Enhanced Sarcoplasmic Reticulum Ca$^{2+}$ Leak and Increased Na$^{+}$-Ca$^{2+}$ Exchanger Function Underlie Delayed Afterdepolarizations in Patients With Chronic Atrial Fibrillation

Niels Voigt, MD; Na Li, PhD; Qiongling Wang, PhD; Wei Wang, PhD; Andrew W. Trafford, PhD; Issam Abu-Taha, BSc; Qiang Sun, PhD; Thomas Wieland, PhD; Ursula Ravens, MD; Stanley Nattel, MD; Xander H.T. Wehrens, MD, PhD; Dobromir Dobrev, MD

**Background**—Delayed afterdepolarizations (DADs) carried by Na$^{+}$-Ca$^{2+}$-exchange current (I_{NCX}) in response to sarcoplasmic reticulum (SR) Ca$^{2+}$ leak can promote atrial fibrillation (AF). The mechanisms leading to delayed afterdepolarizations in AF patients have not been defined.

**Methods and Results**—Protein levels (Western blot), membrane currents and action potentials (patch clamp), and [Ca$^{2+}$]$_i$ were measured in right atrial samples from 76 sinus rhythm (control) and 72 chronic AF (cAF) patients. Diastolic [Ca$^{2+}$]$_i$ and SR Ca$^{2+}$ content (integrated I_{NCX} during caffeine-induced Ca$^{2+}$ transient) were unchanged, whereas diastolic SR Ca$^{2+}$ leak, estimated by blocking ryanodine receptors (RyR2) with tetracaine, was $\approx$50% higher in cAF versus control. Single-channel recordings from atrial RyR2 reconstituted into lipid bilayers revealed enhanced open probability in cAF samples, providing a molecular basis for increased SR Ca$^{2+}$ leak. Calmodulin expression (60%), Ca$^{2+}$/calmodulin-dependent protein kinase-II (CaMKII) autophosphorylation at Thr287 (87%), and RyR2 phosphorylation at Ser2808 (protein kinase A/CaMKII site, 236%) and Ser2814 (CaMKII site, 77%) were increased in cAF. The selective CaMKII blocker KN-93 decreased SR Ca$^{2+}$ leak, the frequency of spontaneous Ca$^{2+}$ release events, and RyR2 open probability in cAF, whereas protein kinase A inhibition with H-89 was ineffective. Knock-in mice with constitutively phosphorylated RyR2 at Ser2814 showed a higher incidence of Ca$^{2+}$ sparks and increased susceptibility to pacing-induced AF compared with controls. The relationship between [Ca$^{2+}$]$_i$ and I_{NCX} density revealed I_{NCX} upregulation in cAF. Spontaneous Ca$^{2+}$ release events accompanied by inward I_{NCX} currents and delayed afterdepolarizations/triggered activity occurred more often and the sensitivity of resting membrane voltage to elevated [Ca$^{2+}$]$_i$ (diastolic [Ca$^{2+}$]$_i$–voltage coupling gain) was higher in cAF compared with control.

**Conclusions**—Enhanced SR Ca$^{2+}$ leak through CaMKII-hyperphosphorylated RyR2, in combination with larger I_{NCX} for a given SR Ca$^{2+}$ release and increased diastolic [Ca$^{2+}$]$_i$–voltage coupling gain, causes AF-promoting atrial delayed afterdepolarizations/triggered activity in cAF patients. (Circulation. 2012;125:2059-2070.)

**Key Words:** atrial fibrillation ■ delayed afterdepolarizations ■ ryanodine receptor calcium release channel ■ sarcoplasmic reticulum ■ sodium-calium exchanger

Atrial fibrillation (AF) induces self-promoting remodeling.1,2 Altered intracellular Ca$^{2+}$ signaling is a key contributor to the AF-maintaining substrate.3,4 In normal hearts, Ca$^{2+}$ enters cells through L-type Ca$^{2+}$ channels (I_{Ca,L}) with each action potential (AP), triggering Ca$^{2+}$ release from sarcoplasmic reticulum (SR) through type 2 ryanodine receptor channels (RyR2). During diastole, cytosolic Ca$^{2+}$ is removed via reuptake through the SR Ca$^{2+}$-ATPase (SERCA; cardiac form, SERCA2a) and transmembrane extrusion through the Na$^{+}$-Ca$^{2+}$ exchanger (NCX1).5

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Reduced I_{Ca,L} contributes to AF-related AP duration (APD) shortening, which promotes reentry.1,6-8 Recent evidence points to RyR2 dysfunction and increased SR Ca$^{2+}$ leak,9,10 providing another arrhythmogenic mechanism.11,12 Hyperphosphorylation...
increases RyR2 sensitivity to cytosolic Ca\(^{2+}\), making RyR2 “leakier.”\(^1\)\(^3\) RyR2 is hyperphosphorylated at Ser2808/2809 (species dependent; protein kinase A [PKA] and Ca\(^{2+}\)/calmodulin-dependent protein kinase-II [CaMKII] site)\(^1\)\(^1\),\(^1\)\(^4\) and Ser2814/2815 (species-dependent; exclusive CaMKII site)\(^1\)\(^5\) in canine and human chronic AF (cAF).\(^1\)\(^0\),\(^1\)\(^3\),\(^1\)\(^6\) CaMKII activity is enhanced in clinical\(^1\)\(^3\),\(^1\)\(^6\) and experimental AF.\(^1\)\(^7\),\(^1\)\(^8\) CaMKII inhibition reduces Ca\(^{2+}\) spark frequency in AF.\(^1\)\(^0\) Although it is widely believed that altered Ca\(^{2+}\) signaling predisposes to delayed afterdepolarizations (DADs) and triggered activity in AF patients, this has not been directly tested, nor have underlying mechanisms been established in humans. The present study evaluated cardiomyocytes and tissues from right atrial samples of patients in sinus rhythm (Ctl) compared with cAF patients to establish the mechanisms underlying Ca\(^{2+}\)-related DADs with the use of complementary biochemical and biophysical techniques and to relate Ca\(^{2+}\) handling abnormalities directly to cellular arrhythmogenesis.

**Methods**

For details, see the online-only Data Supplement.

**Human Tissue Samples**

Right atrial appendages were dissected from 76 Ctl patients and 72 cAF patients (Tables I and II in the online-only Data Supplement). Experimental protocols were approved by the ethics committees of Dresden University of Technology (EK790799) and the Medical Faculty Mannheim, University of Heidelberg (No. 2011–216N-MA). Each patient gave written informed consent. After excision, atrial appendages either were used for myocyte isolation (41 Ctl, 31 cAF patients) or were flash-frozen in liquid nitrogen for biochemical/biophysical studies (35 Ctl, 41 cAF patients).

**Human Myocyte Isolation**

Right atrial myocytes isolated with a standard protocol\(^1\)\(^9\),\(^2\)\(^0\) were suspended in EGTA-free storage solution.

**Human Myocyte Intracellular [Ca\(^{2+}\)] Measurement**

Intracellular [Ca\(^{2+}\)] ([Ca\(^{2+}\)]\(_i\)) was quantified with Fluo-3-acetoxymethyl ester. In addition, Fluo-3 was included into the electrode solution. Fluorescence was excited at 488 nm and emitted light (>520 nm) converted to [Ca\(^{2+}\)]\(_i\) assuming

\[
[Ca^{2+}]_i = k_d \frac{F}{F_{max} - F}
\]

where \(k_d\) is the dissociation constant of Fluo-3 (864 nmol/L), F is fluo-3 fluorescence, and \(F_{max}\) is Ca\(^{2+}\)-saturated fluorescence obtained at the end of each experiment.\(^2\)\(^1\)

**Patch Clamp**

Membrane currents and potentials were measured at 37°C in whole-cell ruptured-patch configuration using voltage-clamp and current-clamp techniques with simultaneous [Ca\(^{2+}\)] measurement. Membrane capacitances did not differ between groups (Ctl: 133.4±10.3 pF, n=40/20 [myocytes/patients] versus Ctl: 114.8±5.9 pF, n=46/26). Currents are expressed as densities (pA/pF).

L-type Ca\(^{2+}\) current (I\(_{Ca,L}\))–triggered [Ca\(^{2+}\)]\(_i\) transients (CaTs) were recorded simultaneously (Figure 1A). Drugs were applied via a rapid-solution exchange system.

**Quantification of Diastolic SR Ca\(^{2+}\) Leak**

SR Ca\(^{2+}\) leak in intact myocytes was measured according to Shannon et al.\(^2\)\(^2\)

**RyR2 Single-Channel Recordings**

Single-channel recordings of Ctl and cAF RyR2 were obtained under voltage-clamp conditions as previously described.\(^2\)\(^3\)
Intracardiac Electrophysiology and Ca\(^{2+}\) Spark Measurements in S2814D Knock-In Mice

RyRS2814D knock-in mice (S2814D) were generated as described.\(^{12}\) In vivo electrophysiology was performed on S2814D and wild-type (WT) littermates 3 to 4 months of age in the presence and absence of KN-93 (10\(^{-8}\) mol/kg IP).\(^{24}\) More than 1 second of AF or atrial flutter was considered an atrial arrhythmia episode.\(^{16,25}\) Atrial arrhythmia inducibility was considered positive if at least 2 of 3 pacing trials induced AF or atrial flutter. Ca\(^{2+}\) sparks were recorded in Fluo-4-AM–loaded mouse atrial myocytes with LSM510 confocal microscope.

Biochemistry

Protein expression of Ca\(^{2+}\)-handling proteins was quantified by immunoblot.\(^{26}\) The mRNA levels of NCX1 and CaMKII isoforms were measured by real-time polymerase chain reaction.

Statistical Analysis

Differences between group means for continuous data were compared by unpaired Student t test. Categorical data were analyzed with the Fisher exact test. Data are given as mean±SEM. \(P<0.05\) was considered statistically significant.

Results

IC\(_{\text{Ca,L}}\)-Triggered [Ca\(^{2+}\)]\(_i\) Transients in Right Atrial Myocytes From cAF Patients

Figure 1A shows simultaneously depolarization-induced IC\(_{\text{Ca,L}}\) and CaTs in Fluo-3–loaded myocytes. The peak IC\(_{\text{Ca,L}}\) amplitude was 42% lower in cAF, and the time integral of IC\(_{\text{Ca,L}}\) was 22% smaller in cAF compared with Ctl (Figure 1B). Systolic CaT amplitude was 50% lower and the CaT decay was 28% slower in cAF compared with Ctl, whereas diastolic [Ca\(^{2+}\)]\(_i\) tended to be increased (Figure 1C and 1D). No differences in the coupling efficiency between Ca\(^{2+}\) influx and Ca\(^{2+}\) release were noted between Ctl and cAF (Figure 1E).

SR Ca\(^{2+}\) Content and NCX Current

Atrial myocytes were preconditioned for 1 minute by the IC\(_{\text{Ca,L}}\) activation protocol in all subsequent voltage-clamp experiments. Caffeine (10 mmol/L) caused a rapid increase in [Ca\(^{2+}\)]\(_i\) as a result of SR Ca\(^{2+}\) release. The subsequent decay of [Ca\(^{2+}\)]\(_i\) results from sarcolemmal Ca\(^{2+}\) extrusion, mainly through NCX. This generates a transient inward current (INCX). INCX amplitude and time course were monitored simultaneously with [Ca\(^{2+}\)]\(_i\) (Figure 2A).

The amplitude of caffeine-induced CaT was comparable in Ctl and cAF, suggesting unchanged SR Ca\(^{2+}\) content (Figure 2B). Systolic CaT amplitude was 50% lower and the CaT decay was 28% slower in cAF compared with Ctl, whereas diastolic [Ca\(^{2+}\)]\(_i\) tended to be increased (Figure 1C and 1D). No differences in the coupling efficiency between Ca\(^{2+}\) influx and Ca\(^{2+}\) release were noted between Ctl and cAF (Figure 1E).

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addition, $I_{\text{NCX}}$ amplitude was larger in cAF (Figure 2C) and inward $I_{\text{NCX}}$ for any given [Ca$^{2+}$], was greater (Figure 2D), indicating enhanced $I_{\text{NCX}}$. NCX1 expression was higher in cAF atria compared with Ctl (Figure 2E), indicating that functional enhancement may be due to protein upregulation.

**Expression and Phosphorylation of RyR2**

Because Ca$^{2+}$ content was unchanged, we studied the expression and phosphorylation of RyR2 and RyR2 regulatory proteins as a potential source of DAD generation (Figure 1 in the online-only Data Supplement). Total RyR2 was unchanged, whereas fractional phosphorylation of RyR2 at Ser2814 (CaMKII site) and Ser2808 (PKA/CaMKII site) was increased by 77% and 236%, respectively, in cAF (Figure IAI in the online-only Data Supplement). Protein expression of calsequestrin, junction, and triadin, major regulators of RyR2 function, also increased by 60% (Figure IIC in the online-only Data Supplement). Calmodulin expression was unchanged, whereas fractional phosphorylation of CaMKII$\alpha$ increased by 92% in cAF (Figure IIA in the online-only Data Supplement) and stimulatory autophosphorylation at Thr287 increased by 87%. Fractional autophosphorylation of CaMKII$\beta$ at its inhibitory Thr306/307 site was reduced by 46%. The mRNA levels of the cytosolic (CaMKII$\alpha$) and nuclear (CaMKII$\beta$) isoforms and the protein levels of CaMKII$\beta$ were unchanged in cAF, suggesting post-transcriptional upregulation of CaMKII$\alpha$. (Figure IIA and IIB in the online-only Data Supplement). Calmodulin expression was also increased by 60% (Figure IIC in the online-only Data Supplement). In contrast, protein levels of PKA$\alpha$ and PKA$\beta$ subunits were similar (Figure IID in the online-only Data Supplement). Overall, these data point to enhanced CaMKII activity underlying RyR2 hyperphosphorylation and previously reported increases in Ca$^{2+}$ spark incidence.9,10,16

**Diastolic SR Ca$^{2+}$ Leak**

Increased Ca$^{2+}$ spark frequency has been reported in cAF patients,9,10 but Ca$^{2+}$ spark estimation with Ca$^{2+}$ spark recordings is semiquantitative because of substantial non-spark SR Ca$^{2+}$ release.28,29 To directly quantify total SR Ca$^{2+}$ leak, we used the tetracaine method of Shannon et al,22 as illustrated in Figure 3A. Rapid application of tetracaine to voltage-clamped myocytes perfused with Na$^+$- and Ca$^{2+}$-free bath solution (to prevent transmembrane fluxes) reflects the magnitude of total SR Ca$^{2+}$ leak.22

The tetracaine-induced Ca$^{2+}$ shift was significantly enhanced in cAF myocytes (Figure 3B). The contributions of...
CaMKII and PKA to enhanced SR Ca\(^{2+}\) leak in cAF were assessed in myocytes pretreated for 30 minutes with KN-93 to inhibit CaMKII or H-89 to inhibit PKA. Pretreatment with KN-93, its inactive analog KN-92, or H-89 did not significantly affect diastolic [Ca\(^{2+}\)]\(_i\) in sinus rhythm, whereas the cAF-related increase in SR Ca\(^{2+}\) leak was abolished by KN-93 but not by KN-92 or H-89 (Figure 3B). Similar results were obtained when diastolic SR Ca\(^{2+}\) leak was related to SR Ca\(^{2+}\) content, estimated by caffeine-induced CaT in each myocyte (Figure 3A and 3C). Moreover, the leak-load relationship was shifted leftward in cAF; this was reversed by KN-93 but not KN-92 (Figure 3D and 3E), indicating increased CaMKII-mediated SR Ca\(^{2+}\) leak at any given SR Ca\(^{2+}\) content.

During decay of I\(_{Ca,L}\)-induced CaT, cytosolic Ca\(^{2+}\) is extruded by Ca\(^{2+}\) reuptake into the SR via SERCA and into extracellular space via NCX and plasmalemmal Ca\(^{2+}\) ATPase. We estimated the Ca\(^{2+}\) transport rate of SERCA, NCX, and plasmalemmal Ca\(^{2+}\) ATPase by the rate constants of exponential curves fitted to I\(_{Ca,L}\) and caffeine-evoked CaT decays as previously described (Figure III in the online-only Data Supplement).\(^{30}\) Ca\(^{2+}\) removal mechanisms switched from a predominance of SERCA over NCX in Ctl to an equal contribution of SERCA and NCX in cAF (Figure IIID in the online-only Data Supplement).

Enhanced Open Probability of RyR2 in cAF

To compare directly the Ca\(^{2+}\) sensitivities of RyR2 channels in Ctl versus cAF, single RyR2 currents were recorded from RyR2 channels obtained from cAF (42 channel recordings from 10 patients) and Ctl patients (38 channel recordings from 11 patients; Figure 4A and 4B). Under conditions that mimic diastole (ie, 150 nmol/L cytosolic [Ca\(^{2+}\)]), cAF patients exhibited enhanced opening frequencies and open probabilities (Po; Figure 4A and 4B) and shorter closed times (cAF, 827 ± 266 milliseconds versus Ctl, 3687 ± 930 milliseconds; P<0.05). Open times were unchanged (cAF, 827 ± 266 milliseconds versus Ctl, 827 ± 266 milliseconds), cAF RyR2 channels had left-shifted Ca\(^{2+}\) dependence so that they were activated at resting cytosolic Ca\(^{2+}\) levels (Figure 4C). The enhanced RyR2 Po in cAF provides a molecular correlate for increased SR Ca\(^{2+}\) leak in intact myocytes (Figure 3).
Next, we tested whether CaMKII and PKA phosphorylation inhibition reduces RyR2 Po in cAF. KN-93 (at 350 nmol/L cytosolic [Ca\(^{2+}\)]) did not alter RyR2 Po for Ctl myocytes but significantly decreased RyR2 Po in cAF, whereas KN-92 and the PKA inhibitors H-89 and PKI were ineffective (Figure 4C–4E and Figure IVB in the online-only Data Supplement). These results suggest that the increased SR Ca\(^{2+}\) leak in cAF results from a CaMKII-mediated increase in Po of RyR2.

Atrial Myocytes From cAF Patients Display Enhanced Frequency of Spontaneous Ca\(^{2+}\) Release Events

To establish the relationship between NCX and arrhythmogenic spontaneous Ca\(^{2+}\) release events (SCaEs), we quantified SCaEs and corresponding inward Na\(^{+}\)-Ca\(^{2+}\)-exchange current (INCX) in myocytes from sinus rhythm (Ctl) and chronic atrial fibrillation (cAF) patients. A, Voltage-clamp protocol (top) and representative recordings of SCaEs (Fluo-3; middle) and corresponding INCX (bottom) from a Ctl (left) and cAF (right) myocytes, respectively, after steady-state stimulation for 1 minute at 0.5 Hz. B, Susceptibility to SCaEs in Ctl and cAF. *P<0.05 vs Ctl (Fisher exact test). C, Mean±SEM frequency (left), latency (middle), and amplitude (right) of SCaEs in Ctl and cAF. D, Mean±SEM amplitude of SCaE-generated INCX. C and D, *P<0.05 and **P<0.01 vs corresponding means in Ctl. Numbers indicate myocytes/patients.

Figure 5. Spontaneous sarcoplasmic reticulum Ca\(^{2+}\) release events (SCaEs) with corresponding inward Na\(^{+}\)-Ca\(^{2+}\)-exchange current (INCX) in myocytes from sinus rhythm (Ctl) and chronic atrial fibrillation (cAF) patients. A, Voltage-clamp protocol (top) and representative recordings of SCaEs (Fluo-3; middle) and corresponding INCX (bottom) from a Ctl (left) and cAF (right) myocytes, respectively, after steady-state stimulation for 1 minute at 0.5 Hz. B, Susceptibility to SCaEs in Ctl and cAF. *P<0.05 vs Ctl (Fisher exact test). C, Mean±SEM frequency (left), latency (middle), and amplitude (right) of SCaEs in Ctl and cAF. D, Mean±SEM amplitude of SCaE-generated INCX. C and D, *P<0.05 and **P<0.01 vs corresponding means in Ctl. Numbers indicate myocytes/patients.

Membrane-Voltage Response to SCaEs and DAD Susceptibility

In normal cardiomyocytes, safety factors limit the ability of SCaEs to cause DADs.\(^{31,32}\) In cAF, however, besides an increased incidence of SCaEs, the same [Ca\(^{2+}\)]\(_i\) generates a larger depolarizing INCX (Figure 2D), pointing to an increase in intracellular [Ca\(^{2+}\)]\(_i\)-membrane voltage (V\(_m\)) coupling gain, enhancing the risk of DADs/trIGGERed activity.

Figure 6A shows representative AP-triggered CaTs in a Ctl and a cAF myocyte, respectively. Resting membrane potential and AP amplitude did not differ between Ctl and cAF, whereas AP duration was significantly shorter in cAF (Figure 6B). Diastolic [Ca\(^{2+}\)]\(_i\) was nonsignificantly increased whereas amplitude of the systolic CaT was 48% smaller in cAF compared with Ctl, consistent with the results of ICa,L-triggered CaTs. Figure 7A shows simultaneous V\(_m\) and CaT recordings in current-clamped myocytes immediately after a 1-minute period of AP-triggered CaTs at 0.5 Hz. DADs were defined as a SCaE-induced V\(_m\) change >20 mV because only DADs of such magnitude cause arrhythmogenic triggered activity in whole hearts.\(^{33}\) Susceptibility to SCaEs and SCaE-induced DADs was significantly increased in cAF (Figure 7B). Triggered activity in cAF was accompanied by prominent diastolic V\(_m\) oscillations that appeared as DADs (6 of 13 myocytes) or triggered APs (2 of 13 myocytes). In the 1 Ctl myocyte showing DADs, triggered APs
were also observed. The $V_m$ oscillations were likely due to INCX upregulation because the NCX blocker Ni$^{2+}$ (10 mmol/L) prevented DADs/triggered activity without changing SCaE incidence ($n=3$; data not shown).

Consistent with Figure 5C, the intrinsic frequency of SCaEs tended to be higher whereas the coupling interval to the first SCaE tended to be shorter in cAF (Figure 7C). The amplitude of SCaEs was nonsignificantly larger whereas the SCaE-induced $V_m$ change was significantly higher in cAF versus Ctl (Figure 7D). Diastolic $[Ca^{2+}]_i-V_m$ coupling gain, defined as the ratio of SCaE-induced $V_m$ change magnitude to SCaE amplitude, was 5-fold greater in cAF than in Ctl, indicating that changes in diastolic $[Ca^{2+}]_i$ produce stronger $V_m$ depolarizations in cAF compared with Ctl. Because of possible changes in cytosolic constituents with the ruptured-patch clamp technique, we repeated selected experiments with perforated-patch methods. Qualitatively similar results were obtained for basic AP properties and CaTs in perforated-patch–clamped myocytes (Figure V in the online-only Data Supplement) compared with tight-seal results (Figure 6).

Perforated-patch–clamped myocytes also showed a cAF-related promotion of SCaEs/DADs (Figure VIA–VIC in the online-only Data Supplement) similar to that of ruptured-patch clamped cells (Figure 7A–7C), and their Ctl-cAF SCaE/DAD frequency differential persisted despite PKA inhibition with H-89, whereas subsequent CaMKII inhibition with KN-93 normalized the frequency of SCaEs/DADs in cAF to Ctl levels (Figure VIIC in the online-only Data Supplement). Similar trends were observed for SCaE-associated $V_m$ change (Figure VID in the online-only Data Supplement).

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**Figure 6.** Simultaneous recordings of membrane voltage ($V_m$) and $[Ca^{2+}]_i$ in atrial myocytes from sinus rhythm (Ctl) and chronic atrial fibrillation (cAF) patients. A. Current-clamp protocol (0.5 Hz; top) together with simultaneous recordings of triggered action potential (AP; middle) and $Ca^{2+}$-transients (CaT) (CaT; Fluo-3; bottom) in a Ctl (left) and a cAF (right) myocyte. B, Mean±SEM resting membrane potential, AP amplitude, APD$_{50}$, and APD$_{90}$. C, Mean±SEM diastolic and systolic $[Ca^{2+}]_i$ levels (left) and resulting CaT amplitude (right). *P<0.05 vs corresponding means in Ctl. Numbers indicate myocytes/patients.
Here, we detailed the Ca$^{2+}$/H11001 signaling mechanisms leading to cellular ectopic activity in cAF. cAF myocytes showed greater SR Ca$^{2+}$/H11001 leak for any given SR Ca$^{2+}$/H11001 content as a consequence of a CaMKII-mediated increase in Po of RyR2. Inhibition of CaMKII normalized RyR2 Po and SR Ca$^{2+}$/H11001 leak, suggesting a direct link between defective RyR2 function and enhanced SR Ca$^{2+}$/H11001 leak. Occurrence of SCaEs and potentially arrhythmogenic DADs was increased in cAF. A given [Ca$^{2+}$]i generated greater depolarizing INCX and a stronger Vm depolarization in cAF, suggesting increased diastolic [Ca$^{2+}$]i-Vm coupling gain as a mechanism of increased ectopic activity in cAF. CaMKII inhibition reduced the AF-related increase in SCaE occurrence. Phosphomimetic S2814D mice showed enhanced SR Ca$^{2+}$/H11001 leak and increased susceptibility to pacing-induced AF, validating the direct mechanistic link between CaMKII-phosphorylated RyR2 and atrial arrhythmogenesis in clinical AF.

Comparison With Previous Studies

In previous work, atrial tissue and myocytes from cAF patients showed abnormal SR Ca$^{2+}$/H11001 handling$^{9,10,13,16,26}$ and increased incidence of Ca$^{2+}$/H11001 sparks$^{9,10}$ pointing to enhanced SR Ca$^{2+}$/H11001 leak, a well-established contributor to cardiac arrhythmogenesis.$^{11,12}$ Because SR Ca$^{2+}$/H11001 content was not increased$^{9,10}$ and RyR2 was hyperphosphorylated at Ser2808 (PKA and CaMKI site) and Ser2814 (CaMKII site)$^{10,13,16}$ it was assumed that the higher Ca$^{2+}$/H11001 spark frequency results from altered RyR2 function. Consistent with this hypothesis, inhibition of CaMKII, which is enhanced in cAF (see below)$^{16}$ reduced Ca$^{2+}$/H11001 spark frequency in cAF myocytes$^{10}$ suggesting RyR2 hyperphosphorylation as a major mechanism of increased Ca$^{2+}$/H11001 sparks frequency in cAF. However, the direct contribution of RyR2 to SR Ca$^{2+}$/H11001 leak and the mechanistic link between CaMKII, RyR2 dysfunction, and SR Ca$^{2+}$/H11001 leak underlying AF pathogenesis were not addressed.

RyR2 hyperphosphorylation sensitizes RyR2 channels to Ca$^{2+}$/H11001, and here, we found increased Ca$^{2+}$/H11001 sensitivity along with enhanced Po of RyR2 in cAF. Increased Po was due to increased frequency of openings, with no change in open time. These data, the first direct demonstration of RyR2-mediated SR Ca$^{2+}$/H11001 leak and increased susceptibility to pacing-induced AF, validating the direct mechanistic link between CaMKII-phosphorylated RyR2 and atrial arrhythmogenesis in clinical AF.
INCX that may produce DADs. Expression of NCX1 is increased in sheep and patients with cAF, and [Ca\(^{2+}\)]\(_i\); I\(_{\text{NCX}}\) coupling gain is increased in cAF sheep, although SR Ca\(^{2+}\) leak was not studied in this model. Here, we show an increase in [Ca\(^{2+}\)]\(_i\)–I\(_{\text{NCX}}\) coupling gain in cAF myocytes, which, along with the higher SR Ca\(^{2+}\) leak, should increase the propensity for arrhythmogenic DADs/triggered activity.

Although the increased incidence of Ca\(^{2+}\) sparks in cAF patients was postulated to predispose to DADs/triggered activity by producing larger depolarizing I\(_{\text{NCX}}\), we are not aware of any study demonstrating higher susceptibility to DADs in clinical AF. In this study, we conducted for the first time simultaneous recordings of Ca\(^{2+}\) and membrane current or potentials, respectively, in human atrial myocytes. We observed 42% lower IC\(_{\text{aL}}\) and 23% shorter APD\(_{90}\), 2 hallmarks of atrial remodeling in cAF patients. In addition, we detected 50% and 48% lower amplitudes of IC\(_{\text{aL}}\) and AP-triggered CaT, respectively, and a tendency of diastolic [Ca\(^{2+}\)]\(_i\) to be higher in cAF than in Ctrl, which was consistent with previous results. Most important, we found that myocytes from cAF patients have increased SCaE frequency and amplitude accompanied by greater I\(_{\text{NCX}}\) currents than Ctrl myocytes. The magnitude of membrane depolarization resulting from SCaE-induced I\(_{\text{NCX}}\) depends not only on the amplitude of SCaEs but also on the latency of the SCaE from the last regular CaT. Consistent with the greater leakiness of RyR2 in cAF, the coupling interval to the first SCaE was significantly shorter in cAF compared with Ctrl myocytes, suggesting that the intrinsic ability of the Ca\(^{2+}\) release mechanism to become refractory after release is impaired in cAF. These findings shed light on the factors that govern the rate of cytosolic SR Ca\(^{2+}\) release during SCaEs.

In normal hearts, plasmalemmal safety factors constrain the ability of SCaEs to promote DADs, protecting the heart against arrhythmias. We demonstrated more frequent burst emergence of SCaEs in cAF, accompanied by V\(_{\text{m}}\) oscillations in the form of DADs and triggered APs. The V\(_{\text{m}}\) oscillations disappeared after NCX inhibition with Ni\(^{2+}\), whereas SCaEs remained unaffected, indicating cause-effect relationships among SCaEs, NCX, and DADs. The size of a DAD depends on at least 2 factors: the latency and amplitude of SCaE and the sensitivity of resting V\(_{\text{m}}\) to [Ca\(^{2+}\)]\(_i\). Here, we detected a 5-fold increase in [Ca\(^{2+}\)]\(_i\)–V\(_{\text{m}}\) coupling gain in cAF compared with Ctrl. The [Ca\(^{2+}\)]\(_i\)–V\(_{\text{m}}\) coupling gain is determined by the amplitude of depolarizing I\(_{\text{NCX}}\) and membrane resistance, set by background conductances like the inward rectifier potassium current I\(_{\text{K1}}\), with enhanced I\(_{\text{NCX}}\) and/or reduced I\(_{\text{K1}}\), both promoting DADs. Expression of NCX1 and [Ca\(^{2+}\)]\(_i\)-corrected I\(_{\text{NCX}}\) amplitude are greater in cAF than in Ctrl; however, I\(_{\text{K1}}\) is upregulated in cAF patients, suggesting that augmented SCaEs and increased I\(_{\text{NCX}}\) rather than reduced I\(_{\text{K1}}\), accounts for the stronger [Ca\(^{2+}\)]\(_i\)–V\(_{\text{m}}\) coupling gain in cAF. In addition, the relative contribution of SERCA to Ca\(^{2+}\) removal from the cytosol was decreased whereas the relative contribution of NCX was increased in cAF, suggesting that a larger part of the SR Ca\(^{2+}\) leak and SCaEs is removed by NCX, which might contribute to the increased NCX function in cAF. Further extensive experimentation is needed to dissect the molecular determinants of increased diastolic [Ca\(^{2+}\)]\(_i\)–V\(_{\text{m}}\) coupling gain in cAF.

**Novel Findings and Potential Significance**

Atrial remodeling is a key element of the AF-maintaining substrate, and emerging evidence suggests a critical involvement of abnormal subcellular Ca\(^{2+}\) signaling. Our study shows the existence of RyR2-mediated SR Ca\(^{2+}\) leak and upregulated NCX in cAF, which predispose to diastolic SCaEs, enhancing the susceptibility to cellular DADs/triggered activity in cAF. Defective RyR2 and upregulated I\(_{\text{NCX}}\) likely contribute to atrial arrhythmogenesis in vivo by creating an arrhythmogenic substrate and acting as triggers for AF. We suggest that the CaMKII-mediated increase in SR Ca\(^{2+}\) leak in cAF creates an arrhythmogenic substrate by increasing the susceptibility to SCaEs. Additionally, due to the increased coupling gain between diastolic [Ca\(^{2+}\)]\(_i\) and membrane potential, SCaEs generate a larger NCX, providing a trigger for AF.
Here, we noted increased protein expression and stimulatory Thr287 phosphorylation and reduced inhibitory Thr306 phosphorylation of CaMKII in cAF compared with Ctl. The mRNA levels of CaMKII\(\delta\)C were unchanged, suggesting a posttranscriptional mechanism. The increased atrial expression and Thr287 phosphorylation of CaMKII\(\delta\)C are typical for dogs with atrial tachycardia remodeling\(^{18}\) and goats with sustained AF,\(^{17}\) suggesting that the greater CaMKII activity might be a consequence of the high atrial rate during AF. Atrial dilatation in goats\(^{17}\) and ventricular tachypacing–induced heart failure in dogs\(^{41,42}\) commonly used models of AF, are also associated with increased expression and Thr287 phosphorylation of CaMKII\(\delta\)C. Thus, it is possible, but not proven, that an increase in CaMKII activity may also be a risk factor for AF, suggesting that CaMKII activation may be both a cause and a consequence of AF. This finding adds to our appreciation of the phenomenon of AF begets AF, indicating that, in addition to the well-established tendency of AF to promote its own reentrant substrate, it can enhance spontaneous ectopic activity, which induces reentry.

Abnormal Ca\(^{2+}\) handling can contribute to atrial arrhythmogenesis through multiple mechanisms. For instance, changes in Ca\(^{2+}\) homeostasis modify the function of ion channels and the shape and dynamics of the AP, creating tissue properties (vulnerable substrate) that may initiate and maintain reentry. SCaEs may cause subcellular Ca\(^{2+}\) alternans and abrupt repolarization changes that may increase the dispersion of atrial refractoriness, facilitating reentry.\(^{42}\) Thus, the Ca\(^{2+}\) handling abnormalities we observed may promote AF by mechanisms additional to triggered activity, a notion that merits assessment in future studies.

**Potential Limitations**

We focused on the role of abnormal subcellular Ca\(^{2+}\) signaling for atrial arrhythmogenesis, but these alterations may critically contribute to AF-related atriohypocontactility and myofila-

ment dysfunction.\(^{43–45}\) SCaEs can impair contractile function by causing dys synchrony of myocyte contraction. The potential link of altered subcellular Ca\(^{2+}\) signaling to impaired myocyte contraction should be a subject of future investigations.

For myocyte isolation, we used only right atrial tissue collected from only 1 region (right atrial appendages). Thus, our findings may not apply fully to other atrial regions. In statistical comparisons, patients may have contributed >1 observation to each subanalysis, suggesting that observations are not necessarily independent. Within-patient correlations were not taken into account in statistical comparisons because of the small sample size, so our results should be interpreted with caution.

We used 5 mmol/L extracellular Ca\(^{2+}\) to provoke SCaE. These conditions test SCaE vulnerability rather than occurrence under normal physiological conditions. However, many AF patients do not immediately show atrial ectopy or AF recurrence immediately after cardioversion, indicating that they likely have a latent predisposition to develop arrhythmia, perhaps related to changes in autonomic tone or other factors. Our results provide, for the first time, insights into the magnitude and dynamics of SCaEs and underlying factors in humans, essential information to understand why an increase in SR Ca\(^{2+}\) leak and SCaE occurrence are potentially arrhythmogenic in AF patients.

In the subanalysis including SCaEs-positive myocytes only, Ca\(^{2+}\) sensitivity of \(I_{\text{SCa}}\) appeared comparable between Ctl and cAF. However, because Ca\(^{2+}\) is a strong modulator of NCX function\(^{46}\) and these experiments were carried out primarily at 5 mmol/L extracellular Ca\(^{2+}\) to test SCaE vulnerability, the complex patterns of Ca\(^{2+}\) modulation of NCX under these conditions might have masked the differences in Ca\(^{2+}\) sensitivity of NCX between Ctl and cAF that we detected using caffeine in unselected atrial myocytes and at physiological extracellular Ca\(^{2+}\). Because our results with caffeine showing increased Ca\(^{2+}\) sensitivity of \(I_{\text{SCa}}\) were consistent with recent work in sheep with persistent AF,\(^{44}\) increased Ca\(^{2+}\) sensitivity of \(I_{\text{SCa}}\) might be a typical finding in myocytes during persistent AF.

Previous results from perforated-patch–clamped human atrial myocytes showed that Ca\(^{2+}\) waves disappear 10 minutes after patch break\(^{47}\) and that H-89 reduces the frequency of \(I_{\text{c}}\) currents,\(^{48}\) suggesting a role for baseline cAMP formation and PKA activation.\(^{49,50}\) Here, we did not detect a contribution of PKA to the increased SR Ca\(^{2+}\) leak in cAF. One reason could be that cAMP was washed out of the myocyte in the ruptured-patch whole-cell configuration. However, we detected a 2-fold increase in cAMP levels in cAF (Figure VIII in the online-only Data Supplement) and found that application of isoprenaline and inhibition of phosphodiesterases with isobutylmethylxanthine increased \(I_{\text{Ca,L}}\) and CaT amplitudes in Ctl and cAF myocytes without significant differences in magnitude between the rhythm groups (Figures VIII and IX in the online-only Data Supplement). Because cAMP and PKA are essential for both signals, washout of cAMP is an unlikely explanation for the lack of any effect of PKA inhibition in Figures 3 and 4. In addition, we directly tested the specific contributions of CaMKII and PKA to the enhanced susceptibility to SCaEs and the generation of DADs in perforated-patch–clamped myocytes with preserved cAMP levels. We found that enhanced SCaEs/DADs persisted in cAF myocytes in the presence of PKA inhibition in Figures 3 and 4. In addition, we directly tested the specific contributions of CaMKII and PKA to the enhanced susceptibility to SCaEs and the generation of DADs in perforated-patch–clamped myocytes with preserved cAMP levels. We found that enhanced SCaEs/DADs persisted in cAF myocytes in the presence of PKA inhibition in H-89 and that subsequent CaMKII inhibition with KN-93 reduced the frequency of SCaEs/DADs. Perhaps because of the limited number of cells available for these experiments, although these studies all provided results consistent with the more extensive experiments shown elsewhere in this article, some differences between groups did not achieve formal statistical significance (Figures V and VI in the online-only Data Supplement), which needs to be considered in their interpretation.

Our approach of preincubating the myocytes with H-89 did not allow us to establish baseline SCaE incidence, preventing an evaluation of the immediate effects of PKA inhibition on SCaEs. However, because SCaEs were seen in \(\approx 83\%\) of cAF myocytes incubated in H-89 (Figure VI in the online-only Data Supplement), PKA activity is clearly not essential to SCaE expression. A more systematic approach and much larger number of cells are needed to determine precisely the role of PKA in SCaE occurrence under physiological conditions in human atrial myocytes. Although interesting and relevant, the required work is beyond the scope of the present study. Furthermore, our finding of a primary role for CaMKII in the RyR2 abnormalities and in the specific AF-related DAD substrate notwithstanding, cAMP and PKA contribute importantly to determining atrial myocyte Ca\(^{2+}\) entry and SR Ca\(^{2+}\) load. Thus, although PKA phosphorylation may not importantly control intrinsic RyR2 abnormalities, it could certainly contribute to DAD generation and arrhythmias in
AF patients by enhancing SR Ca\textsuperscript{2+} loading and unmasking their vulnerability to DADs/triggered activity.

Finally, transgenic mouse models are useful research tools, but they do not phenocopy all important aspects of clinical AF. Nevertheless, they are quite useful because it is difficult to assess directly the specific mechanisms controlling CaMKII and PKA phosphorylation of RyR2 and their consequences in AF patients. Further extensive work in large-animal AF models is required to define the specific contributions of CaMKII and PKA to AF pathophysiology under conditions similar to those causing AF in humans.

Conclusions

Increased diastolic RyR2-mediated SR Ca\textsuperscript{2+} leak, together with upregulated NCX and enhanced diastolic [Ca\textsuperscript{2+}]\textsubscript{i},\textsubscript{V_m} coupling gain, predisposes to cellular DADs/triggered activity, contributing to the pathogenesis of human AF. Recent\textsuperscript{16,25} and present work validated the mechanistic link between dysfunctional RyR2, along with underlying CaMKII hyperphosphorylation, and susceptibility to AF, highlighting the importance of molecular RyR2 defects for AF pathobiology. Although the contribution of these cellular Ca\textsuperscript{2+}-mediated proarrhythmic events to atrial foci in AF patients in vivo has to be established, the development of new drugs specifically targeting diastolic SR Ca\textsuperscript{2+} leak might open novel therapeutic avenues to prevent atrial arrhythmogenesis by normalizing SR Ca\textsuperscript{2+} cycling.

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Disclosures

None.

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Enhanced Sarcoplasmic Reticulum Ca\textsuperscript{2+} Leak and Increased Na\textsuperscript{+}-Ca\textsuperscript{2+} Exchanger Function Underlie Delayed Afterdepolarizations in Patients With Chronic Atrial Fibrillation

Niels Voigt, Na Li, Qiongling Wang, Wei Wang, Andrew W. Trafford, Issam Abu-Taha, Qiang Sun, Thomas Wieland, Ursula Ravens, Stanley Nattel, Xander H.T. Wehrens and Dobromir Dobrev

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Enhanced Sarcoplasmic Reticulum Ca\(^{2+}\)-Leak and Increased Na\(^+\)-Ca\(^{2+}\)-Exchanger Function Underlie Delayed Afterdepolarizations in Patients with Chronic Atrial Fibrillation

Niels Voigt, MD; Na Li, PhD, Qiongling Wang, PhD; Wei Wang, PhD; Andrew W. Trafford, PhD; Issam Abu-Taha, BSc; Qiang Sun, PhD; Thomas Wieland, PhD; Ursula Ravens, MD; Stanley Nattel, MD; Xander HT. Wehrens, MD PhD; Dobromir Dobrev, MD

SUPPLEMENTAL MATERIAL

Methods

Measurement of Intracellular [Ca\(^{2+}\)] and Patch-clamp Experiments

Membrane currents and potentials were measured at 37°C in whole-cell ruptured-patch configuration using voltage-clamp and current-clamp techniques with simultaneous intracellular [Ca\(^{2+}\)] measurement. pClamp-Software (V10.2, Molecular Devices, Sunnyvale, CA) was used for data acquisition and analysis.

Intracellular [Ca\(^{2+}\)] was quantified using Fluo-3-acetoxymethyl ester (Fluo-3 AM; Invitrogen, Carlsbad, CA; 10 µmol/L, 10 min loading and 30 min de-esterification). In addition Fluo-3 was included into the electrode solution containing (in mmol/L): EGTA 0.02, Fluo-3 0.1 (Invitrogen), GTP-Tris 0.1, HEPES 10, K-aspartate 92, KCl 48, Mg-ATP 1, Na\(_2\)-ATP 4; pH=7.2.

Borosilicate glass microelectrodes had tip resistances of 2-5 MΩ when filled with pipette solution. Seal-resistances were 4-8 GΩ. For voltage-clamp experiments series resistance and cell capacitance were compensated.
During experiments myocytes were superfused at 37°C with a bath solution containing (in mmol/L): CaCl$_2$ 2, glucose 10, HEPES 10, KCl 4, MgCl$_2$ 1, NaCl 140, probenecid 2; pH=7.4. For voltage-clamp experiments, K$^+$ currents were blocked by adding 4-aminopyridine (5 mmol/L) and BaCl$_2$ (0.1 mmol/L) to the bath solution.

L-type Ca$^{2+}$-current ($I_{Ca,L}$) and corresponding triggered [Ca$^{2+}$]-transients (CaTs) were recorded simultaneously, using a holding potential of -80 mV and a 100-ms ramp-pulse to -40 mV to inactivate the fast Na$^+$-current followed by a 100-ms test-pulse to +10 mV at 0.5 Hz. Action potentials were stimulated at 0.5 Hz using 1 ms current pulses of 1.2x threshold strength. Caffeine (10 mmol/L) was used for quantification of SR Ca$^{2+}$-content.$^2$ Isoprenaline (1 µmol/L) and the phosphodiesterase-inhibitor 3-isobutyl-1-methylxanthine (IBMX, 10 µmol/L) were used to increase cAMP-formation.

**Current-Clamp Recordings in Perforated-patch Configuration**

In **Online-Figures VI and VII** the amphotericin-perforated-patch technique was used to avoid dialysis of cytosolic components and concomitant changes in Ca$^{2+}$ handling properties. The composition of the pipette solution was (mmol/L): EGTA 0.02, GTP-Tris 0.1, HEPES 10, K-aspartate 92, KCl 48, Mg-ATP 1, Na$_2$-ATP 4; pH=7.2. On experiment days amphotericin (Sigma-Aldrich, St. Louis, MO) was added to the pipette solution at a final concentration of 240 µg/mL from a 60 mg/mL DMSO stock solution. Intracellular [Ca$^{2+}$] was quantified using Fluo-3-acetoxymethyl ester (Fluo-3 AM; 10 µmol/L, 10 min loading and 30 min de-esterification).$^1$ During experiments myocytes were superfused at 37°C with a bath solution containing (in mmol/L): CaCl$_2$ 2, glucose 10, HEPES 10, KCl 4, MgCl$_2$ 1, NaCl 140, probenecid 2; pH=7.4. Action potentials were stimulated in current-clamp configuration at 0.5 Hz using 1 ms current pulses of 1.2x threshold strength. No series resistance compensation was employed.
Quantification of Diastolic SR Ca\textsuperscript{2+} Leak with Tetracaine

SR Ca\textsuperscript{2+}-leak in intact myocytes was measured according to Shannon et al.\textsuperscript{3} using Na\textsuperscript+- and Ca\textsuperscript{2+}-free bath solution (in mmol/L: 4-aminopyridine 5, BaCl\textsubscript{2} 0.1, EGTA 10, glucose 10, HEPES 10, KCl 4, LiCl\textsubscript{2} 140, Mg\textsubscript{2}Cl 1, probenecid 2) and tetracaine (1 mmol/L). KN-93 (1 µmol/L; EMD Chemicals, Gibbstown, NJ) and H-89 (1 µmol/L EMD Chemicals) were used as blockers of CaMKII and PKA, respectively. The inactive KN-93 analogue KN-92 (1 µmol/L; EMD Chemicals) was used as negative control.

RyR2 Single-channel Recordings

Single-channel recordings were obtained under voltage-clamp conditions at 0 mV, as previously described.\textsuperscript{4} Atrial SR membrane-preparations were incorporated into lipid-bilayer membranes comprised of a mixture of phosphatidylethanolamine and phosphatidylserine at a ratio of 3:1 (Avanti Polar Lipids, Alabaster, AL) dissolved in n-decane (25 mg/ml). Bilayers were formed across a 150 µm aperture of a polystyrene cuvette. The cis and trans chambers correspond to the cytosolic and the luminal sides of the SR, respectively. The trans chamber contained (in mmol/L) HEPES 250, KCl 50 and Ca(OH)\textsubscript{2} 53. The cis chamber contained (in mmol/L) HEPES 250, Tris-base 125, KCl 50, EGTA 1, CaCl\textsubscript{2} 0.5, pH=7.35. Ca\textsuperscript{2+}-activation curves were generated by varying [Ca\textsuperscript{2+}] in the cis compartment. Ryanodine (5 µmol/L) was applied to the cis chamber to confirm identity of RyR2 channels at the end of each experiment. KN-93 (10 µmol/L, EMD Chemicals), KN-92 (10 µmol/L, EMD Chemicals), H-89 (10 µmol/L, Sigma-Aldrich), and PKI (10 µmol/L, EMD Chemicals) were applied to the cis chamber and mixed with the buffer for 5 min before channel tracings were recorded. Data were collected using Digidata 1322A (Molecular Devices, Sunnyvale, CA) and Warner Bilayer Clamp Amplifier BC-535 (Warner Instruments, Hamden, CT) under voltage-clamp conditions. Cytosolic free [Ca\textsuperscript{2+}] was calculated with WinMax32. Data were analyzed...
from digitized current recordings using pCLAMP-9.2 software (Molecular Devices). Clampfit-9.2 was used to create amplitude histograms from one representative single-channel trace (10 kHz sampling rate, 0.2 pA bin-width) from a Ctl and a cAF patient, respectively.

**Ca^{2+}-spark Measurements in Atrial Myocytes From S2814D Knock-in Mice**

Animal studies were performed according to protocols approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine. Mouse atrial myocytes were isolated as previously described. The heart was removed and blood rinsed out with 0-Ca^{2+} Tyrode solution (in mmol/L: NaCl 137, KCl 5.4, MgCl_{2} 1, HEPES 5, glucose 10, NaOH 3, pH=7.4). Hearts were cannulated through the aorta and perfused on a Langendorff apparatus with 0-Ca^{2+} Tyrode for 3-5 minutes at 37°C, followed by 0-Ca^{2+} Tyrode solution containing 20 μg/mL Liberase (Roche, Indianapolis, IN) for 10-15 minutes at 37°C. After digestion, hearts were perfused with 5 mL KB solution (in mmol/L: KCl 90, K_{2}HPO_{4} 30, MgSO_{4} 5, pyruvic acid 5, β-hydroxybutyric acid 5, creatine 5, taurine, glucose 10, EGTA 0.5, HEPES 5, pH=7.2). Both left and right atrium were minced in KB solution and gently agitated, then filtered through a 210 µm polyethylene mesh. Atrial myocytes were stored in KB solution at room temperature before use.

Only rod-shaped myocytes showing clear striations were studied. Atrial myocytes were loaded with 2 μmol/L Fluo-4-AM (Invitrogen, Carlsbad, CA) in normal Tyrode solution containing 1.8 mmol/L Ca^{2+} for 30 minutes at room temperature. Cells were then washed with dye-free Tyrode solution for 15 minutes for de-esterification and transferred to a chamber equipped with a pair of parallel platinum electrodes. The chamber was placed on a LSM510 confocal microscope (Carl Zeiss, Thornwood, NY). Fluorescence images were recorded in line-scan mode with 1024 pixels per line at 500 Hz. Once steady-state Ca^{2+}-transients during 1 Hz-pacing (20 V) were observed, pacing
was stopped for 45 seconds and Ca\(^{2+}\) sparks were counted. KN-93 (10 \(\mu\)mol/L, EMD Chemicals) was applied to inhibit CaMKII. Steady-state SR Ca\(^{2+}\)-content was assessed by rapid application of 10 mmol/L caffeine.

**Immunoblot Analysis**

The protein levels of calmodulin (1:1000; abcam, Cambridge, UK), total CaMKII\(\delta\) (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), Thr287 and Thr306/Thr307 phosphorylated CaMKII\(\delta\) (1:5000; Promega, Madison, WI and 1:2500; biomol, Hamburg, Germany, respectively), calsequestrin (CSQ, 1:2500; Dianova, Hamburg, Germany), GAPDH (1:100000; HyTest, Turku, Finland), junctin and triadin (JNC, 1:100; TRD, 1:1000; kind gifts from Dr. Uwe Kirchhefer, Münster, Germany), NCX1 (1:500; Fitzgerald, Concord, MA); catalytic PKA-subunit (PKAc, 1:1000; BD Biosciences, Franklin Lakes, NJ); regulatory PKA\(\text{II}\)\(\alpha\)-subunit (PKA\(\text{II}\)\(\alpha\), 1:500, Santa Cruz Biotechnology), total RyR2 (1:3000, Affinity BioReagents, Golden, CO), Ser2808 and Ser2814 phosphorylated RyR2 (1:3000 and 1:1000, respectively)\(^6,7\) were quantified by Western blotting as described.\(^8,9\) The RyR2-Ser2808 and RyR2-Ser2814 phosphoepitope-specific antibodies were custom generated using the peptide C-RTRRI-(pS)-QTSQV corresponding to the PKA phosphorylation site region at Ser2808 on RyR2 and peptide CSQTSQV-(pS)-VD corresponding to CaMKII phosphorylated RyR2 at Ser2814. Appropriate peroxidase-conjugated goat anti-rabbit (Sigma-Aldrich), goat anti-mouse (Sigma-Aldrich) and donkey anti-goat (Santa-Cruz) were used as secondary antibodies and visualized by chemiluorescense (GE Healthcare, Chalifont St. Giles, UK). Quantity One Software (Bio-Rad, Hercules, CA) was used for quantification.\(^8\) Protein expression was normalized to CSQ and GAPDH, respectively, which were unchanged in cAF compared to SR samples.
Reverse Transcription and Quantitative Real-time PCR

Total RNA was isolated from human heart tissue samples using a RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. cDNA was synthesized with RevertAid First-Strand cDNA synthesis kit (Fermentas, St. Leon-Rot, Germany). The reaction mix consisted of 10 U/μL of reverse transcriptase, 1 U/μL of RNase inhibitor, 1 mmol/L dNTP, 5 μmol/L random primers, and 0.2 μg RNA in 20 μL total volume. The reaction mixture was incubated at 25°C for 10 min and then at 42°C for 60 min. Finally, the mixture was heated at 70°C for 5 min. Real-time PCR was performed by using 2X Taqman Universal PCR mix (Applied Biosystems, Foster City, CA) with commercial primers (NCX1: Hs01062258_m1; CaMKIIβ: Hs00945363_m1; CaMKIIα: Hs00949946_m1; HPRT1: Hs01003267_m1; Applied Biosystems, Foster City, CA) on an ABI7500 Fast real-time PCR system for 20 sec at 95°C, followed by 40 cycles for 3 sec at 95°C and 30 sec at 60°C. Relative amount of target gene in each sample was calculated by the PCR system using a series 1:2 dilution of sample pool as standard curve. Mean value of relative amount for each gene was calculated from duplicate measure of each sample and normalization is performed to get ratio of mean value of the target gene to that of housekeeping gene in corresponding sample.

cAMP Assay

The cAMP content of frozen atria was determined as described before. In brief, after homogenization of the tissue in the presence of 5% trichloroacetic acid (TCA) and 1 mmol/L 3-isobutyl-1-methylxanthine (IBMX), the insoluble debris pellets were removed by centrifugation at 1,500 x g for 10 min. Thereafter, the remaining TCA was extracted from the supernatant by using water-saturated ether. Twenty-five μl of the supernatant were then used for the competitive cAMP enzyme immunoassay (Cayman Chemical Company, Ann Arbor, MI) according to the manufacturer's recommendations. Protein
concentrations were determined by the bicinechonic acid (BCA) protein assay kit (Pierce, Rockford, IL) after neutralization of the protein containing debris pellets with 0.1 mol/L NaOH.

**Ca\textsuperscript{2+} Transport via SERCA, NCX and Plasmalemmal Ca\textsuperscript{2+} ATPase (PMCA)**

During decay of I\textsubscript{Ca,L}-induced Ca\textsuperscript{2+}-transient (CaT) the Ca\textsuperscript{2+} is extruded from the cytosol by Ca\textsuperscript{2+} reuptake into the SR mediated via SR Ca\textsuperscript{2+}-ATPase (SERCA) and by Ca\textsuperscript{2+} extrusion into extracellular space via forward-mode Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (NCX) and plasmalemmal Ca\textsuperscript{2+} ATPase (PMCA). We estimated the relative contribution of these three mechanisms from the rate constants (k) of single exponential curves fitted to decaying parts of the I\textsubscript{Ca,L}- and caffeine-evoked CaTs, as described by Choi and Eisner.\textsuperscript{11}

The decay of I\textsubscript{Ca,L}-induced CaT (Online-Figure IIIA and B) was fitted by an exponential curve:

\[
[\text{Ca}^{2+}]_{t}(t) = ([\text{Ca}^{2+}]_{\text{Amplitude}} \cdot e^{-k_{\text{sys}} \cdot t}) + [\text{Ca}^{2+}]_{\text{diast}} \tag{1}
\]

where \([\text{Ca}^{2+}]_{t}(t), [\text{Ca}^{2+}]_{\text{Amplitude}} \text{ and } [\text{Ca}^{2+}]_{\text{diast}}\) represent the Ca\textsuperscript{2+} concentration at the timepoint \(t\), the CaT amplitude and the diastolic Ca\textsuperscript{2+} level, respectively. The rate constant of decay \(k_{\text{sys}}\) is the reciprocal value of the time-constant of decay \((\tau), \quad k_{\text{sys}} = \frac{1}{\tau}\) and reflects the rate of combined Ca\textsuperscript{2+} transport by SERCA, NCX and PMCA. Therefore \(k_{\text{sys}}\) may be also expressed as the sum of the three separate rate constants:

\[
k_{\text{sys}} = k_{\text{SERCA}} + k_{\text{NCX}} + k_{\text{PMCA}} \tag{2}
\]

Application of caffeine leads to rapid depletion of SR Ca\textsuperscript{2+}, which is reflected by the caffeine-induced CaT (cCaT, Online-Figure IIIA and B):

\[
[\text{Ca}^{2+}]_{t}(t) = ([\text{Ca}^{2+}]_{\text{cCaT-Ampl}} \cdot e^{-k_{\text{caff}} \cdot t}) + [\text{Ca}^{2+}]_{\text{diast}} \tag{3}
\]

with “\([\text{Ca}^{2+}]_{\text{cCaT-Ampl}}\)” representing the amplitude of cCaT and “\(k_{\text{caff}}\)” the rate constant of
decay of cCaT. Since caffeine prevents SR Ca\(^{2+}\) reaccumulation via SERCA (Ca\(^{2+}\) transport by SERCA under caffeine = 0, Ref.), the decay of cCaT depends largely on Ca\(^{2+}\) extrusion via NCX and PMCA:

\[
k_{\text{caff}} = k_{\text{NCX}} + k_{\text{PMCA}}
\]  

Using this approach the contribution of SERCA to the decay of \(I_{\text{Ca,L}}\)-induced CaT can be estimated by subtracting the rate constant of cCaT (4) from the rate constant of \(I_{\text{Ca,L}}\)-induced CaT (Formula 2, Online-Figure III):  

\[
k_{\text{SERCA}} = k_{\text{syst}} - k_{\text{caff}}
\]  

For further evaluation of NCX and PMCA transport rates, contribution of NCX was additionally blocked by perfusion with Na\(^+\) and Ca\(^{2+}\) free bath solution. Under these conditions the rate constant of cCaTs “\(k_{\text{Na0Ca0}}\)” reflects the rate of Ca\(^{2+}\) transport by PMCA only (Online-Figure IIIA and B):  

\[
k_{\text{Na0Ca0}} = k_{\text{PMCA}}
\]  

The rate constant of NCX can now be estimated by subtracting \(k_{\text{Na0Ca0}}\) (6) from \(k_{\text{caff}}\) (Online-Figure IIII):

\[
k_{\text{NCX}} = k_{\text{caff}} - k_{\text{Na0Ca0}}
\]  

Relative contributions (Online-Figure IIII) were calculated by dividing the rate constant of the respective Ca\(^{2+}\) removal mechanism \((k_{\text{SERCA}}, k_{\text{NCX}}, k_{\text{PMCA}})\) by the rate constant of the \(I_{\text{Ca,L}}\)-induced CaT \((k_{\text{syst}})\).
## Tables

### Online-Table I: Characteristics of patients used for functional studies

<table>
<thead>
<tr>
<th></th>
<th>Ctl</th>
<th>cAF</th>
</tr>
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<tbody>
<tr>
<td>Patients, n</td>
<td>41</td>
<td>31</td>
</tr>
<tr>
<td>Gender, m/f</td>
<td>33/8</td>
<td>22/9</td>
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<tr>
<td>Age, y</td>
<td>69.6±1.5</td>
<td>69.7±1.4</td>
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<tr>
<td>Body mass index, kg/m²</td>
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<td>27.4±0.8</td>
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<tr>
<td>CAD, n</td>
<td>28</td>
<td>9**</td>
</tr>
<tr>
<td>MVD/AVD, n</td>
<td>7</td>
<td>13*</td>
</tr>
<tr>
<td>CAD+MVD/AVD, n</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>Hypertension, n</td>
<td>39</td>
<td>29</td>
</tr>
<tr>
<td>Diabetes, n</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>Hyperlipidemia, n</td>
<td>26</td>
<td>15</td>
</tr>
<tr>
<td>LVEF, %</td>
<td>49.3±2.6</td>
<td>48.3±2.5</td>
</tr>
<tr>
<td>Digitalis, n</td>
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<td>3</td>
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<tr>
<td>ACE inhibitors, n</td>
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<td>24</td>
</tr>
<tr>
<td>AT1 blockers, n</td>
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<td>2</td>
</tr>
<tr>
<td>β-Blockers, n</td>
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<td>25</td>
</tr>
<tr>
<td>Dihydropyridines, n</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>Diuretics, n</td>
<td>10</td>
<td>14</td>
</tr>
<tr>
<td>Nitrates, n</td>
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<td>4</td>
</tr>
<tr>
<td>Lipid-lowering drugs, n</td>
<td>25</td>
<td>13</td>
</tr>
</tbody>
</table>

CAD, coronary artery disease; MVD/AVD, mitral/aortic valve disease; LVEF, left ventricular ejection fraction; ACE, angiotensin-converting enzyme; AT, angiotensin receptor.

*P<0.05 and **P<0.01 vs. SR from Fisher’s exact test for categorical variables.
### Online-Table II: Characteristics of patients used for biochemistry

<table>
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<tr>
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<th>Ctl</th>
<th>cAF</th>
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</thead>
<tbody>
<tr>
<td>Patients, n</td>
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</tr>
<tr>
<td>Gender, m/f</td>
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<td>24/17</td>
</tr>
<tr>
<td>Age, y</td>
<td>66.5±1.7</td>
<td>68.9±1.3</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>29.5±1.0</td>
<td>28.5±0.7</td>
</tr>
<tr>
<td>CAD, n</td>
<td>18</td>
<td>7**</td>
</tr>
<tr>
<td>MVD/AVD, n</td>
<td>11</td>
<td>17</td>
</tr>
<tr>
<td>CAD+MVD/AVD, n</td>
<td>6</td>
<td>17*</td>
</tr>
<tr>
<td>Hypertension, n</td>
<td>27</td>
<td>36</td>
</tr>
<tr>
<td>Diabetes, n</td>
<td>12</td>
<td>14</td>
</tr>
<tr>
<td>Hyperlipidemia, n</td>
<td>26</td>
<td>29</td>
</tr>
<tr>
<td>LVEF, %</td>
<td>52.3±1.9</td>
<td>47.3±2.5</td>
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<tr>
<td>Digitalis, n</td>
<td>3</td>
<td>12*</td>
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<tr>
<td>ACE inhibitors, n</td>
<td>22</td>
<td>25</td>
</tr>
<tr>
<td>AT1 blockers, n</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>β-Blockers, n</td>
<td>21</td>
<td>34*</td>
</tr>
<tr>
<td>Dihydropyridines, n</td>
<td>5</td>
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<tr>
<td>Diuretics, n</td>
<td>13</td>
<td>26*</td>
</tr>
<tr>
<td>Nitrates, n</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>Lipid-lowering drugs, n</td>
<td>23</td>
<td>19</td>
</tr>
</tbody>
</table>

CAD, coronary artery disease; MVD/AVD, mitral/aortic valve disease; LVEF, left ventricular ejection fraction; ACE, angiotensin-converting enzyme; AT, angiotensin receptor.

*P<0.05 and **P<0.01 vs. SR from Fisher’s exact test for categorical variables.
Online-Figures and Figure Legends

**Online-Figure I.** **A,** Protein levels of total, Ser2814 and Ser2808 phosphorylated ryanodine receptor channels (RyR2) and **B,** Protein levels of calsequestrin, junctin, and triadin in atria of cAF vs. Ctl patients. *P*<0.05 vs. corresponding Ctl.
Online-Figure II. Protein levels and autophosphorylation status of Ca\(^{2+}\)/calmodulin-dependent protein-kinase II (CaMKII) at stimulatory (Thr287) and inhibitory (Thr306/Thr307) phosphorylation sites, and of calmodulin, and catalytic and regulatory protein-kinase A isoforms (PKA\(_c\) and PKA\(_\alpha\)_I\(_\kappa\), respectively) in atria of cAF vs. Ctl patients. *P<0.05 and **P<0.01 vs. corresponding Ctl.
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A

\[ [\text{Ca}^{2+}] \ (\mu M) \]

\[ k_{\text{syst}} \]

\[ k_{\text{caff}} \]

\[ k_{0\text{Na}0\text{Ca}} \]

B

\[ k_{\text{syst}} = k_{\text{SERCA}} + k_{\text{NCX}} + k_{\text{PMCA}} \]

\[ k_{\text{caff}} = k_{\text{NCX}} + k_{\text{PMCA}} \]

\[ k_{0\text{Na}0\text{Ca}} = k_{\text{PMCA}} \]

C

\[ k_{\text{SERCA}} = k_{\text{syst}} - k_{\text{caff}} \]

\[ k_{\text{NCX}} = k_{\text{caff}} - k_{0\text{Na}0\text{Ca}} \]

D

\begin{align*}
\text{Ctl} & \quad \text{cAF} \\
66\pm7\% & \quad 46\pm5\% \\
25\pm6\% & \quad 44\pm6\% \\
9\pm2\% & \quad 9\pm1\% \\
\end{align*}

\[ \text{SERCA} \ (p<0.05) \]

\[ \text{NCX} \ (p<0.05) \]

\[ \text{PMCA} \]
Online-Figure III: Estimation of Ca\textsuperscript{2+} transport rates of SERCA, NCX and PMCA. A, Representative recording to illustrate the experimental protocol used to estimate Ca\textsuperscript{2+} transport by SERCA, NCX and PMCA. Caffeine (10 mmol/L) application following steady-state stimulation (*) with I\textsubscript{Ca,L} activation at 0.5 Hz (1 minute) resulted in SR Ca\textsuperscript{2+} depletion reflected by caffeine induced Ca\textsuperscript{2+} transient (cCaT). After washout the same myocyte was stimulated again at 0.5 Hz with I\textsubscript{Ca,L} activation to regain SR Ca\textsuperscript{2+} load. Finally, caffeine (10 mmol/L) was applied again while perfusion with Na\textsuperscript{+}- and Ca\textsuperscript{2+}-free bath solution to quantify SERCA and NCX independent Ca\textsuperscript{2+} removal mechanisms, which are suggested to be largely mediated by PMCA. B, Mean±SEM of rate constants of monoexponential curves fitted to the decay of electrically stimulated CaT (k\textsubscript{syst}, left panel), caffeine evoked CaT (k\textsubscript{caff}, middle panel) and caffeine evoked CaT in absence of Na\textsuperscript{+} and Ca\textsuperscript{2+} in the bath solution (k\textsubscript{0Na0Ca}, right panel). C, Mean±SEM of calculated rate constants of SERCA (k\textsubscript{SERCA}) and NCX (k\textsubscript{NCX}). D, Relative contributions of SERCA, NCX and PMCA to diastolic Ca\textsuperscript{2+} removal. *P<0.05 vs. corresponding values in Ctl myocytes. Numbers indicate myocytes/patients. For further details see Supplemental methods section.
Online-Figure IV. A, Representative current amplitude histograms of RyR2 single-channel recordings from a Ctl and a cAF patient. B, Mean±SEM for open probability ($P_o$) of RyR2 before and during perfusion with the PKA-inhibitory peptide PKI (10 µmol/L). Numbers indicate channels/patients.
Online-Figure V. Simultaneous recordings of membrane voltage ($V_m$) and $[\text{Ca}^{2+}]_i$ in atrial myocytes from Ctl and cAF patients in perforated-patch configuration after incubation with the PKA-inhibitor H-89 (1 µmol/L; 30 mins). A, Current-clamp protocol (0.5 Hz, top) together with simultaneous recordings of triggered AP (middle) and CaT (Fluo-3, bottom) in a Ctl (left) and in a cAF (right) myocyte. B, Mean±SEM resting membrane potential, AP amplitude, APD$_{50}$ and APD$_{90}$, respectively. C, Mean±SEM diastolic and systolic $[\text{Ca}^{2+}]_i$ levels (left) and resulting CaT-amplitude (right). Numbers indicate myocytes/patients.
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Online-Figure VI. Incidence of SCaEs and corresponding DADs in atrial myocytes from Ctl and cAF patients in perforated-patch configuration after incubation with the selective PKA-inhibitor H-89 (1 µmol/L, 30 mins). A, Representative recordings of [Ca^{2+}]_i (Fluo-3) and corresponding membrane-voltage (V_m) oscillations (DADs/triggered APs) in a Ctl and a cAF-myocyte, respectively, following steady-state stimulation for 1-minute at 0.5 Hz. After 1-minute follow-up period the selective CaMKII-inhibitor KN-93 (10 µmol/L) was applied to cAF myocytes. B, Enhanced susceptibility to spontaneous Ca^{2+}-release events (SCaEs) and SCaE-induced DADs in cAF vs. Ctl. C, Mean±SEM for frequency (left) and latency (right) of SCaEs. D, Mean±SEM amplitude of SCaEs (top left), magnitude of corresponding V_m-change (top right), and the calculated [Ca^{2+}]-membrane voltage coupling gain (bottom). *P<0.05 vs. corresponding mean in Ctl. Numbers indicate myocytes/patients.
Online-Figure VII. Characteristics of Ca\(^{2+}\) sparks in atrial myocytes from WT and S2814D knock-in mice. Mean±SEM of SR Ca\(^{2+}\) content (F/F0) measured by rapid application of caffeine (10 mmol/L), spark amplitude (F/F0), full-width half-maximum (FWHM) and full-duration half-maximum (FDHM) of Ca\(^{2+}\) sparks in WT myocytes, S2814D myocytes and S2814D myocytes treated with KN-93 (10 μmol/L). Numbers indicate numbers of myocytes.
Online-Figure VIII. Effect of 3-isobutyl-1-methylxanthine (IBMX, 10 µmol/L) on \( I_{\text{Ca,L}} \)-triggered \( \text{Ca}^{2+} \) transients (CaT) in sinus rhythm (Ctl) and atrial fibrillation (AF). A, Voltage-clamp protocol (0.5 Hz, top) together with simultaneous recordings of \( I_{\text{Ca,L}} \) (middle) and triggered CaT (bottom) in atrial myocytes from Ctl (left) and cAF (right) patients before (baseline) and after application of IBMX (1µmol/L). A-D, Corresponding mean±SEM for Peak-\( I_{\text{Ca,L}} \) (B, left) and integrated \( I_{\text{Ca,L}} \) (B, right), diastolic and systolic \([\text{Ca}^{2+}]_i\) levels (C, left), CaT amplitude (C, right) and the time-constant (\( \tau \)) of decay of \( I_{\text{Ca,L}} \)-triggered CaT (D). E, Mean±SEM of cAMP levels in right atrial biopsies from sinus rhythm and cAF patients. *P<0.05 and **P<0.01 vs. corresponding mean baseline values and Ctl myocytes, respectively. Numbers indicate myocytes/patients.
Online-Figure IX. Effect of isoprenaline (1 µmol/L) on $I_{\text{Ca,L}}$-triggered Ca$^{2+}$ transients (CaT) in sinus rhythm (Ctl) and atrial fibrillation (AF). A, Voltage-clamp protocol (0.5 Hz, top) together with simultaneous recording of $I_{\text{Ca,L}}$ (middle) and triggered CaT (bottom) in atrial myocytes from Ctl (left) and cAF (right) patients before (baseline) and after application of isoprenaline. A-D, Corresponding mean±SEM for Peak-$I_{\text{Ca,L}}$ (B, left) and integrated $I_{\text{Ca,L}}$ (B, right), diastolic and systolic [Ca$^{2+}$]$_{i}$ levels (C, left), CaT amplitude (C, right) and the time-constant ($\tau$) of decay of $I_{\text{Ca,L}}$-triggered CaT (D). *P<0.05, **P<0.01, ***P<0.001, #P<0.05 and ##P<0.01 vs. corresponding mean baseline values and Ctl myocytes, respectively. Numbers indicate myocytes/patients.
References


