

Trigger-Specific Remodeling of $K_{Ca}2$ Potassium Channels in Models of Atrial Fibrillation

Ann-Kathrin Rahm¹⁻³
 Dominik Gramlich¹⁻³
 Teresa Wieder^{1,2}
 Mara Elena Müller¹⁻³
 Axel Schoeffel^{1,2}
 Fadwa A El Tahry¹
 Patrick Most^{1,2}
 Tanja Heimberger^{1,3}
 Steffi Sandke^{1,3}
 Tanja Weis^{1,3}
 Nina D Ullrich⁴
 Thomas Korff^{4,5}
 Patrick Lugenbiel¹⁻³
 Hugo A Katus¹⁻³
 Dierk Thomas¹⁻³

¹Department of Cardiology, Medical University Hospital Heidelberg, Heidelberg, 69120, Germany; ²HCR (Heidelberg Center for Heart Rhythm Disorders), University Hospital Heidelberg, Heidelberg, 69120, Germany; ³DZHK (German Centre for Cardiovascular Research), Partner Site Heidelberg/Mannheim, University of Heidelberg, Heidelberg, 69120, Germany; ⁴Institute of Physiology and Pathophysiology, Department of Cardiovascular Physiology, Heidelberg University, Heidelberg, 69120, Germany; ⁵European Center for Angioscience (ECAS), Medical Faculty Mannheim, Heidelberg University, Mannheim, 68167, Germany

Correspondence: Dierk Thomas
 Department of Cardiology, University of Heidelberg, Im Neuenheimer Feld 410, Heidelberg, 69120, Germany
 Tel +49 6221 568855
 Fax +49 6221 565514
 Email dierk.thomas@med.uni-heidelberg.de

Aim: Effective antiarrhythmic treatment of atrial fibrillation (AF) constitutes a major challenge, in particular, when concomitant heart failure (HF) is present. HF-associated atrial arrhythmogenesis is distinctly characterized by prolonged atrial refractoriness. Small-conductance, calcium-activated K^+ (K_{Ca} , SK, *KCNN*) channels contribute to cardiac action potential repolarization and are implicated in AF susceptibility and therapy. The mechanistic impact of AF/HF-related triggers on atrial K_{Ca} channels is not known. We hypothesized that tachycardia, stretch, β -adrenergic stimulation, and hypoxia differentially determine K_{Ca} 2.1–2.3 channel remodeling in atrial cells.

Methods: *KCNN1-3* transcript levels were assessed in AF/HF patients and in a pig model of atrial tachypacing-induced AF with reduced left ventricular function. HL-1 atrial myocytes were subjected to proarrhythmic triggers to investigate the effects on *Kcnn* mRNA and K_{Ca} channel protein.

Results: Atrial *KCNN1-3* expression was reduced in AF/HF patients. *KCNN2* and *KCNN3* suppression was recapitulated in the corresponding pig model. In contrast to human AF, *KCNN1* remained unchanged in pigs. Channel- and stressor-specific remodeling was revealed in vitro. Lower expression levels of *KCNN1/KCa2.1* were linked to stretch and β -adrenergic stimulation. Furthermore, *KCNN3/KCa2.3* expression was suppressed upon tachypacing and hypoxia. Finally, *KCNN2/KCa2.2* abundance was specifically enhanced by hypoxia.

Conclusion: Reduction of $K_{Ca}2.1-2.3$ channel expression might contribute to the action potential prolongation in AF complicated by HF. Subtype-specific $K_{Ca}2$ channel remodeling induced by tachypacing, stretch, β -adrenergic stimulation, or hypoxia is expected to differentially determine atrial remodeling, depending on patient-specific activation of each triggering factor. Stressor-dependent $K_{Ca}2$ regulation in atrial myocytes provides a starting point for mechanism-based antiarrhythmic therapy.

Keywords: atrial fibrillation, calcium, K_{Ca} channel, *KCNN*, remodeling, SK channel

Introduction

Atrial fibrillation (AF) is the most common cardiac arrhythmia and accounts for significant morbidity and mortality. Effective antiarrhythmic treatment of AF still exhibits suboptimal effectiveness due to patient-specific characteristics that individually determine atrial arrhythmogenesis. Among these, heart failure (HF) worsens the prognosis of AF patients and poses a particular therapeutic challenge that is mechanistically attributed to a distinct atrial substrate. Atrial action potential duration (APD) and effective refractory period (AERP) are prolonged in AF complicated by HF in humans and in animal models,¹ requiring tailored therapeutic approaches. During AF and HF, multiple mechanisms including tachycardia,

stretch, β -adrenergic stimulation, and hypoxia are activated and may differentially affect APD through remodeling of repolarizing atrial ion channels.

Atrial APD is determined by the combined action of ionic currents. The role of small-conductance, calcium-activated K^+ (K_{Ca} , SK) channels in atrial action potential repolarization has recently been delineated.^{2,3} K_{Ca} channels conduct the cardiac $I_{K_{Ca}}$ current and are primarily activated by intracellular Ca^{2+} via binding of calmodulin to the channel C-terminus, while their voltage sensitivity is negligible.⁴ Three K_{Ca2} channels ($K_{Ca2.1}$ – 2.3) and their respective genes *KCNI-3* have been identified in the heart.^{5–9} $K_{Ca2.2}$ and $K_{Ca2.3}$ exhibit higher expression levels than $K_{Ca2.1}$ in human atrial tissue.^{7,8} $K_{Ca2.1}$ and $K_{Ca2.2}$ are expressed in human and murine atria with predominance over ventricular tissue,^{3,5,6} indicating a potential advantage as atrial-selective therapeutic targets. K_{Ca2} channel inhibition induces prolongation of atrial refractoriness, APD duration, and suppression of atrial arrhythmia.^{5,8,10,11} The mechanistic role of K_{Ca2} channels in AF has been suggested in genome-wide association studies (GWAS) and candidate gene-based approaches, revealing the relationship between *KCNN2* and *KCNN3* variants and AF risk in humans.¹² Furthermore, reduced expression of $K_{Ca2.1}$, $K_{Ca2.2}$, and $K_{Ca2.3}$ has been detected in patients with persistent or permanent AF and preserved cardiac function when compared to sinus rhythm (SR) subjects.^{7,8,13} Of note, either inappropriate shortening or excessive prolongation of atrial APD may increase AF susceptibility.^{3,11,14,15} Thus, the therapeutic efficacy of interventions targeting K_{Ca2} channels will depend on the functional K_{Ca2} homeostasis, which is affected by individual patient characteristics and by environmental factors that determine specific K_{Ca2} channel remodeling. However, the mechanistic impact of proarrhythmic triggers on K_{Ca2} channel expression in AF and HF as basis for patient-specific antiarrhythmic therapy is poorly understood. This study was designed to assess $K_{Ca2.1}$ – 2.3 channel remodeling in patients and in a porcine model of AF with concomitant HF, and to elucidate differential effects of tachycardia, stretch, β -adrenergic stimulation, and hypoxia on $K_{Ca2.1}$ – 2.3 expression in atrial cells.

Methods

Ethics Statement

The study involving human tissue samples was conducted in accordance with the Declaration of Helsinki, and the

study protocol was approved by the University of Heidelberg Ethics Committee (Germany; institutional approval number S-390/2011). Written informed consent was obtained from all patients. Animal experiments have been carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health (NIH publication No. 86–23, revised 1985) and with EU Directive 2010/63/EU, and the current version of the German Law on the Protection of Animals was followed. Experiments involving pigs (institutional approval numbers G-106/10 and G-165/12) have been approved by the local animal welfare authority (Regierungspräsidium Karlsruhe, Karlsruhe, Germany).

Patients and Human Tissue Handling

The study comprised a total of 30 patients (66.7% male, mean age 51.0 ± 12.2 years) with sinus rhythm (SR; $n = 10$), paroxysmal (p)AF ($n = 10$), and chronic (c)AF (ie, persistent, long-standing persistent or permanent AF; $n = 10$) undergoing heart transplantation were included ([Supplementary Table 1](#)). The patient cohort with detailed characteristics has been reported previously.^{1,16–18} Atrial tissue samples were obtained from the Heidelberg CardioBiobank (Department of Cardiology, University Hospital Heidelberg, Heidelberg, Germany) and quality controlled by the tissue bank of the National Center for Tumor Diseases (NCT, Heidelberg, Germany) in accordance with the regulations of the tissue bank. Cardiac tissue samples were dissected immediately following explantation of the recipient's heart in the operating room. The atrial tissue sections were shock-frozen in liquid nitrogen and stored at -80°C .

AF Pig Model

Remodeling of study proteins was evaluated using an established porcine AF model.¹ AF was induced in domestic swine by right atrial burst pacing via an implanted cardiac pacemaker. High-rate atrial pacing and AF with rapid ventricular rate response resulted in reduced left ventricular function. Animals receiving inactive pacemakers served as controls. Cardiac tissue was obtained from previously reported pigs 7 days ($n = 5$)¹⁹ or 14 days ($n = 5$)¹ after the initiation of atrial burst pacing or from corresponding control pigs not subjected to AF induction ($n = 5$ each). Animal characteristics were previously described in detail.^{1,19}

HL-1 Cell Culture

HL-1 cells (kindly provided by Dr. William Claycomb, Louisiana State University Health Science Center, New Orleans, LA, USA) originate from atrial tumor cells of transgenic mice and exhibit cardiomyocyte-specific ion channel expression.^{20,21} K_{Ca2} channel expression in HL-1 cells has been shown previously.^{9,16} Cells were cultured in a supplemented Claycomb medium (Sigma-Aldrich, Steinheim, Germany) containing 0.1 mM norepinephrine.²⁰ A total of $4\text{--}5 \times 10^6$ HL-1 cells per well were seeded on gelatin/fibronectin-coated 6-well dishes and subjected to experimental procedures, unless stated otherwise. For β -adrenergic stimulation 50 μ M isoproterenol was added to the culture medium for 24 h. Similar doses of isoprenaline have been employed previously in experimental studies.^{22–24} For HL-1 cell authentication, Short Tandem Repeat (STR) profiling of HL-1 cells used in this study (obtained directly from Dr. Claycomb) and of commercially available HL-1 cells (Sigma-Aldrich, Steinheim, Germany) for comparison was performed by American Type Culture Collection (ATCC) cell-line authentication service (Wesel, Germany). This approach confirmed a high degree of similarity between cell lines ([Supplementary Table 2](#)). In addition, human and/or African green monkey has not been detected in these samples.

Rapid Electrical Stimulation of HL-1 Cells

Cells were subjected to electrical stimulation as reported using the C-Pace EP system (IonOptix, Westwood, MA, USA).²⁵ The cells were stimulated with 10 V/10 ms pulses at 5 Hz stimulation rate. Following rapid electrical stimulation for 24 h, cell viability was visually assessed by microscopic examination and TUNEL assays. Cells were harvested and subjected to RNA and protein isolation. Control cells not subjected to stimulation were otherwise maintained and handled similarly.

HL-1 Cardiomyocyte Stretch Experiments

Prior to stretching, amino-coated FlexCell dishes or amino-coated membranes in 6-well tissue culture plates were coated for 24 h with gelatin/fibronectin, and 1×10^7 cells were seeded into each well. HL-1 cells were stretched for 24 h with 13% stretch at 1 Hz using the FlexCell Tension system (FlexCell International Corporation, Burlington, NC, USA). Control cells plated on similar

control plates were maintained at identical conditions (without stretch) for the same duration.

Hypoxia

Hypoxia was induced by maintaining HL-1 cells in an incubator with 1% oxygen for 24 h (HERAcell150i, ThermoFisher Scientific, Waltham, MA, USA). Cells receiving 21% oxygen served as controls.

RNA Isolation and Quantitative RT-PCR

Total RNA was isolated from tissue samples and from HL-1 cells with QiaZol-Reagent (Qiagen, Hilden, Germany), followed by chloroform extraction, isopropanol precipitation, and assessment of quantity and integrity using a NanoDrop2000 (Thermo Fisher Scientific, Waltham, MA, USA). Genomic DNA was digested using TurboDNase-Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Complementary DNA was synthesized using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Fisher Scientific) using 3 μ g of total RNA. Quantitative real-time PCR (qRT-qPCR) was performed with 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. Ninety-six-well optical detection plates (Applied Biosystems) were loaded to a total volume of 10 μ L per well, consisting of 0.5 μ L cDNA, 5 μ L TaqMan Fast Universal Master Mix (Applied Biosystems), and 6-carboxyfluorescein (FAM)-labeled TaqMan probes and primers (TaqMan Gene Expression Assays; Applied Biosystems) detecting human, porcine, and murine *KCNKI-3* ([Supplementary Table 3](#)). Primers and probes detecting glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA or ribosomal protein L32 (Rpl32) mRNA were used for normalization. All qRT-PCR reactions were performed in duplicates or higher replicates, and non-template control (NTC) and dilution series were included on each plate for quantification. Data are expressed as averages of replicates. Data analyses were performed using the second derivative method. All measurements were adjusted using a standard probe, and quantification was corrected for efficiency calculated with the standard curves.

Protein Isolation and Western Blotting

HL-1 cells were lysed in a radioimmunoprecipitation (RIPA) buffer consisting of 20 mM Tris-HCl, 0.5% NP-40, 0.5% sodium-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4 , 1 mM NaF and inhibitors of

proteases (CompleteMini, Roche Applied Science, Indianapolis, IN, USA). After centrifugation of homogenates for 30 min at 14,000 g supernatants were collected and the protein concentration was determined using the bicinchoninic acid (BCA) protein assay (Thermo Scientific, Rockford, IL, USA) and proteins were diluted to equal concentrations with sterile water. Protein immunodetection was performed using sodium dodecyl sulfate (SDS) gel electrophoresis and Western blotting as described previously.²⁵ Equal amounts of protein were separated on 10% SDS polyacrylamide gels and transferred onto polyvinylidene difluoride membranes. Membranes were blocked with 5% milk in PBS-T for 2 hours at room temperature and developed using primary antibodies directed against K_{Ca}2.1 (1:1000, APC-039), K_{Ca}2.2 (1:1000, APC-028) and K_{Ca}2.3 (1:1000, APC-025, all from Alomone Lab, Jerusalem, Israel). Primary antibodies were incubated overnight at 4°C. Horseradish peroxidase (HRP)-conjugated donkey anti-rabbit (ab6802; Abcam, Cambridge, UK) secondary antibody was used. Signals were developed using an enhanced chemiluminescence assay (ECL Western Blotting Reagents, GE Healthcare, Buckinghamshire, UK). After removal of primary and secondary antibodies (ReBlot Strong Stripping Solution, Merck, Germany), the membranes were re-probed with anti-GAPDH (1:10,000, ab181602, Abcam) or anti- β -actin (1:1000, ab 8227, Abcam) antibody and corresponding secondary antibodies (ab6802; Abcam). Protein content was normalized to GAPDH or β -actin for quantification of optical density with ImageJ 1.50i Software (National Institutes of Health, Bethesda, MD, USA).

TUNEL Staining

HL-1 cells were seeded on glass coverslips before treatment with tachypacing or hypoxia, respectively. Apoptosis was detected by TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) according to the manufacturer's instructions (TMR red In Situ Cell Death Detection Kit, Roche Applied Science). Negative controls (omitting terminal transferase) and positive controls (using recombinant DNase I) were included. Cells were mounted with Fluoroshield Mounting Medium with DAPI (ab10433, Abcam) and sealed on object slides with nail polish. TUNEL-positive cells were counted using a fluorescence microscope (Zeiss, Oberkochen, Germany), and ImageJ 1.41 software was used to calculate the total cell number in 3×3 tiles. The percentage of

TUNEL-positive cells was calculated by dividing TUNEL-positive cells by total cell count.

Statistics

Patient data are presented as mean \pm standard deviation (SD) or number and percentage. Experimental data are presented as box plots with dots representing raw data. Statistical differences of continuous variables were determined using unpaired Student's *t* tests (two-sided tests). Categorical data were analyzed using the chi-square test. Multiple comparisons were performed using ANOVA. Statistical analyses were performed with Origin 2020 (OriginLab, Northampton, MA, USA). *P* < 0.05 was considered statistically significant.

Results

AF-Related K_{Ca}2 Channel Remodeling in HF Patients

K_{Ca}2 channel mRNA expression was analyzed in the right atrium of patients with severe heart failure (mean left ventricular ejection fraction, 17%-21%) and SR, pAF, or cAF, respectively (Figure 1A and B). AF and concomitant HF were associated with macroscopic atrial alteration, reflected by increased LA size that was numerically more pronounced in pAF and cAF subjects compared with SR patients (Figure 1A). Paroxysmal AF was associated with reduced expression of *KCNN1* (−73%, *n* = 10, *P* = 0.010), *KCNN2* (−62%, *n* = 10, *P* = 0.010), and *KCNN3* (−50%, *n* = 10, *P* = 0.003) compared to HF patients with SR (*n* = 10) (Figure 1C), as reported earlier.^{16,18} In HF patients with cAF, *KCNN* expression was similarly suppressed by 78% (*KCNN1*, *n* = 10, *P* < 0.0001), 55% (*KCNN2*, *n* = 10, *P* = 0.010), and 48% (*KCNN3*, *n* = 10, *P* = 0.037), respectively (Figure 1C).^{16,18}

K_{Ca}2 Channel Remodeling in a Porcine AF/HF Model

To assess K_{Ca}2 channel expression changes in an animal model, previously described domestic pigs subjected to induction of atrial fibrillation for 7 days (*n* = 5)¹⁹ and 14-day duration (*n* = 5)¹ by pacemaker-induced right atrial burst stimulation were studied (Figure 2A). Control pigs exhibiting sinus rhythm (SR) during 14 days (*n* = 5)¹ served as controls. The pigs exhibited a reduction in LV function and atrial dilation due to rapid ventricular rate response during AF.¹ We observed a trend towards reduced expression of the right atrial *KCNN2* mRNA levels 7 days (−30%, *P* = 0.105)

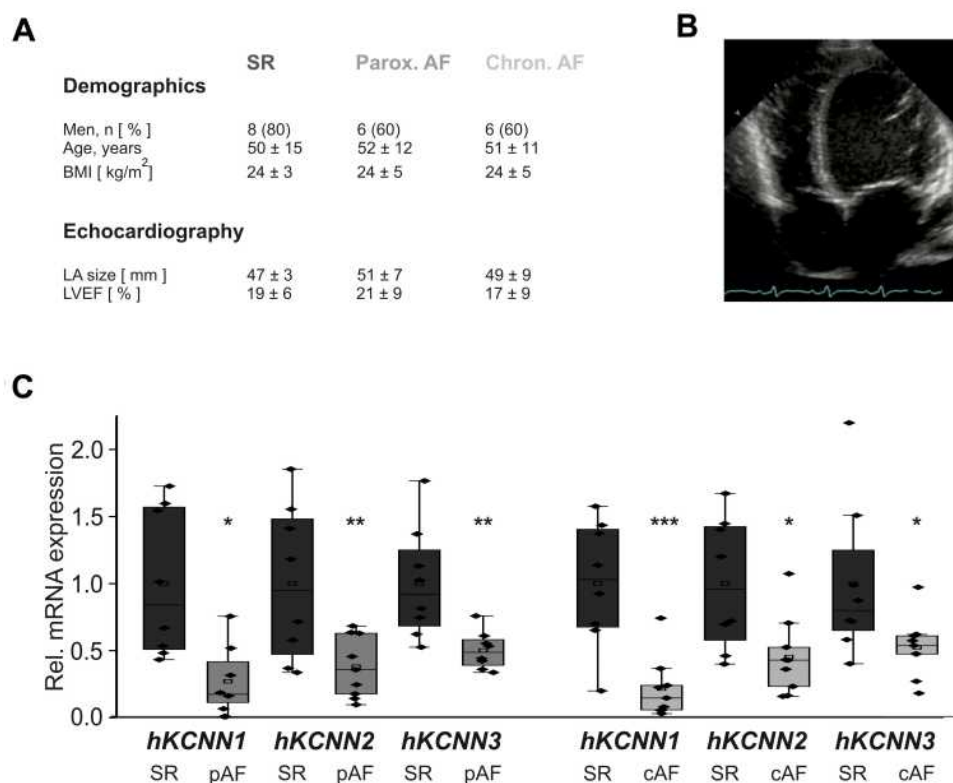


Figure 1 Remodeling of K_{Ca2} channels in atrial fibrillation (AF) patients with concomitant heart failure. **(A)** Key characteristics of study patients (LA, left atrium; LVEF, left ventricular ejection fraction; see Supplementary Table 1 for details). Please note that patient characteristics have been published previously.^{1,16–18} **(B)** Representative apical 4-chamber echocardiography view of a patient with severely reduced LVEF and sinus rhythm (SR). **(C)** *KCNN* mRNA levels normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and respective SR control, obtained from patients with SR, paroxysmal (pAF), or chronic AF (cAF). *KCNN1*¹⁸ and *KCNN2/3*¹⁶ mRNA expression data were reported previously and are shown here for reference. Data are presented as box plots with underlying dots representing raw data; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus SR controls.

and 14 days (-34% , $P = 0.063$) after AF induction compared to SR control animals, while *KCNN1* mRNA abundance did not differ between the study groups (Figure 2B and C). *KCNN3* transcript levels showed trends towards reduced abundance after 14 days AF (-32% , $P = 0.22$) and towards enhanced expression after 7 days AF ($+19\%$, $P = 0.39$), respectively (Figure 2D). Previous protein analyses were confined to animals studied after 14 days atrial burst pacing due to limited sample availability and indicated a tendency towards decreased $K_{Ca2.1}$ protein expression in RA tissue.¹⁸ Furthermore, reduced expression of the right atrial $K_{Ca2.2}$ and $K_{Ca2.3}$ protein after 14 days AF in pigs has been reported previously.¹⁶

Tachypacing-Related Remodeling of K_{Ca2} Channels in vitro

To test the hypothesis that potential proarrhythmic triggers (ie high-rate electrical activity, myocardial wall stress, β -adrenergic activation, hypoxia) differentially affect K_{Ca2} channel expression, we next analyzed their effects in vitro using immortalized murine atrial cardiomyocytes (HL-1

cells). Rapid electrical pacing of cultured cells provides an established model of cardiac tachyarrhythmia that may induce electrophysiological remodeling similar to findings obtained in humans or animal models. HL-1 cells were subjected to tachypacing (TP; 24 h) by electric field stimulation (Figure 3A). TP resulted in a significant reduction of $K_{Ca2.3}$ at mRNA (-56% , $P = 0.029$, $n = 6$; Figure 3B) and protein levels (-63% , $P = 0.024$, $n = 6$; Figure 3C and D). $K_{Ca2.1}$ or $K_{Ca2.2}$ expression was not significantly affected by TP (Figure 3C and 4). To exclude tachypacing-related apoptosis as an unspecific mechanism underlying $K_{Ca2.3}$ expression changes, apoptosis was quantified using TUNEL staining (Figure 3E). Apoptosis rates were low and not significantly different between HL-1 cells following TP ($3.7 \pm 0.3\%$) or maintained under control conditions ($3.2 \pm 0.4\%$, $P = 0.294$, $n = 3$; Figure 3F).

Effects of Cell Stretch on K_{Ca2} Channel Expression

Membrane stretch is another key factor in pathologic atrial remodeling following pressure and volume overload. To

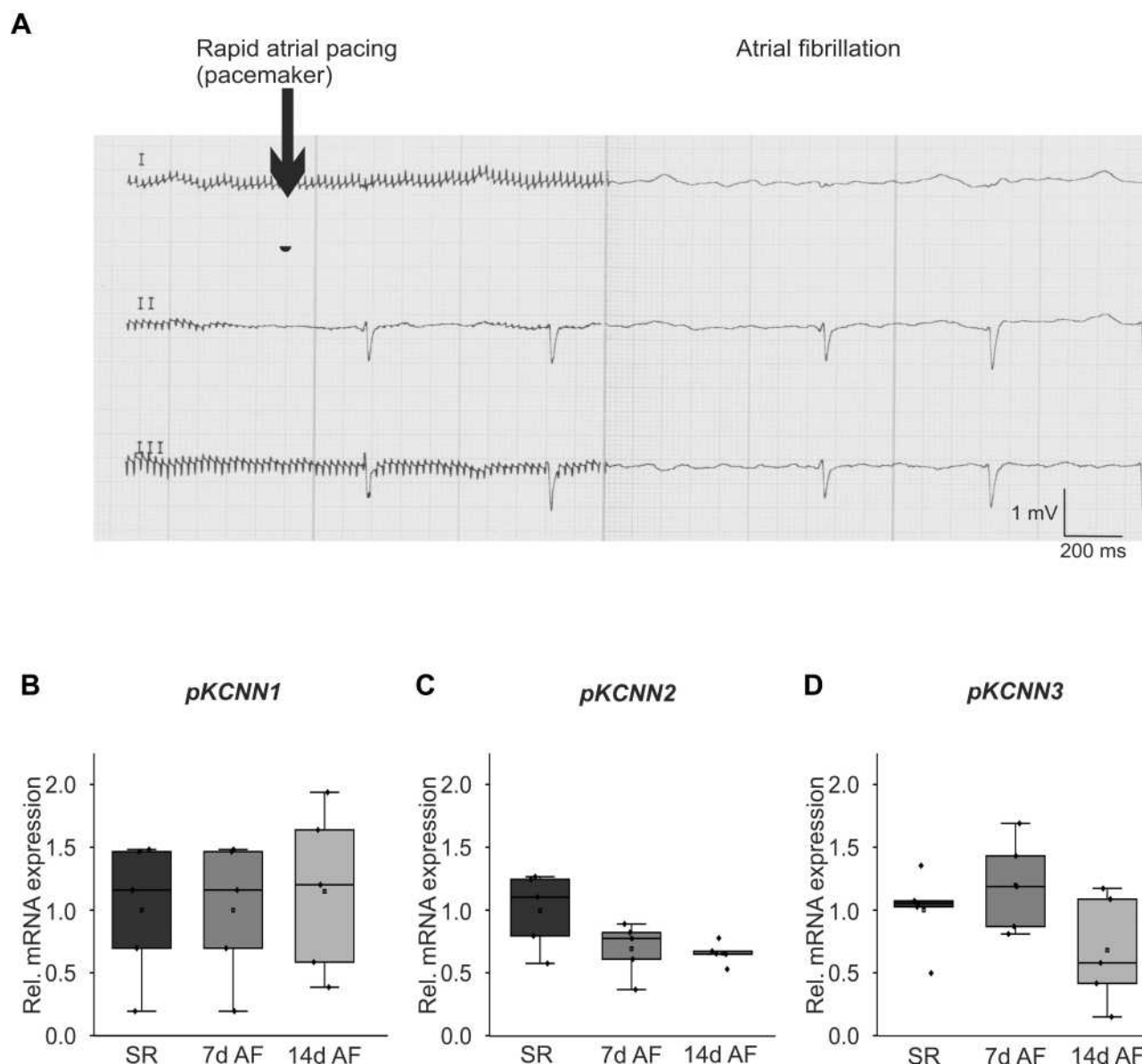


Figure 2 Remodeling of $K_{Ca}2$ channels in a porcine atrial fibrillation (AF)/heart failure model. **(A)** Representative ECG recordings illustrate AF (right) induced by rapid atrial pacing (left). **(B–D)** Relative right atrial mRNA expression levels of *KCNN1* **(B)**, *KCNN2* **(C)**, and *KCNN3* **(D)** are provided for AF pigs after 7 and 14 days of atrial burst pacing compared to sinus rhythm (SR) controls.

evaluate its effects on electrical remodeling, HL-1 cells were exposed to 24 h membrane stretch mimicking atrial dilatation (Figure 4A). Contrary to effects of TP on $K_{Ca}2.3$ channel expression, stretch reduced $K_{Ca}2.1$ channel abundance without affecting $K_{Ca}2.2$ or $K_{Ca}2.3$ levels (Figure 4B–D). *Kcnn1* mRNA was lowered by 38% ($P = 0.015$, $n = 6$) compared to untreated controls ($n = 6$), while $K_{Ca}2.1$ protein was reduced by 51% ($P = 0.019$, $n = 6$) compared with controls ($n = 6$).

$K_{Ca}2$ Channel Remodeling Upon β -Adrenergic Stimulation

We next assessed the potential effect of β -adrenergic stress as a proarrhythmic mechanism during AF/HF. To this end, HL-1 cells were maintained for 24 h in culture medium supplemented with 50 μ M isoproterenol (Figure 5A). Adrenergic stimulation reduced *Kcnn1* mRNA levels by 38% ($P < 0.001$, $n = 6$) compared to non-paced control cells ($n = 6$) (Figure 5B). $K_{Ca}2.1$ protein was not

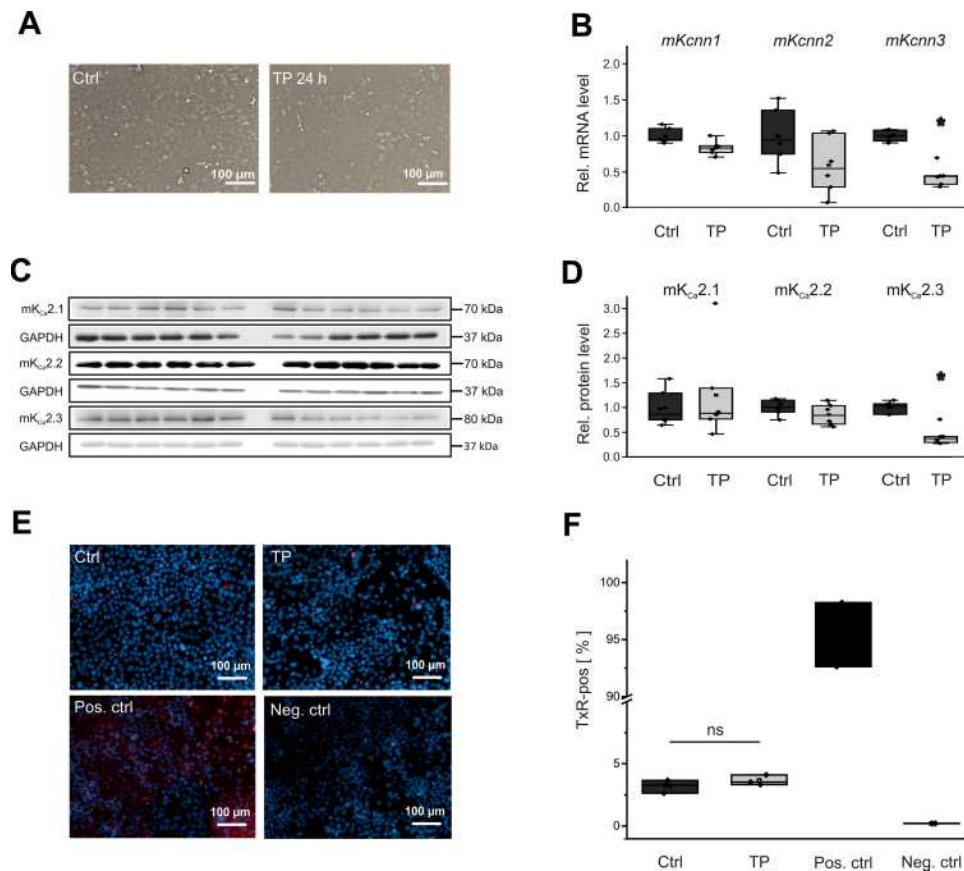


Figure 3 Effects of electrical tachypacing on K_{Ca2} channel expression and viability in HL-1 atrial cells. **(A)** Representative microscopic images under control conditions (Ctrl) and after 24 h tachypacing (TP), respectively (scale bar, 100 μ m). **(B)** *Kcnn1* ($n = 6$), *Kcnn2* ($n = 6$), and *Kcnn3* mRNA expression ($n = 6$) calculated relative to untreated controls ($n = 6$ each). **(C, D)** Remodeling of $K_{Ca2.1}$ ($n = 6$), $K_{Ca2.2}$ ($n = 6$), and $K_{Ca2.3}$ protein ($n = 6$), evaluated using Western blot analyses and compared to controls ($n = 6$ each). **(E, F)** Apoptosis of HL-1 cells subjected to tachypacing ($n = 3$), including untreated (Ctrl; $n = 3$), positive (Pos. ctrl; $n = 2$), and negative controls (Neg. ctrl; $n = 2$). **(E)** Representative fluorescence microphotographs corresponding to terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays (scale bar, 100 μ m). Red nuclear fluorescence reflects endonucleolytic DNA degradation and apoptosis. **(F)** Mean apoptosis rates. TUNEL (Texas Red, TxR)-positive cells are expressed in relation to the total number of cells. Data are provided as box plots with underlying dots representing original data; * $P < 0.05$ versus HL-1 cells not subjected to tachypacing, ns= non significant.

significantly affected by isoproterenol (Figure 5C and D). Furthermore, β -adrenergic stimulation did not change K_{Ca} 2.2 and K_{Ca} 2.3 transcript and protein levels, respectively (Figure 5B–D).

Effects of Hypoxia on K_{Ca2} Channel Expression

High rates of electrical activity and HF may be associated with regional myocardial hypoxia. To examine the impact of hypoxia on K_{Ca2} expression, HL-1 cells were maintained at 1% oxygen for 24 h (Figure 6A). Hypoxia increased *Kcnn2* mRNA (+46%, $P = 0.013$, $n = 6$; Figure 3B) and $K_{Ca2.2}$ protein (+66%, $P = 0.059$, $n = 6$; Figure 3C and D) compared with controls receiving 21% oxygen ($n = 6$ each). By contrast, hypoxia resulted in reduction of $K_{Ca2.3}$ expression at mRNA (–42%, $P < 0.001$, $n = 6$; Figure 6B) and protein levels (–63%, $P = 0.001$, $n = 6$; Figure 6C and D) compared

with controls ($n = 6$ each). $K_{Ca2.1}$ expression was not significantly altered upon hypoxia. Finally, we observed low apoptosis rates that did not differ between HL-1 cells maintained at 1% oxygen ($1.9 \pm 0.3\%$, $n = 3$) or 21% oxygen ($1.1 \pm 0.1\%$, $P = 0.064$, $n = 3$), indicating that K_{Ca2} channel expression changes were not triggered by potential hypoxia-related apoptosis (Figure 6E and F).

Discussion

Remodeling of $K_{Ca2.1}$, $K_{Ca2.2}$, and $K_{Ca2.3}$ Channels During AF Complicated by HF

Paroxysmal and chronic atrial fibrillation was associated with reduced atrial $K_{Ca2.1}$, $K_{Ca2.2}$, and $K_{Ca2.3}$ transcript levels in patients with severe HF. In pigs subjected to AF induction for a limited time (ie, 7 days and 14 days, respectively), suppression of K_{Ca2} channel expression occurred in time- and channel-dependent manner. While $K_{Ca2.1}$ mRNA

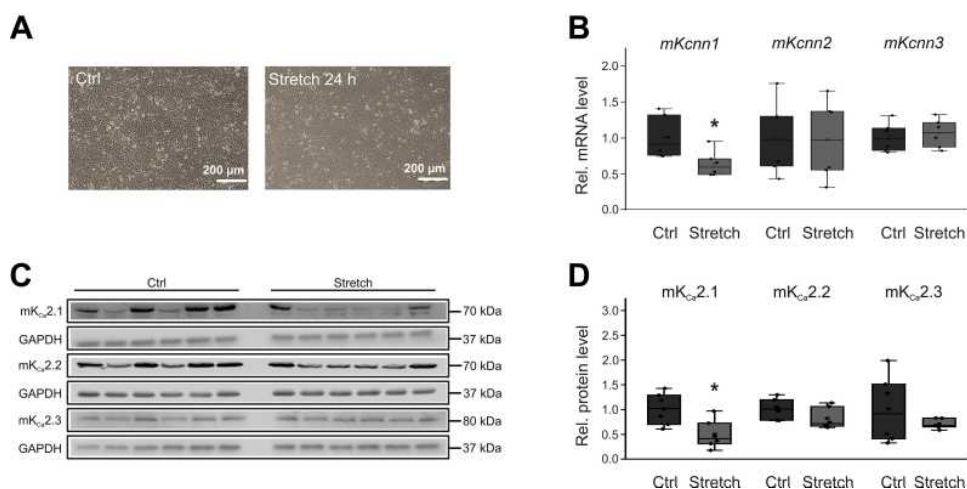


Figure 4 Stretch-related $K_{Ca}2$ channel remodeling in HL-1 cells. **(A)** Representative microphotographs under control conditions (Ctrl) and after 24 h cell stretch, respectively (scale bar, 200 μ m). **(B)** *Kcnn1* ($n = 6$), *Kcnn2* ($n = 6$), and *Kcnn3* mRNA expression ($n = 6$) relative to untreated controls ($n = 6$ each). **(C, D)** Expression of $K_{Ca}2.1$ ($n = 6$), $K_{Ca}2.2$ ($n = 6$), and $K_{Ca}2.3$ protein ($n = 6$) compared with controls ($n = 6$ each). Data are shown as box plots with underlying dots representing original data; * $P < 0.05$ versus HL-1 cells not subjected to stretch.

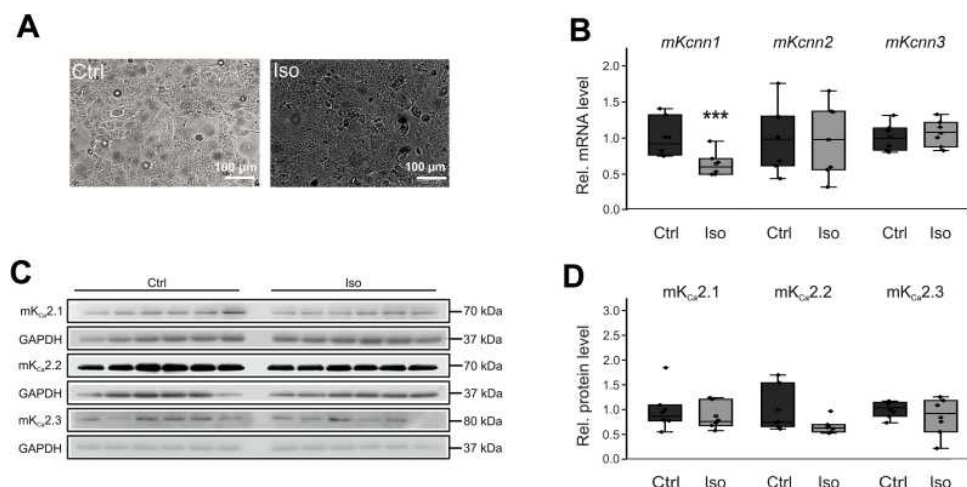


Figure 5 Effects of β -adrenergic stimulation on $K_{Ca}2$ channel expression in HL-1 cells. **(A)** Representative microphotographs under control conditions (Ctrl) and after incubation with 50 μ M isoproterenol for 24 h, respectively (scale bar, 100 μ m). **(B)** *Kcnn1* ($n = 6$), *Kcnn2* ($n = 6$), and *Kcnn3* mRNA expression ($n = 6$) relative to untreated controls ($n = 6$ each). **(C, D)** Relative $K_{Ca}2.1$ ($n = 6$), $K_{Ca}2.2$ ($n = 6$), and $K_{Ca}2.3$ protein levels ($n = 6$) compared with controls ($n = 6$ each). Data are shown as box plots with underlying dots representing original data; *** $P < 0.001$ versus HL-1 cells not subjected to isoproterenol administration.

levels were not significantly affected, $K_{Ca}2.2$ transcripts were reduced after 7 and 14 days. $K_{Ca}2.3$ mRNA exhibited an intermediate phenotype characterized by reduction requiring 14 days of AF, whereas expression was not changed after 7 days AF. These observations suggest that $K_{Ca}2$ channel suppression may be more pronounced in cases with long AF duration. Reduced expression of $K_{Ca}2.1$, $K_{Ca}2.2$, and $K_{Ca}2.3$ in patients with preserved cardiac function and persistent or permanent AF has previously been reported.^{7,8,13} The present work extends AF-related $K_{Ca}2$ downregulation to patients with severely impaired left ventricular function, highlighting a broader mechanism of atrial

electrophysiological remodeling. While these results suggest uniform $K_{Ca}2$ remodeling during AF, a more complex picture with species- and mechanism-based remodeling of the channels is supported by data obtained from a canine model of AF induced by 7 days atrial tachypacing.¹¹ In dogs, left atrial $K_{Ca}2.1$ channel protein was increased without changes in the corresponding mRNA. This observation may correspond to a tendency towards higher *KCNK1* mRNA in the present pig model that did not reach statistical significance (Figure 2B). Importantly, there was no remodeling of $K_{Ca}2.2$ or $K_{Ca}2.3$ in the canine model. In case of $K_{Ca}2.3$ this may be due to the duration of AF that was

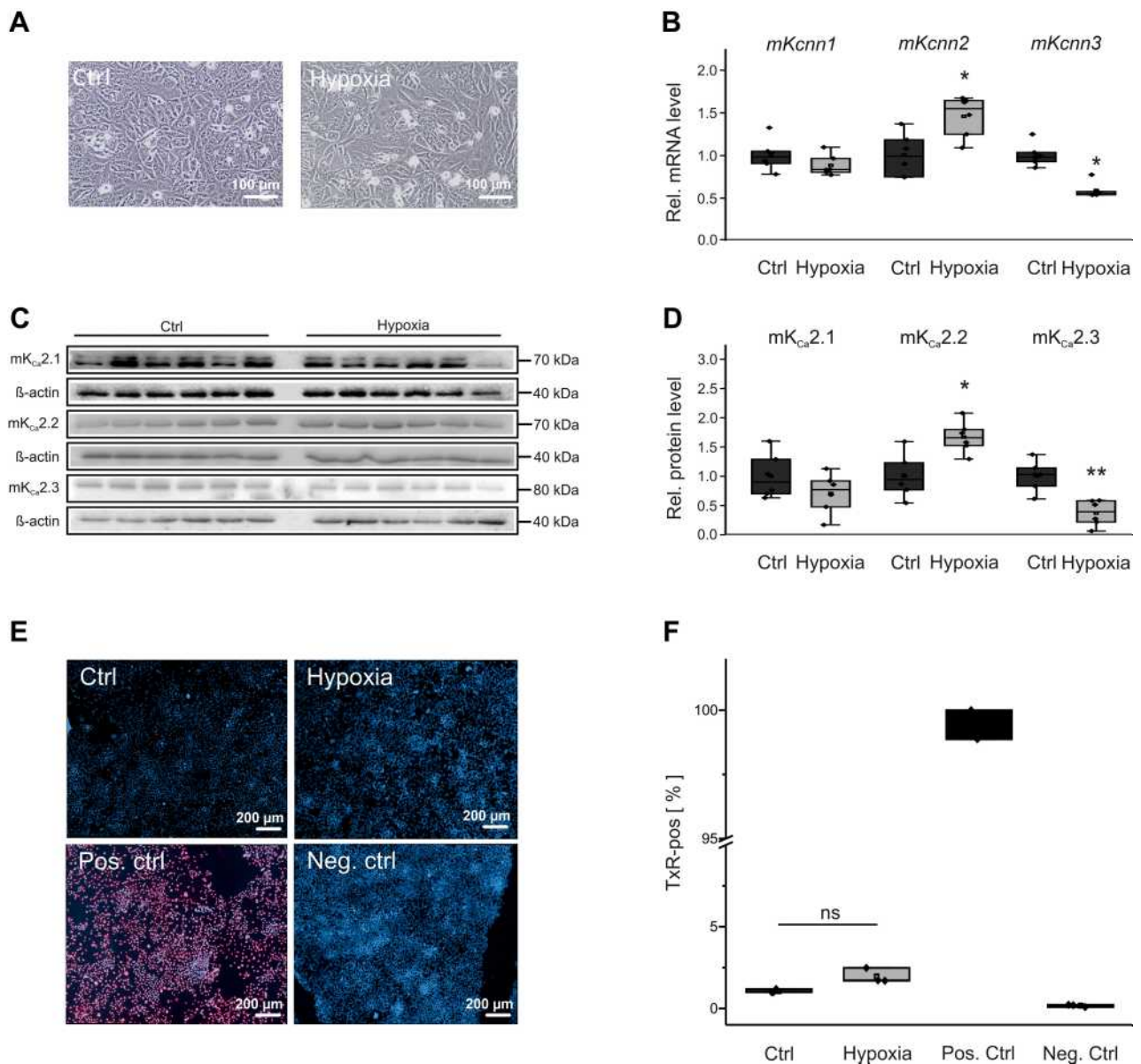


Figure 6 K_{Ca2} channel expression and viability of HL-I atrial cells during hypoxia. **(A)** Representative microscopic images under control conditions (Ctrl, 21% O_2) and after 24 h hypoxia (1% O_2), respectively (scale bar, 100 μm). **(B)** *Kcnn1* ($n = 6$), *Kcnn2* ($n = 6$), and *Kcnn3* mRNA expression ($n = 6$) calculated relative to untreated controls ($n = 6$). **(C, D)** Relative $K_{Ca2.1}$ ($n = 6$), $K_{Ca2.2}$ ($n = 6$), and $K_{Ca2.3}$ protein expression ($n = 6$) during hypoxia and under control conditions ($n = 6$ each). **(E, F)** Apoptosis of HL-I cells subjected to hypoxia, including untreated (Ctrl), positive (Pos. ctrl), and negative controls (Neg. ctrl). **(E)** Representative fluorescence microphotographs corresponding to TUNEL assays (scale bar, 200 μm). **(F)** Mean apoptosis ratios. TUNEL (Texas Red, TxR)-positive cells are provided in relation to the total number of cells. Data are given as box plots with underlying dots representing original data; * $P < 0.05$, ** $P < 0.01$ versus HL-I cells not exposed to hypoxia.

reduced only after 14 days AF in pigs. In addition, species-dependent differences could explain the lack of $K_{Ca2.2}$ remodeling in dogs vs pigs. Considering that right atrial tissue was analyzed in the present work, whereas Qi et al¹¹ obtained left atrial samples for analyses, K_{Ca2} channel remodeling may exhibit side-specific components. Finally, different degrees of proarrhythmic trigger activity may account for differential K_{Ca2} channel remodeling and were investigated further in this study.

Differential Mechanistic Effects of AF/HF-Related Triggers on K_{Ca2} Channel Abundance

The aim of the present approach was to dissect individual mechanisms leading to electrical remodeling in AF. Potential proarrhythmic triggers that may be differentially activated during AF and/or HF comprise high-rate electrical activity, myocardial wall stress, β -adrenergic activation, and hypoxia.

In HL-1 atrial myocytes, $K_{Ca2.1}$ channel suppression was mediated by cell stretch and β -adrenergic stress, whereas $K_{Ca2.3}$ expression was reduced by electrical tachypacing and hypoxia. Thus, either of these mechanisms (or a combination thereof) may contribute to the downregulation of both channels observed in humans and in pigs. Considering the lack of $K_{Ca2.3}$ reduction after 7 days AF in the porcine model, we may speculate that high-rate electrical activity and hypoxia play a less important role in short-term AF in these animals. In contrast, $K_{Ca2.2}$ channel reduction was not detected under any of these conditions, suggesting additional triggers of $K_{Ca2.2}$ downregulation in human AF and in pigs. However, $K_{Ca2.2}$ was enhanced by hypoxia, representing a previously unrecognized regulatory mechanism in cardiomyocytes, with no apparent impact on the present patient subgroup or on the animal model studied here. Of note, upregulation or activation of $K_{Ca2.2}$ is associated with reduction of reactive oxygen species and neuroprotection or reduction of arrhythmias in neuronal and cardiac cell models, respectively.^{26–28}

In summary, this work provides a comprehensive analysis of the effects of different AF triggers on *Kcnn1-3* mRNA expression in a cellular model. We acknowledge that this approach cannot yet yield a complete assessment of *KCNV* remodeling observed in AF patients or in animal models. Rather, it provides initially novel insights into the mechanistic effects of AF-related triggers on atrial K_{Ca} channels. Further studies are required to integrate these individual mechanisms and to assess their functional, in vivo significance in more detail. To further delineate the specific impact of individual proarrhythmic triggers in humans, future studies that include larger patient cohorts are required to establish significant associations between clinical characteristics, such as heart rate, left atrial size, or catecholamine levels and K_{Ca2} channel expression, and to elucidate underlying signal transduction pathways.

Clinical Implications, Limitations, and Future Directions

We specifically investigated potentially proarrhythmic mechanisms in models of a clinically relevant subgroup of AF patients with concomitant HF. Atrial APD prolongation and increased AERPs due to distinct changes in ion channel expression are characteristic of HF patients with reduced left ventricular ejection fraction (LVEF).¹⁶ The porcine model employed in this work is

characterized by a reduction in LV function and prolonged AERP, thus partially resembling findings in human AF/HF patients.¹⁶ Antiarrhythmic strategies that specifically target electrical remodeling to improve and personalize AF treatment are limited. Downregulation of repolarizing K_{Ca2} potassium channels observed here is expected to contribute to prolonged atrial refractoriness. Reversal of K_{Ca2} channel remodeling by channel activation or overexpression could represent an individualized strategy for rhythm control in this specific AF patient entity. Trigger-specific remodeling mechanisms reflect the need for channel-specific pharmacological modulation. Unfortunately, no subtype-specific modulators of $K_{Ca2.1–2.3}$ channels have been identified to date, preventing further evaluation of the channels' individual contributions to the atrial action potential and of their potential role in antiarrhythmic therapy. Furthermore, current K_{Ca2} channel blockers exhibit off-target effects on sodium channels.² It cannot be expected that a single antiarrhythmic intervention will be sufficient to treat all types of AF, as both shortening and prolongation of atrial APD have been suggested to increase AF susceptibility.^{3,11,14,15} Rather, the mechanism-based understanding that is advanced by the present work suggests that tailored, individualized antiarrhythmic strategies are required based on patient-specific mechanisms. Inhibition of K_{Ca2} channels and prolongation of atrial refractoriness by the compound AP30663 is currently evaluated in a Phase II clinical trial for termination of AF (EudraCT Number: 2018–004445-17).²⁹ While this intervention may be effective in a subset of patients, we suggest that the therapeutic efficacy of interventions targeting K_{Ca2} channels may require channel activation in other subjects, depending on the individual K_{Ca2} expression and function.

We acknowledge that small sample sizes due to the large animal model and limitations in human tissue acquisition resulted in relatively low statistical power. In addition, this study was designed to specifically address K_{Ca2} channel expression upon different conditions. Functional analyses were not performed due to pharmacological limitations mentioned above and require future studies when specific inhibitors are available. Additional effects of comorbidities with potential effects on K_{Ca2} channel remodeling such as diabetes⁹ were beyond the scope of the present work and therefore need to be addressed in separate approaches.

Conclusions

This study advances the understanding of the role of K_{Ca2} channels in AF by providing evidence for trigger- and channel-specific remodeling. Suppression of $K_{Ca2.1}$, $K_{Ca2.2}$, and $K_{Ca2.3}$ channel expression in human AF and in pigs is expected to facilitate action potential prolongation that is characteristic of AF with concomitant HF. Differential effects of proarrhythmic triggers resulting in a reduction of $K_{Ca2.1}$ (stretch, β -adrenergic activation) and $K_{Ca2.3}$ (electrical tachypacing, hypoxia) or in an enhanced expression of $K_{Ca2.2}$ (hypoxia) contribute to complex K_{Ca2} channel remodeling. Trigger-dependent K_{Ca2} regulation in atrial myocytes that is determined by individual patient characteristics and by environmental factors provides a new basis for personalized antiarrhythmic therapy.

Data Sharing Statement

The data underlying this article are available in the article and supplementary material.

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