in the initiation and propagation of Ca$^{2+}$ waves in cardiac myocytes and the elimination of cytosolic Ca$^{2+}$ via the Na$^+$/Ca$^{2+}$ exchanger (NCX) generates a depolarizing current (transient inward current, $I_{Ti}$), which can give rise to delayed afterdepolarizations (DADs).11 Increased Ca$^{2+}$ influx attributable to the increased rate of $I_{Ca,L}$ activation in AF may contribute to the increased diastolic Ca$^{2+}$ levels, although $I_{Ca,L}$ itself is reduced. In addition, other causes for elevated diastolic Ca$^{2+}$ levels are possible. One major mechanism in failing ventricular myocardium is a Ca$^{2+}$ leak from the SR via the ryanodine receptor type 2 (RyR2). In fact, increased SR Ca$^{2+}$ spark frequency has been observed in patients with AF.
atrial myocytes from human AF patients, as well as increased RyR2 open probability in an AF animal model.\textsuperscript{12,13} The cause for this SR Ca\textsuperscript{2+} leak remained unknown.

It is suggested that hyperphosphorylation of the RyR2 is a mechanism causing “leakiness” of the channel.\textsuperscript{14–16} In AF, compartmentalization of kinase/phosphatase activity is presumed to occur and upregulation of inhibitor-1 is believed to reduce protein phosphatase (PP) activity at the SR, leading to enhanced phosphorylation levels of RyR2.\textsuperscript{13,17,18} PP activity along the sarcolemmal appears to be increased. Phospholamban (PLB) phosphorylation levels have been described both as increased as well as reduced.\textsuperscript{17,18} Mice in a model with hyperphosphorylation of PLB and RyR2 developed AF and other arrhythmias.\textsuperscript{19}

Ca\textsuperscript{2+}/calmodulin-dependent protein kinase (CaMKII) is a serine/threonine protein kinase that regulates several Ca\textsuperscript{2+}-handling proteins (eg, RyR2, PLB, L-type Ca\textsuperscript{2+} channel) and has been shown to be a central regulator of excitation–contraction coupling and a critical mediator of adrenergic signaling.\textsuperscript{20} SR Ca\textsuperscript{2+} leak is believed to be a possible mechanism for arrhythmia through I\textsubscript{f} and DADs. CaMKII-overexpressing mice display SR Ca\textsuperscript{2+} leak and are more susceptible to arrhythmias.\textsuperscript{20,21} CaMKII is activated in a frequency-dependent manner, and increased CaMKII expression was found in AF.\textsuperscript{14,22}

We hypothesized that increased CaMKII expression in AF leads to hyperphosphorylation of RyR2 at the CaMKII site, inducing SR Ca\textsuperscript{2+} leak and increased diastolic [Ca\textsuperscript{2+}]. We further speculated that this could be normalized by CaMKII inhibition thus reducing proarrrhythmic mechanisms in AF.

### Methods

#### Human Samples

Right atrial appendages from patients with AF (n=20) and in sinus rhythm (n=27) were obtained during coronary artery bypass or cardiac valve surgery. All procedures were in compliance with the ethical committee of the institution.

#### Cell Isolation

Obtained tissue was put into St. Thomas Hospital cardioplegic solution containing (mmol/L): 110 NaCl, 16 KCl, 16 MgCl\textsubscript{2}, 16 NaHCO\textsubscript{3}, 1.2 CaCl\textsubscript{2}, 11 glucose. Appendages were rinsed, cut into small pieces, and incubated (30 minutes) at room temperature with a Ca\textsuperscript{2+}-free solution containing 2 mg/mL collagenase (Worthington type 2, 290 U/mg) and 50 μg/mL protease (Sigma type XXIV, 9 μg/mL). Experimental solutions contained (mmol/L): 136 NaCl, 4 KCl, 0.33 NaH2PO\textsubscript{4}, 4 NaHCO\textsubscript{3}, 2 CaCl\textsubscript{2}, 1.6 MgCl\textsubscript{2}, 10 HEPES, 10 glucose (23°C, pH 7.4, NaOH), as well as KN-93 or KN-92. To wash out the loading buffer and remove any extracellular dyes, cells were washed with solution for 5 minutes before starting measurements.

Ca\textsuperscript{2+} spark measurements were performed with a laser-scanning confocal microscope (LSM 5 Pascal, Zeiss). Following preconditioning stimulation at 1 Hz, Fluo-3 was excited at 488 nm, and emitted fluorescence was collected through a 505 nm long-pass emission filter. Fluorescence images were recorded in line scan mode with 512 pixels per line (width of each scan line, 38.4 μm) pixel time 0.64 μs. Background fluorescence was corrected for. Ca\textsuperscript{2+} spark frequency was measured at resting conditions and normalized to scan volume and rate (pl.	extsuperscript{-1}·sec\textsuperscript{-1}) assuming a pixel volume of 0.75×0.75×0.75 μm\textsuperscript{3}. Absolute [Ca\textsuperscript{2+}]\textsubscript{L} levels were calculated from Fluo-3 signals based on known diastolic Ca\textsuperscript{2+} levels from the ratiometric measurements using the equation: [Ca\textsuperscript{2+}]\textsubscript{L}=K\textsubscript{c}×F\textsubscript{Fl}/(K\textsubscript{c}×F\textsubscript{Fl}+[Ca\textsuperscript{2+}]\textsubscript{L}+1−F\textsubscript{Fo}),\textsuperscript{23} where F\textsubscript{Fo} being fluorescence normalized to baseline fluorescence and K\textsubscript{c}=1100 μmol/L the dissociation constant of Fluo-3.\textsuperscript{24}

#### Measurement of Cell Dimensions

Cell dimensions were determined by obtaining 2D scans using the confocal microscope and measuring cell length and width.

#### Measurements of Diastolic [Ca\textsuperscript{2+}] and SR Ca\textsuperscript{2+} Content

Measurements were performed using an epifluorescence setup (IonOptix, Milton, Mass) mounted to a Nikon microscope. Diastolic Ca\textsuperscript{2+} was measured in myocytes loaded with 10 μmol/L Fura-2AM and again either CaMKII inhibitor KN-93 or KN-92 for 15 minutes at room temperature in loading buffer. Cells were field stimulated (1 Hz) and superfused with experimental solution with either KN-93 or KN-92 for 15 minutes to wash out and to obtain deesterification before starting measurements. The fluorescence ratio was calculated by division of the signals obtained by excitation at 340 nm by those at 380 nm. These Fura ratio values were used to calculate intracellular diastolic Ca\textsuperscript{2+} concentrations using the Grynkiewicz equation: {[Ca\textsuperscript{2+}]\textsubscript{L}}=K\textsubscript{c}×F\textsubscript{Fl}/(R−R\textsubscript{min}/(R−R\textsubscript{max})).\textsuperscript{25} To obtain the values for R\textsubscript{min} and R\textsubscript{max} for our setup, Fura-2–loaded cells were stored for at least 1 hour in Kraftbrühe medium before division of the signals obtained by excitation at 340 nm by those at 380 nm. These values were used to calculate intracellular concentrations of Ca\textsuperscript{2+}.
myocytes were subjected to 5 μmol/L ionomycin and 1.34 μmol/L nigericin in a solution containing (mmol/L): 10 NaCl, 125 KCl, 25 HEPES, 1 MgSO4 (pH 7.4, KOH). For R_{min}, the solution contained 5 mmol/L EGTA, whereas for R_{max} it contained 5 mmol/L CaCl2.

To investigate SR Ca^{2+} content, myocytes were loaded for 20 minutes with 10 μmol/L Fluo-3AM containing KN-92 and superfused for 5 minutes with experimental solution again containing KN-92 before starting experiments. The dye was excited at 480±15 nm and emitted fluorescence collected at 535±20 nm. Caffeine (10 mmol/L) was applied causing complete SR Ca^{2+} release. The amplitude of the Ca^{2+} transient was used as a measure of SR Ca^{2+} content by evaluating F/F0 and calculating absolute Ca^{2+} values from this as described for the Ca^{2+} sparks. The time constant (τ) of the decay of the transient was used as an estimate of NCX function.26

**Patch-Clamp Investigation of L-type Ca^{2+} Current and Action Potentials**

Patch-clamp experiments were performed using the EPC-10 amplifier and Patchmaster software (HEKA, Germany). The myocytes were placed in a recording chamber mounted on the stage of a microscope (Nikon). Both action potentials and I_{Ca,L} were recorded using the ruptured-patchwhole-cell-patch clamp technique. After rupture, 5 minutes was allowed for equilibration of intracellular solution and cytosol before starting recordings. Fast and slow capacitance as well as series resistance were compensated for using the built-in functions of Patchmaster.

For action potential measurements, the bath solution contained (mmol/L): 135 NaCl, 5.4 KCl, 2 CaCl2, 1 MgCl2, 10 glucose, 10 HEPES, 0.33 NaH2PO4 (23°C, pH 7.4, NaOH). Pipettes were pulled to resistances of 2 to 3 MΩ when filled with intracellular solution consisting of (mmol/L): 120 K-aspartic acid, 8 KCl, 7 NaCl, 1 MgCl2, 5 MgATP, 10 HEPES (23°C, pH 7.2, Tris). A small holding current (∼50 pA; on average 44.5±1.8 pA in AF and 38.3±5.1 in sinus rhythm; P=0.3) was used to clamp cells to a diastolic potential of −75 mV and was kept constant in each myocyte. Action potentials were stimulated at 0.5 Hz and 2 Hz with 4- to 6-ms current pulses with a 40- to 60-pulse preconditioning train.

For I_{Ca,L} recordings, the bath solution contained (mmol/L): 140 NaCl, 4 CsCl, 1 MgCl2, 5 HEPES, 10 glucose, 2 CaCl2 (23°C, pH 7.4, NaOH) and either KN-93 or KN-92 (1 μmol/L). Cells incubated with KN by superfusion for 20 minutes before starting experiments. Pipettes for I_{Ca,L} experiments were pulled to resistances of 2 to 2.5 MΩ when filled with intracellular solution containing (mmol/L): 105 CsCl, 20 HEPES, 5 BAPTA*4Cs, 2 di-bromo-BAPTA, 5 MgATP, 1.49 CaCl2 (23°C, pH 7.2, CsOH). From a holding potential of −70 mV, cells were briefly depolarized to −40 mV for 50 ms to inactivate Na+ currents, then clamped to test potentials steps between −30 mV and +40 mV for a duration of 200 ms in 10 mV step increases with an interval of 1 s. Measured currents were normalized to membrane capacitance.

**Western Blot Analysis**

Atrial tissue samples were homogenized in Tris buffer containing (mmol/L): 20 Tris-Cl, 200 NaCl, 20 NaF, 1 Na3VO4, 1 DTT, 1% Triton X-100 (pH 7.4), and complete protease inhibitor cocktail (Roche Diagnostics). Protein concentration was determined by BCA assay (Pierce Biotechnology). Denatured tissue homogenates were subjected to Western blotting (4% to 15% gradient and 10% SDS-polyacrylamide gels) using anti-CaMKII (1:12000, gift from D. M. Bers, University of California, Davis, Calif) or anti-phospho-CaMKII (1:1000, Affinity Bioreagents), anti-RyR2 (1:2500), anti-phospho-RyR2 (1:2500, Upstate, anti-phospho-Thr17 (1:5000, Biotrend Chemikalien) antibodies. Chemiluminescent detection was done with SuperSignal West Pico Substrate (Pierce).

**Statistics**

All data are presented as means±SEM. Statistical analyses were performed using Student’s t test for unpaired values, Fisher’s exact test, or repeated-measures 2-way ANOVA, as appropriate. Values P>0.05 were considered significant.
Results

Patient Characteristics
All patients in the AF group were in documented AF immediately before the operation. Patients did not show significant differences with respect to left ventricular function (50% to 60%), severe mitral valve insufficiency (10% to 11%), incidence of diabetes (25% to 41%), or pharmacological treatment received. Of note, none of them received antiarrhythmics.

Single Cell Dimensions
Single myocytes were measured to investigate possible cell hypertrophy. We found that cells from AF patients were longer (113.3±2.3 μm, n=106; versus 101.1±1.5 μm, n=115; P<0.05) and wider (17.1±0.4 versus 14.8±0.3 μm; P<0.05) compared to those from sinus rhythm patients, demonstrating cell hypertrophy (Figure 1a).

CaMKII Expression and Phosphorylation Is Increased in AF
CaMKII expression and activation have previously been found to be increased in ventricular myocardium in human heart failure. Our Western blot analysis showed significantly higher CaMKII protein levels in samples from AF patients as compared to those in sinus rhythm by 40±14% (AF, n=9; versus sinus rhythm, n=11; P<0.05; Figure 1b). Similarly to CaMKII protein expression, phosphorylated
CaMKII (normalized to CaMKII protein expression) was significantly increased by 33±12% (AF, n=11; versus sinus rhythm, n=13; P<0.05; data not shown).

Expression Levels of RyR2, SERCA2a, and PLB
We further investigated possible changes in expression levels of Ca$^{2+}$ handling proteins. As shown in Figure 2a, we found significantly reduced expression of RyR2 by 44±5% (n=10 each; P<0.05). Whereas SERCA2a (AF, n=8; versus sinus rhythm, n=9) and PLB (n=8 versus n=9) expression levels were unaltered in AF compared to sinus rhythm patients (data not shown).

PLB Phosphorylation at the CaMKII Site Is Unaltered
To investigate possible effects of CaMKII on SERCA2a function, we measured phosphorylation of PLB (AF, n=8; versus sinus rhythm, n=9) at the CaMKII site (Thr17) as normalized to PLB expression and found that CaMKII-dependent PLB phosphorylation was unaltered in the AF patients investigated (87±20%; data not shown).

CaMKII-Dependent Phosphorylation of RyR2 Is Increased
RyR2 contains multiple phosphorylation sites including Ser2814, phosphorylated by CaMKII, and Ser2808, which may be phosphorylated by both CaMKII and protein kinase A.14,28 The degree to which phosphorylation of both sites induces “leakiness” of the channel is subject to debate, but recent results suggest a critical role of Ser2814.15 RyR2 phosphorylation status at both sites was normalized to RyR2 protein levels. Phosphorylation levels of RyR2 were significantly increased by 110±53% at the CaMKII specific Ser2814 site (AF, n=9; versus sinus rhythm, n=10; P<0.05). At the Ser2808 site, phosphorylation was still increased by 29±14%, but this was not significant (n=9 versus n=10, P=0.13; Figure 2b and 2c).

Increased SR Ca$^{2+}$ Leak Leads to Elevated Free Cytosolic Ca$^{2+}$ Levels in AF
Total SR Ca$^{2+}$ leak can be estimated by the size of single Ca$^{2+}$ sparks and the frequency of these events. We found that
in cells from AF patients, spark frequency (316.7±63.1 versus 142.4±16.9 pL^{-1}·sec^{-1}, n=19 versus n=32 cells; P<0.001) and size (1.43±0.11 versus 0.89±0.09 nmol·ps^{-1}, n=96 versus n=72 sparks; P<0.05) were significantly increased compared to those from sinus rhythm patients. This means a significant increase in total SR Ca^{2+} leak in AF patient cells by 3.9-fold compared to cells from sinus rhythm patients (Figure 3a and 3b).

Absolute free cytosolic Ca^{2+} levels were investigated using Fura-2. We found that in the absence of CaMKII inhibition (KN-92), free cytosolic Ca^{2+} levels were significantly increased in cells from AF patients as compared to sinus rhythm (229.4±19.6 versus 163.5±7.9 nmol/L, n=33 and n=43 for AF and sinus rhythm, P<0.05; Figure 3c).

**Ca^{2+} Sparks Are Not Attributable to Higher SR Ca^{2+} Load**

Another reason for increased Ca^{2+} spark frequency besides increased RyR2 phosphorylation could be a higher SR Ca^{2+} load, which has been shown to provoke spontaneous SR Ca^{2+} release ("leak–load relationship"). To exclude this, we investigated SR Ca^{2+} load. We found F/F_0 of the caffeine-induced transient to be significantly reduced in AF (2.68±0.29 versus 3.65±0.33 F/F_0 in SR, n=22 versus n=21). When calculating absolute [Ca^{2+}] values this difference was not significant any more (714.3±57.3 versus 822.0±48.5 nmol/L in SR, P=0.16; Figure 4) most likely because of the elevated diastolic [Ca^{2+}] levels in AF. No change or slight decreases in SR Ca^{2+} load has also been reported by others both for human AF as well as an animal model for AF.

**Elevated Diastolic Ca^{2+} Levels Are Attributable to SR Ca^{2+} Leak**

To investigate whether Ca^{2+} sparks were the cause for elevated cytosolic Ca^{2+}, we performed measurements with tetracaine. Tetracaine leads to complete closing of the RyR2, so this should remove all Ca^{2+} sparks and SR Ca^{2+} leak as well as normalize the associated increases in cytosolic Ca^{2+}. We found that tetracaine completely abolished Ca^{2+} sparks both in cells from AF, as well as sinus rhythm patients, compared to cells from the same patients in normal Tyrode’s (NT) solution (NT: 12 of 19 cells investigated showing sparks in sinus rhythm, 7 of 16 cells in AF; compared to tetracaine: 0 of 21 cells in sinus rhythm, 0 of 14 cells in AF; P<0.05, Fisher’s test). Furthermore, tetracaine also led to normalization of diastolic [Ca^{2+}] in AF (NT, 285.7±36.3; versus tetracaine 189.9±25.4 nmol/L, n=16 versus n=22, P<0.05) compared to sinus rhythm NT (208.4±28.8, n=15), as well as KN-92. In cells from sinus rhythm patients, tetracaine had no significant effect on diastolic Ca^{2+} levels (185.7±29.0 nmol/L, n=16; Figure 5).

**SR Ca^{2+} Leak and Diastolic Ca^{2+} Levels Can Be Normalized by CaMKII Inhibition**

Again, Ca^{2+} spark frequency and size were measured to investigate SR Ca^{2+} leak. We found that CaMKII inhibition with KN-93 dramatically reduced Ca^{2+} spark frequency in AF from 316.7±63.1 pL^{-1}·sec^{-1} (n=19) to 120.0±21.5 pL^{-1}·sec^{-1} (n=32, P<0.05; Figure 3b), meaning a normalization to control levels in sinus rhythm. This CaMKII inhibition effect was still present, but with only a minor reduction of spark frequency in sinus rhythm to 88.7±19.5 pL^{-1}·sec^{-1} (n=19, P<0.05). Spark size was not altered by CaMKII inhibition (Figure 3b).

The net effect was a 4-fold reduction in total SR Ca^{2+} leak in AF myocytes meaning a total normalization compared to sinus rhythm cells. In sinus rhythm myocytes, CaMKII inhibition had a much smaller but still significant reduction of SR Ca^{2+} leak (Figure 3c).

This reduction of SR Ca^{2+} leak by CaMKII inhibition was accompanied by a complete normalization of free cytosolic Ca^{2+} levels in AF cells from 229.4±19.6 nmol/L (KN-92) down to 166.1±9.9 nmol/L (n=36, P<0.05 versus AF KN-92, P=0.84 versus sinus rhythm KN-92) with KN-93. In cells from sinus rhythm patients, KN-93 does not lead to a further reduction in cytosolic [Ca^{2+}] levels (161.4±9.8 nmol/L, n=34, P=0.86; Figure 3c).

**I_{Ca,L} Is Not Reduced by CaMKII Inhibition in AF**

CaMKII is known to be a regulator of L-type Ca^{2+} channel function. CaMKII inhibition has been shown to reduce I_{Ca,L} in ventricular myocytes. Whereas, in line with this, it has been demonstrated that CaMKII inhibition with KN-93 reduces I_{Ca,L} in human right atrial cells from sinus rhythm patients, in contrast, it did not further reduce I_{Ca,L} in AF cells. To confirm these findings, we investigated I_{Ca,L} in AF cells from our patients (Figure 6) and found...
that KN-93 did not reduce $I_{\text{Ca,L}}$ compared to KN-92 (peak currents at +10 mV for KN-93 1.39±0.28 pA/pF versus KN-92 1.27±0.19 pA/pF, n=6 versus n=8, P=0.73). These currents are within the range reported for AF cells with KN-drugs.\textsuperscript{33} Estimated leaks were similarly low for both groups with 1.04±0.58 nS for KN-92-measurements and 1.08±0.44 nS for KN-93.

NCX Expression and Function in AF
NCX function has been reported to be increased in an animal model of AF.\textsuperscript{30} We found that this was also true for human AF, as the decay of the caffeine-induced transients as estimated by their time constant tau showed a trend toward faster NCX Ca$^{2+}$ outward function in cells from AF patients, (1.71±0.12 sec versus 2.25±0.27 sec in sinus rhythm, n=18 versus n=21, P=0.10). This correlated with significantly increased expression of NCX by 43±14\% in AF as shown by Western blotting (n=5 versus n=6, P<0.05; Figure 7).

Action Potentials Are Shortened in AF
Shortening of APD is typical for electrophysiological remodeling in AF. To verify this in our samples, we investigated APD. As expected,\textsuperscript{1} APD was shorter in AF samples. In particular, the later phase of repolarization was shorter in AF myocytes, whereas the very early phase was largely unaltered (Figure 8). This can also be seen in the mean data with an APD90 in AF of 179.5±21.8 ms at 0.5 Hz (n=7) and 144.4±14.6 ms at 2 Hz. In sinus rhythm myocytes, APD90 was significantly longer with 279.2±37.0 ms at (n=7) 0.5 Hz and 234.2±32.3 ms at 2 Hz (P<0.05). APD30, however, was not altered in AF compared to sinus rhythm (P=0.5 and P=0.8).

Discussion
The results of the present study show that AF leads to elevated diastolic Ca$^{2+}$ levels in human atrial myocytes and demonstrate that CaMKII may be central to this disordered Ca$^{2+}$-homeostasis: Molecular and functional data show consistently that CaMKII-dependent hyperphosphorylation of the RyR2 leads to elevated SR Ca$^{2+}$ leak and increased diastolic [Ca$^{2+}$]$_{\text{i}}$ in human AF. These effects could be reversed by CaMKII inhibition and by closing the SR Ca$^{2+}$ leak with tetracaine.

Altered Ca$^{2+}$ Handling in AF
In AF, there is electric and contractile remodeling of the myocardium. Abnormalities in intracellular Ca$^{2+}$ handling are believed to contribute to atrial arrhythmogenesis during AF. Although it has been speculated that diastolic [Ca$^{2+}$] might be elevated in AF,\textsuperscript{34} this has never been investigated in human myocytes.

One important mechanism that can lead to elevated diastolic [Ca$^{2+}$] levels is Ca$^{2+}$ leak from the SR via the RyR2. In fact, Hove-Madsen et al have shown increased SR Ca$^{2+}$ sparks in human AF.\textsuperscript{12} However, the cause for this and whether it

Figure 5. Tetracaine abolishes the occurrence of Ca$^{2+}$ sparks and normalizes diastolic Ca$^{2+}$ concentration. Twelve of 19 cells from sinus rhythm patients investigated showed sparks without tetracaine, with tetracaine none of 21 cells showed sparks. In cells from AF patients, there were sparks in 7 of 16 cells without tetracaine, but none of 14 cells showed sparks with tetracaine. Diastolic Ca$^{2+}$ in AF cells in NT solution (n=16 from 2 patients) was normalized down to sinus rhythm levels by tetracaine (n=22). Whereas in cells from sinus rhythm patients, tetracaine (n=16) had no effect on Ca$^{2+}$ levels compared to NT (n=15 from 2 patients).

Figure 6. $I_{\text{Ca,L}}$ is not reduced by CaMKII inhibition in AF. CaMKII inhibition (KN-93) does not decrease L-type Ca$^{2+}$ current in AF compared to control conditions (KN-92), as shown for mean values (n=6 vs n=8 myocytes from 4 patients) and demonstrated by original recordings from currents at +10 mV.
influences diastolic \([\text{Ca}^{2+}]\) remained unknown. Increased RyR2 open probability has been shown in an animal model of AF and in human samples,\(^{13}\) suggesting that direct regulation of the channel (eg, by phosphorylation through protein kinase A) might play a role. Recent reports provided evidence that CaMKII is associated with RyR2 and that it can phosphorylate the receptor.\(^{15,28,35–38}\) This CaMKII-dependent RyR2 phosphorylation increases fractional SR \([\text{Ca}^{2+}]\) release as well as \([\text{Ca}^{2+}]\) spark frequency.\(^{24,39,40}\)

**CaMKII-Dependent SR \([\text{Ca}^{2+}]\) Leak and Elevated Diastolic \([\text{Ca}^{2+}]\) in AF**

We demonstrate that cytosolic \([\text{Ca}^{2+}]\) is increased in atrial myocytes from AF patients. This was associated with an increased SR \([\text{Ca}^{2+}]\) leak as measured by \([\text{Ca}^{2+}]\) sparks. Tetracaine completely abolished the occurrence of \([\text{Ca}^{2+}]\) sparks and normalized cytosolic \([\text{Ca}^{2+}]\) levels. This demonstrates that the RyR2 was the source of these spontaneous \([\text{Ca}^{2+}]\) release events and the elevation of \([\text{Ca}^{2+}]\). Although we found RyR2 expression to be reduced in AF (which could be expected to lead to reduced SR \([\text{Ca}^{2+}]\) leak), we found that the RyR2 is more phosphorylated. Most importantly, we saw strong hyperphosphorylation of the RyR2 at the CaMKII specific site, which correlates with the increased CaMKII expression we found in AF (in accordance with previous studies)\(^{22}\) as well as the increased CaMKII autophosphorylation (activation) that has recently been demonstrated in AF.\(^{41}\) Phosphorylation of the RyR2 at this site is increasingly being believed to be the critical mechanism inducing diastolic SR \([\text{Ca}^{2+}]\) leak in failing ventricular myocardium. But does it have the same consequences in atrial myocytes as well?

In fact, our studies showed that the SR \([\text{Ca}^{2+}]\) leak, as measured by the frequency and size of \([\text{Ca}^{2+}]\) sparks, was strongly increased in cells from AF patients. Increased SR \([\text{Ca}^{2+}]\) content might have been a trigger for the increased SR \([\text{Ca}^{2+}]\) leak observed by us. However, we could confirm previous findings that SR \([\text{Ca}^{2+}]\) content is not increased in AF,\(^{12,18,30}\) which corroborates our hypothesis that hyperphosphorylation of the RyR2 is the cause for this increased leak. This is further
strengthened by our observation that in the presence of CaMKII inhibition, SR Ca\(^{2+}\) leak was completely normalized in AF cells and that in addition, CaMKII inhibition also lead to a normalization of diastolic [Ca\(^{2+}\)] to levels seen in cells from sinus rhythm patients. On the other hand, in cells from sinus rhythm patients where SR Ca\(^{2+}\) leak is already low, CaMKII inhibition did not lead to a further reduction of cytosolic free Ca\(^{2+}\). To exclude the possibility that a reduction of \(I_{\text{Ca,L}}\) attributable to CaMKII inhibition rather than the reduction of SR Ca\(^{2+}\) leak was the cause for these effects, we investigated the effect of CaMKII inhibition on \(I_{\text{Ca,L}}\). Our results confirm previous findings that in human AF,\(^{33}\) in contrast to sinus rhythm, CaMKII inhibition does not further reduce the already diminished \(I_{\text{Ca,L}}\). This further strengthens our hypothesis that CaMKII-mediated SR Ca\(^{2+}\) leak was the main cause of elevated cytosolic Ca\(^{2+}\) levels in AF. Furthermore we as well as others found NCX function to be slightly increased in human AF in the presence of significantly increased NCX expression.\(^{30}\) We suggest that this might be a compensatory mechanism in the face of the elevated diastolic Ca\(^{2+}\) levels.

CaMKII-Dependent SR Ca\(^{2+}\) Leak As Potential Arrhythmogenic Substrate

Increased SR Ca\(^{2+}\) leak is regarded as a potential arrhythmogenic mechanism, because elimination of Ca\(^{2+}\) via the NCX would lead to depolarization of the cell and \(I_{\text{TI}}\), which could cause DADs as shown previously by Song et al in ventricular guinea pig myocytes.\(^{42}\) Also, experiments in dog pulmonary veins have already demonstrated that ectopy may result from Ca\(^{2+}\)-mediated triggered activity arising from DADs.\(^{43}\) Moreover, transgenic mice with increased SR Ca\(^{2+}\) leak attributable to FKBP12.6 deficiency have been shown to be more prone to induced AF.\(^{44}\) Also, mice overexpressing CaMKI\(\delta\) display an increased propensity to arrhythmias;\(^{21}\) whether they also develop AF is not known. Furthermore, very recent findings by Chelu et al from elegant studies in knock-in mice with a RyR2 which is not phosphorylatable by CaMKII suggest that CaMKII SR Ca\(^{2+}\) leak through the RyR2 might be relevant for AF induction.\(^{45}\)

Consequently, we suggest that CaMKII-dependent SR Ca\(^{2+}\) leak in AF caused by hyperphosphorylation of the RyR2 could be an arrhythmogenic mechanism and that CaMKII inhibition may reduce the propensity for atrial arrhythmias and might provide a novel therapeutic target.

CaMKII Inhibition Targets SR Ca\(^{2+}\) Leak but Not \(I_{\text{Ca,L}}\) in AF

That CaMKII inhibition does not further decrease \(I_{\text{Ca,L}}\) in AF is also important with respect to potential therapeutic use; importantly, it means that CaMKII inhibition can target one arrhythmogenic mechanism (SR Ca\(^{2+}\) leak) while not further aggravating another (reduced APD attributable to reduced \(I_{\text{Ca,L}}\)). Although these differential effects of CaMKII inhibition on \(I_{\text{Ca,L}}\) in AF versus sinus rhythm are quite surprising at first, they can be explained by findings by El-Armouche et al, which suggest that in AF, PP activity becomes differentially regulated in subcellular compartments; whereas PP1 activity is suppressed around the SR, PP1 and PP2a activity are increased at the cell membrane. This means that in AF CaMKII effects would be on one hand amplified around the SR, where the RyR2 is located, they would on the other hand be suppressed at the cell membrane, where the L-type Ca\(^{2+}\) channel is located. Thus, CaMKII inhibition can rather specifically target SR Ca\(^{2+}\) leak while not having significant effects on \(I_{\text{Ca,L}}\).

Limitations of the Study

It has to be mentioned that for the in vivo data, differences in heart rate might have played a role with respect to CaMKII-effects, because CaMKII can be activated in a frequency dependent manner.\(^{15}\) However, for our in vitro data, such a confounder can be ruled out, as myocytes from both groups were stimulated at the same frequencies. Therefore, we may rather have underestimated CaMKII-dependent effects in AF cells by performing experiments using the same stimulation rates in AF as well as sinus rhythm.

Because of the methodology of Ca\(^{2+}\) sparks, only those cells could be analyzed for SR Ca\(^{2+}\) leak that showed any Ca\(^{2+}\) sparks. However, one effect of CaMKII inhibition was that the percentage of cells that actually showed Ca\(^{2+}\) sparks was considerably reduced. This means that the real effect of CaMKII inhibition on SR Ca\(^{2+}\) leak in AF will probably be even stronger than what is already presented in our investigation.

Summary and Perspectives

We present data showing that CaMKII-dependent phosphorylation of the RyR2 leads to increased SR Ca\(^{2+}\) leak in AF. This causes elevated cytosolic Ca\(^{2+}\) levels, which might provide an arrhythmogenic substrate. Consequently, CaMKII inhibition should be investigated with respect to it possibly providing novel antiarrhythmogenic strategies and therapies in AF. With respect to established pharmacological treatments in AF, our results might explain a part of the antiarrhythmic effect of \(\beta\)-blockers, which work upstream of CaMKII and which have been shown to reduce hyperphosphorylation of the RyR2 and reduce SR Ca\(^{2+}\) leak.\(^{45,46}\)

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Disclosures

None.

References


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Novelty and Significance

What Is Known?

- Ca$^{2+}$ leak (Ca$^{2+}$ sparks) is increased in human AF.
- CaMKII activity is increased in AF.
- Protein phosphatase activity at the SR is decreased in AF.

What New Information Does This Article Contribute?

- Diastolic Ca$^{2+}$ concentration is increased in human AF.
- CaMKII inhibition can normalize increased Ca$^{2+}$ spark frequency and diastolic Ca$^{2+}$ concentration; this is not attributable to effects on $\ell_{\text{Ca,L}}$ or SR Ca$^{2+}$ load, but to effects on the RyR2.
- CaMKII-mediated SR Ca$^{2+}$ leak appears to be a novel arrhythmogenic mechanism in AF; its inhibition could be a therapeutic strategy.

Sarcoplasmic reticulum (SR) Ca$^{2+}$ leak and elevated diastolic Ca$^{2+}$ are emerging as possible important arrhythmogenic mechanisms and therapeutic targets in the heart. In atrial fibrillation (AF), increased SR Ca$^{2+}$ leak (Ca$^{2+}$ sparks) has previously been reported, but its mechanisms are unknown and diastolic Ca$^{2+}$ has not been investigated. In this study of human AF, we show that SR Ca$^{2+}$ leak is attributable to CaMKII-dependent hyperphosphorylation of the SR Ca$^{2+}$ release channel (RyR2) and that this leads to elevation of diastolic Ca$^{2+}$. CaMKII inhibition can normalize both SR Ca$^{2+}$ leak and diastolic Ca$^{2+}$ levels. Because CaMKII inhibition does not further reduce the already diminished L-type Ca$^{2+}$ current in AF, it might provide a novel, specifically targeted antiarrhythmic strategy in AF that should be investigated in the future. Furthermore, AF and heart failure are often associated and can aggravate each other. CaMKII-dependent pathophysiological mechanisms have been proposed as therapeutic targets for hypertrophy and heart failure. Our results suggest that CaMKII inhibition could be beneficial with respect to AF, as well as heart failure, thus offering a novel combined therapeutic approach for these two conditions.
CaMKII-Dependent Diastolic SR Ca\textsuperscript{2+} Leak and Elevated Diastolic Ca\textsuperscript{2+} Levels in Right Atrial Myocardium of Patients With Atrial Fibrillation

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