**Article**

\( I_{KACH} \) is constitutively active via PKC epsilon in aging mediated atrial fibrillation

**Highlights**

Atrial fibrillation (AF) is highly prevalent in aging and is a major cause of stroke

A potassium current (\( I_{KACH} \)) is aberrantly active in atrial myocytes from aged hearts

Knocking out PKCe prevents the development of this aberrant current

Blocking \( I_{KACH} \) or knocking out PKCe abrogates the effects of aging on AF
**SUMMARY**

Atrial fibrillation (AF), the most common abnormal heart rhythm, is a major cause for stroke. Aging is a significant risk factor for AF; however, specific ionic pathways that can elucidate how aging leads to AF remain elusive. We used young and old wild-type and PKC epsilon- (PKCε) knockout mice, whole animal, and cellular electrophysiology, as well as whole heart, and cellular imaging to investigate how aging leads to the aberrant functioning of a potassium current, and consequently to AF facilitation. Our experiments showed that knocking out PKCε abrogates the effects of aging on AF by preventing the development of a constitutively active acetylcholine sensitive inward rectifier potassium current (I_{KACH}). Moreover, blocking this abnormal current in the old heart reduces AF inducibility. Our studies demonstrate that in the aging heart, I_{KACH} is constitutively active in a PKCε-dependent manner, contributing to the perpetuation of AF.

**INTRODUCTION**

Atrial fibrillation (AF) is the most common arrhythmia in clinical practice and its management has become a global challenge. AF is characterized by rapid and irregular electrical and mechanical activation of the atria. AF is a major risk factor for stroke, thromboembolism, heart failure, and myocardial infarction. Consequently, AF constitutes a major public health burden.

Epidemiological studies have shown that the natural history of AF is progressive, and that aging is the single greatest risk factor for AF, with each decade of life after the age of 40 conferring up to a 2-fold higher risk. Unfortunately, the underlying mechanisms of aging-mediated AF are not well understood, and the current pharmacological and more invasive therapeutic interventions have numerous limitations and remain inadequate in treating this disease.

Cardiac aging is a complex process, characterized by a gradual increase in cardiac remodeling, as well as increased generation and/or accumulation of reactive oxygen species (ROS). For instance, during aging, changes in atrial structure and function take place, including changes in atrial refractoriness, atrial conduction, aberrant impulse formation, and fibrosis. In addition, oxidative stress has been proposed to play a role in the pathogenesis of AF where increased levels of myocardial ROS such as superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) have been found to be associated with AF. These factors constitute the appropriate electrophysiological and anatomical substrates conducive for the initiation and perpetuation of AF.

Electrical remodeling promotes AF by abbreviating atrial refractoriness. This involves shortening of the action potential duration (APD), in part, via increased inwardly rectifying potassium currents such as the background current I$_K$ and the constitutively active form of the acetylcholine-dependent inwardly rectifying potassium current (I_{KACH}). Cardiac I_{KACH} is a heterotetramer of G protein-regulated inwardly rectifying potassium channel subunits, known as Kir3.1 and Kir3.4. In normal physiology, I_{KACH} is activated by vagally released acetylcholine (ACh) which in turn activates type 2 muscarinic (M2) receptors. This results in the dissociation of the α and the β subunits of the inhibitory Gi protein and the subsequent activation of I_{KACH} because of the direct interaction of the β-subunits with the I_{KACH} channel proteins. The I_{KACH} gating is tightly regulated as this ionic current is important in mediating the chronotropic parasympathetic effects resulting in the decrease of heart rate. However, in AF, I_{KACH} is constitutively active via PKC epsilon in aging mediated atrial fibrillation.
active, independent of parasympathetic signaling. This constitutively active current subsequently works as a background current, promoting atrial APD abbreviation, and shortening of the refractory period, which contributes to the initiation and perpetuation of AF.\textsuperscript{27,29,40–43}

It is thought that a specific isoform of protein kinase C, PKC\textsubscript{ε}, is active and plays a role in the electrical remodeling of the fibrillating atria.\textsuperscript{44} More specifically, animal and human studies have postulated that the activated PKC\textsubscript{ε} in chronic AF may be responsible for I\textsubscript{K\textsubscript{ACh}} constitutive activity.\textsuperscript{30} PKC activation involves translocation of the protein from the cytosolic autoinhibited latent form to the membrane associated active form.\textsuperscript{45,46} Of interest, oxidative stress has been demonstrated to lead to PKC\textsubscript{ε} activation,\textsuperscript{47} and it was recently shown by Yoo and colleagues\textsuperscript{48} that oxidative stress because of mitochondrial ROS and NOX2 underlies PKC\textsubscript{ε} activation in canine atria with pacing induced AF. They further showed that NOX2 knockdown suppressed ROS production and therefore PKC\textsubscript{ε} activation.

Presently, it is not known whether the PKC\textsubscript{ε}/I\textsubscript{K\textsubscript{ACh}} axis plays a role in aging-mediated AF. Therefore, in this study, we directly examined if the PKC\textsubscript{ε}/I\textsubscript{K\textsubscript{ACh}} interplay is important in the mechanism of aging-mediated AF. We set out to test the hypothesis that in the aging heart, I\textsubscript{K\textsubscript{ACh}} is constitutively active via a PKC\textsubscript{ε} mechanism, leading to generation of AF.

RESULTS

PKC\textsubscript{ε} contributes to AF initiation and perpetuation in the old heart

To investigate the role of aging in AF initiation and perpetuation, we conducted a set of in vivo electrophysiological studies with intracardiac programmed electrical stimulation in wild-type (WT) young and old mouse hearts. Figure 1A shows the significantly different age (left) and weight (right) of the old compared to the young WT mice. When both genders were considered, the inducibility of AF in old WT mice was not significantly different from that in young WT mice (Figure 1B left panel). Similarly, the inducibility of AF in female mice was not different between old and young WT animals (Figure 1B middle). However, AF inducibility was statistically higher in old WT compared to young WT male mice (Figure 1B right). The duration of induced AF adjusted for gender was significantly longer in old WT mice compared to young (Figure 1C), suggesting a gender specific difference in AF inducibility.
To investigate the possible role of PKCε in AF initiation and perpetuation in the old heart, we assessed AF inducibility and duration in old PKCε knock-out (KO) versus old WT animals. The age of old WT and old KO animals was not different, but there was a difference in body weight (Figure 2A). AF inducibility was significantly reduced in old KO mice compared to old WT mice of both genders (Figure 2B). Additionally, the duration of induced AF in the old KO mice was significantly shorter than that in the old WT mice (Figure 2C). Knocking out PKCε abrogated the effects of aging on AF inducibility and duration. These results suggest that PKCε plays an important role in the inducibility and stability of AF in the aging heart.

Constitutively active IKACh is arrhythmogenic in the old heart

To investigate whether constitutively active IKACh plays a role in aging-mediated AF, we assessed the in vivo inducibility and duration of AF in old WT mice after jugular vein administration of the IKACh blocker tertiapinQ (TPQ). TPQ is a 21 amino acid synthetic peptide originally isolated from the European Honey bee venom. TPQ is atrial-selective in the heart, blocking IKACh without affecting other inward rectifier potassium currents such as IK1 or IKATP. However, in the kidney, TPQ blocks Kir1.1, a renal outer medullary potassium channel.49,50 Moreover, TPQ prolongs the effective refractory period and terminates AF that is dependent on constitutively active IKACh, or AF that is dependent on IKACh activated with an M2 agonist.27,29,40–43 Here, TPQ administration significantly reduced the duration and the inducibility of AF in the old WT mice compared to saline vehicle control (Figure 3A), suggesting that IKACh is constitutively active in the old heart. However, in KO old mice, TPQ did not affect AF duration and inducibility, indicating that IKACh is not constitutively active in the atria of old KO mice (Figure 3B).

Subsequently, we used patch clamp in atrial myocytes isolated from WT and KO mice to further explore constitutively active IKACh in the old atrial myocytes, and whether knocking out PKCε would abrogate the effects of aging on the development of constitutively active IKACh. The background inwardly rectifying currents were measured in 50 mM [K]o, before and after addition of 100 nM TPQ to the bath solution as previously reported13 (Figure 4). In the young WT (Panel A), and young KO atrial myocytes (Panel B), TPQ did not affect the background currents, suggesting that IKACh was not constitutively active. In the old WT atrial myocytes (Panel C), application of TPQ resulted in a significant decrease in the background current, indicating that IKACh was constitutively active in the old heart, and that the constitutively active IKACh works as a component of the background current in the old cardiomyocytes. In the old KO atrial myocytes (Panel...
D), TPQ did not reduce significantly the background current, implying that knocking out PKCε diminishes the aging effect on constitutively active I_{KACH}. TPQ-sensitive, agonist-independent, constitutively active I_{KACH} was quantified at /C_0/140 mV and shown in the graph of Figure 4E. The constitutively active I_{KACH} was significantly larger in the old WT myocytes compared to young WT, young and old PKCε KO myocytes.

Knocking out PKCε prevents APD shortening in the old heart

To further investigate whether I_{KACH} was constitutively active in the old atria, we tested the effects of TPQ on the atrial action potential duration (APD) in the isolated Langendorff-perfused mouse heart in a PKCε-dependent manner using optical mapping. Our data indicate that 200 nM TPQ prolonged the APD in the old WT but not old PKCε KO hearts (Figure 5). Figure 5A is an APD map at 60% repolarization (APD_{60}) of the right atrium in an old WT (top) and old KO (bottom) heart in control, and after TPQ treatment. TPQ caused an appreciable prolongation of the APD in the WT, but not in the KO hearts. Single-pixel optical tracings of voltage from the maps in Figure 5A show that TPQ prolonged APD in WT old but not in KO old hearts (Figures 5B and 5C). Figure 5D is a compilation of APD_{60} in 4 old WT and 4 old KO hearts. TPQ caused significant APD prolongation in WT, but not in KO. Additionally, at baseline control, the APD in WT hearts was significantly shorter than the baseline APD in KO hearts. This experiment shows that I_{KACH} is constitutively active in the old WT heart at the tissue level, in a PKCε-dependent manner.

Oxidative stress induces cell membrane translocation of PKCε, leading to increased I_{KACH}

Increased levels of myocardial ROS such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2) have been found to be associated with AF. Additionally, the ability of oxidative stress to recruit PKC isoforms to the cell surface has been shown. To assess if H_2O_2 recruits PKCε to the cell surface, we used the catalytic domain of PKCε tagged with mCherry (mCherry-CRY2-mPKCεCAT-HA) in total internal reflection fluorescence microscopy (TIRFM) experiments. This technique allows imaging of fluorescent proteins localized to the cell membrane. Cells transfected with mCherry-CRY2-mPKCεCAT-HA were incubated with H_2O_2 and imaged at baseline and 5, 15, and 30 min after H_2O_2 treatment. mCherry was excited with a 561 nm, ‘red’ laser before and after H_2O_2 treatment (Figure 6A). These data, summarized in Figure 6B, show a significant increase in mCherry fluorescence at the cell surface after a 30-min incubation with...
100 μM H₂O₂ compared to surface fluorescence at baseline, demonstrating that H₂O₂-mediated oxidative stress induced PKCε translocation to the cell surface.

To provide a mechanistic link between aging, PKCε and constitutively active IKACh, we measured the basal IKACh current in HEK cells stably transfected with Kir3.1 and Kir3.4, with PKCε knockdown, in the presence or absence of 100 μM H₂O₂, as a surrogate exposure to simulate oxidative stress associated with aging. The basal IKACh current was recorded in 50 mM extracellular potassium. Figure 6C shows in representative I-V curves that 100 μM H₂O₂ for 1-h resulted in a larger IKACh (red trace) compared to control (black). Silencing PKCε (green trace) prevented the increase of the current brought about by 100 μM H₂O₂. In Figure 6D, data were compiled showing that H₂O₂ significantly increased the maximum inward current, and silencing PKCε prevented this H₂O₂ induced increase in IKACh.

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Transcriptomic changes in old KO versus WT mouse atria

We conducted RNAseq on three old KO and three old WT mouse atrial samples in order to investigate whether knocking out PKCε resulted in transcriptomic changes of major ion channels that underlie the action potential, and thus could shed light on the reduced arrhythmogenesis in the old KO compared to old WT mice. Analysis revealed that out of the 8,631 genes detected above threshold, a total of 38 genes were differentially regulated, with 19 downregulated and 19 upregulated genes in the old KO compared to old WT mice (Tables S1 and S2). None of these differentially regulated genes were for sarcolemmal ion channels. The differentially expressed genes between KO and WT mice were then evaluated for gene ontology (GO) enrichment against all detected genes (Figure 7 and Table S3). There were 22 significantly upregulated and 9 significantly downregulated biological processes (Figure 7). Interestingly, biological processes related to regulation of fat cell differentiation, thermogenesis, and metabolic processes were enriched. This enrichment in transcriptomic programs that control metabolism and fat-cell development converge with our finding that body weight of KO old was significantly reduced compared to the WT old animals (Figure 2B), suggesting that a total knock out of PKCε may affect body weight through pathways relevant to metabolism and adipose tissue regulation. It should be mentioned that obesity is a risk factor for AF,52 and thus the reduction in the body weight of KO mice might be a confounder that affects atrial arrhythmogenesis. Future studies in cardiac-specific PKCε knock out animals are required to address such confounders.

Proteomic differences in female versus male mouse atria

Since results suggested that there are sex-specific differences in the inducibility of AF (Figure 1), we explored in female versus male atrial samples from young WT animals the differentially abundant proteins that could be relevant to AF using proteomics approaches. In our samples, 3008 proteins were detected above threshold across all samples and were assessed for differential expression (Table S4). Of these, 55 proteins were significantly differentially abundant between the sexes, where 32 proteins were increased, and 23 were decreased in females compared to males (Table S5). Differentially regulated biological processes included pathways important for steroid processes, cardiac development, regeneration, immune response related pathways, and components of the gene regulatory apparatus including the mRNA splicing machinery (Figure 8; Table S6). These
pathways may contribute to differential modulation excitability in female versus male atria as well as in the atrial anatomy and can thus affect the dynamics of the arrhythmia.53–56

DISCUSSION

This work provides direct evidence that the PKCε/IKACh pathway plays an important role in aging-mediated AF where constitutively active IKACh contributes to AF perpetuation in the aging heart in a PKCε-dependent manner. This arrhythmogenic pathway has not been explored before in the aging heart. Our experiments showed that IKACh is constitutively active in old atrial myocytes, and knocking out PKCε prevented the constitutive activity of this current, (2)- knocking out PKCε or blocking constitutively active IKACh reduces atrial arrhythmogenesis in the old heart, and (3)- oxidative stress leads to the generation of a constitutively active IKACh via PKCε activation. Our results correspond with epidemiological studies suggesting that clinically, AF prevalence increases with advanced age; in fact, aging is the single greatest risk factor for AF.15–17

Our data indicated sex-specific differences in AF inducibility in female versus male WT mice (Figure 1). Although sex-based differences in cardiac electrophysiology are known, they are still poorly understood. For instance, atrial refractory period in response to rapid atrial pacing was significantly less in premenopausal compared with postmenopausal women and age-matched men, suggesting that estrogen and testosterone play important roles in modulating atrial repolarization.58,59 This finding is consistent with the sex-differentiated enrichment of biological processes relevant to steroid pathways we found in the atria of female versus male WT mice (Figure 8). Sex-based differences in AF-related atrial remodeling is also observed in tissue samples from men and women with long-standing, persistent AF. A significantly higher degree of fibrotic remodeling was found in women, which may contribute to higher incidence and recurrence rates of AF. In addition, sex-specific differences in immune system biology have been found, as we have observed in our proteomics analysis. Such differences may lead to possible enhanced inflammatory responses in the female myocardium and subsequent modulation of fibrotic remodeling and arrhythmogenesis.60 Consistent with these studies, our proteomics analysis (Figure 8) suggests the presence of sex-specific differences in immune response-related pathways. Sex is also suggested to modulate various AF risk factors. Obesity appears to increase the AF risk more in men than in women, coronary disease and

Figure 7. mRNAseq analysis in old KO versus WT atria

Transcript levels of 38 genes were significantly changed, where 19 genes were upregulated, and 19 were downregulated. The bubble plots show the biological processes associated with the differentially regulated genes. Enrichment indicates the ratio of genes with that GO term that are differentially expressed over what would be expected by chance. The size of the dots corresponds to the percentage of genes in that category that are differentially expressed, and the color corresponds to the p value on a log10 scale (more negative is more statistically significant).
sleep apnea have a higher prevalence in men with AF than women, while hypertension and heart failure with preserved ejection fraction are more prevalent in women with AF. Overall, sex-based differences in arrhythmogenesis are multifaceted and complex, and thus, they are being actively investigated from the bench to the bedside. It is becoming clear that such differences may affect the clinical profile and even management of arrhythmias. However, a unified understanding of the sex-based differences in normal and abnormal cardiac excitability remains a work in progress, and this area deserves further study.

The generation and accumulation of ROS have been intimately linked to the cellular processes which underlie aging. By promoting oxidative damage to multiple subcellular and cellular structures, ROS have been suggested to promote the pathogenesis of AF by inducing structural and electrical remodeling including shortening of APD. Our cellular studies support this interpretation and directly implicate PKCe as a key mediator of the relationship between oxidative stress and AF pathogenesis. Our data showed that ROS activate PKCe, induce its translocation to the cell membrane and increase I_{K_{ACh}}. Additionally, our patch clamp data showed that I_{K_{ACh}} is constitutively active in the old atrial myocytes via a mechanism that depends, at least in part, on PKCe. It has been proposed that in chronic AF, I_{K_{ACh}} is constitutively active and could be modulated by PKCe. Makary et al. showed that PKC isoforms differentially modulate I_{K_{ACh}}, with the conventional Ca²⁺-dependent PKCa isoform inhibiting and the novel PKCe isoform enhancing activity. Here, we provide evidence that aging-mediated AF inducibility and duration are reduced by abrogating constitutively active I_{K_{ACh}} via knocking out PKCe or by direct block with TPQ.

Studies have shown that ROS could also activate the ATP-sensitive inwardly rectifying potassium current (I_{K_{ATP}}). I_{K_{ATP}} is another inwardly rectifying potassium current involved in AP repolarization and can mediate APD shortening and stabilization of arrhythmias and could thus be a target of PKCe. Additionally, oxidative stress could also lead to phosphorylation mediated decrease of the L type calcium current (LTCC). Electrophysiological studies revealed that LTCC plays a crucial role in the electrical remodeling of AF, where there is a reduction of LTCC contributing to APD shortening and possibly to AF initiation and perpetuation. Therefore, the LTCC and I_{K_{ATP}} can also be ionic mechanisms that are targets of PKCe and participate in promoting aging-mediated AF.

It has been demonstrated earlier by us and others that I_{K_{ACh}} block with TPQ or with chloroquine reduces the occurrence of persistent AF and restores sinus rhythm in animal models and in a patient with persistent AF. It has been reported that the small molecules NTC-801 and AZD2927 were developed as selective inhibitors of I_{K_{ACh}}, which do not prolong the QT interval. However, these drugs failed to cardiovert paroxysmal AF and atrial flutter in patients. This could be due to the differences in the ionic bases of

![Figure 8. Proteomic analysis in WT female versus male atria](image_url)

Protein levels of 55 genes were significantly different between the sexes, with 32 proteins increased and 23 proteins decreased in abundance in females compared to males. The bar graph shows enriched biological processes associated with the differentially regulated proteins. Enrichment indicates the ratio of genes with that GO term that are differentially expressed over what would be expected by chance.
paroxysmal and persistent AF. For instance, $I_{KACH}$ is not constitutively active in patients with paroxysmal AF, and the mechanisms of atrial flutter differ from those of persistent AF.

LIMITATIONS OF THE STUDY

The pulmonary veins have been recognized as critically important sites of arrhythmogenic foci that trigger AF. In the present study, we performed programmed electrical stimulation in the right atria to induce AF. Thus, our data may not reflect the spontaneous initiation mechanisms of aging-mediated AF in patients. We used a mouse model of PKCε global knockout which might result in confounders. For instance, the body weight of old KO mice is significantly smaller compared to old WT mice. Our RNA-seq data suggested that this could be due, in part, to the role that PKCε might play in metabolism and adipose tissue biology (Figure 7; Tables S1, S2, and S3). An inducible model of cardiac-specific PKCε knockdown could be used in the future to assess, in a cardiac-specific manner, the role of PKCε and $I_{KACH}$ in aging-mediated AF.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Material availability
  - Data and code availability
- METHOD DETAILS
  - Animals
  - Single cell isolation
  - Cellular electrophysiology
  - In-vivo electrophysiology
  - Optical mapping studies
  - Total internal reflection fluorescence microscopy
  - RNAseq
  - Proteomics
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105442.

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AUTHOR CONTRIBUTIONS

MC, DEL, JO, and SFN conceived experiments. MC, KDG, BC, AT, JG, ERW, JO, DEL, and SFN performed experiments and analyzed data. MC, KDG, JG, ERW, JO, DEL, and SFN wrote the manuscript. DEL, and SFN secured funding.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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# STAR Methods

## Key Resources Table

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Chemicals, peptides, and recombinant proteins** | | |
| Trypsin/Lys-C Mix | Promega | Cat#V5073 |
| Collagenase A | Roche Diagnostics GmbH | Cat#10103578001, CAS#9001-12-1 |
| Protease | Sigma-Aldrich | Cat#P5147, CAS#9038-06-0 |
| Tertiapin-Q | Alomone Labs | Cat#STT-170, CAS#910044-56-3 |
| Di-4-ANEEPPS | Sigma | CAT# D8064, CAS# 90134-00-2 |
| (±)Blebbistatin | Abcam | Cat#ab120425, CAS#674289-55-5 |
| RIPA buffer | Sigma-Aldrich | CAT#R0278 |
| Protease and phosphatase inhibitor cocktail | Sigma-Aldrich | CAT#PPC1010 |
| **Critical commercial assays** | | |
| S-Trap micro columns | Protifi | CAT#C02-micro-10 |
| RNeasy Mini kit | QIAGEN | CAT#74104 |
| TruSeq RNA Library Prep Kit v2 | Illumina | CAT#RS-122-2001 |
| **Deposited data** | | |
| RNA-seq (old KO vs. WT hearts) | This paper | GEO: GSE211472 |
| Proteomics (WT female vs. male hearts) | This paper | MassIVE: MSV000090489 |
| **Experimental models: Cell lines** | | |
| HEK 293 Cells | Dr. Dougass Bayliss, University of Virginia | NA |
| **Experimental models: Organisms/strains** | | |
| Mouse: B6.129S4-Prkce<sup>tm1Msg</sup>/J | The Jackson Laboratory | Strain ID: 004,189, RRID: IMSR_JAX:004,189 |
| **Oligonucleotides** | | |
| PKC ε siRNA | Santa Cruz Biotechnology | Cat#sc-36251 |
| **Recombinant DNA** | | |
| mCherry-CRY2-PKCε-CAT-HA | Dr. Diomedes E. Logothetis, Northeastern University | NA |
| **Software and algorithms** | | |
| ImageJ | National Institute of Health, [https://imagej.nih.gov/ij/](https://imagej.nih.gov/ij/) | NA |
| Micromanager | University of California at San Francisco, [https://micro-manager.org](https://micro-manager.org) | NA |
| Multiclamp 700B amplifier | Molecular Devices | NA |
| Axon Digidata 1550B | Molecular Devices | NA |
| pClamp 10.6 PC software | Molecular Devices | NA |
| Clampfit 10.6 | Molecular Devices | NA |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Dr. Sami F. Noujaim (snoujaim@usf.edu).

Material availability
The study did not generate new unique reagents.

Data and code availability
RNA seq data are available through the Gene Expression Omnibus: GSE211472, and the proteomics data are available through MassIVE: MSV000090489. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request. This paper does not report original code.

METHOD DETAILS

Animals
All animal care procedures followed the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Committee on Use and Care of Animals of the University of South Florida. Both male and female mice were used. The PKCε knockout (KO) mouse was purchased from the Jackson Laboratory. Experiments were performed in male and female young and old WT and KO animals. The average age of young animals was 3.9 ± 0.08 months and that of old mice was 20.9 ± 0.43 months. For euthanasia, CO2 was used to induce loss of consciousness, and after loss of pinch reflex, swift cervical dislocation was performed, followed by rapid harvesting of the heart via thoracotomy. This method is consistent with the guidelines of the American Veterinary Medical Association.
Single cell isolation

Atrial myocytes were enzymatically dissociated from young and old WT and KO mice. In short, immediately after cardiac excision, the heart was cleaned, and the aorta was cannulated. The heart was then retrogradely perfused at 2 mL/min at 36 ± 0.5°C, for 3 min with Ca²⁺ free Tyrode solution (in mM): NaCl 137, KCl 5.4, HEPES 10, MgCl₂ 1, and glucose 10 (pH 7.3) until the effluent was clear of blood. Then the heart was perfused with the same solution containing collagenase 1 mg/mL collagenase Type A (Roche, Germany), and 0.08 mg/mL protease Type XIV (Sigma-Aldrich, USA) for 8 to 11 min, followed by Tyrode solution containing 0.2 mM CaCl₂ for 5 min. Single cells were then obtained by dissociation via gentle agitation of digested atrial tissues. Atrial myocytes suspensions were filtered through a nylon mesh, and cells were stored at room temperature in Tyrode solution. All solutions used for dissection and perfusion were continuously bubbled with 100% O₂.

Cellular electrophysiology

Atrial myocytes

The I_{KACH} current in atrial myocytes was measured with standard whole-cell voltage-clamp technique. The Multiclamp 700B (Molecular Devices) amplifier, an A/D converter (Digidata 1550B plus Hum Silencer, Molecular Devices), and the pClamp 10.6 PC software (Molecular Devices) were used for current acquisition. Clampfit 10.6 (pClamp, Molecular Devices) and OriginPro 2018b software packages (OriginLab Corp) were used for data analysis. Whole-cell measurements were performed at room temperature using borosilicate glass microelectrodes with tip resistances of 2.5–3 MΩ filled with pipette solution (in mM): KCl 140, MgCl₂ 1, HEPES 10, EGTA 5, Mg₂ATP 5, and GTP 0.1, pH adjusted to 7.2 with KOH. Basal current was recorded by holding the atrial myocyte at −40 mV, followed by 1000 ms steps from −140 mV to +40 mV in 20 mV increments. The extracellular solution contained (in mM): NaCl 100, KCl 50, MgCl₂ 1, HEPES 5, and D-glucose 5.5, adjusted to pH 7.4. To measure the constitutively active I_{KACH} current in atrial cardiomyocytes, 100 nM Tertiapin-Q (TPQ, Alomone Labs) was applied to the extracellular solution.

HEK cells

HEK293 cells stably transfected with Kir3.1/Kir3.4 were a gift from Dr. Bayliss, University of Virginia. This cell line displays a basal I_{KACH} without the need for muscarinic stimulation. I_{KACH} was recorded in these cells and the internal pipette solution contained (in mM): K-aspartate 100, NaCl 10, KCl 40, Mg-2ATP 5, EGTA 2, GTP-Tris 0.1, HEPES 10, pH 7.4. The bath solution contained (in mM): NaCl 90, KCl 50, CaCl₂ 1, MgCl₂ 2, HEPES 10, glucose 10, pH was adjusted to 7.4 with NaOH. The current was evoked in response to a ramp from −130 mV to 40 mV, from a holding potential of 0 mV, and subtracted from the residual current remaining after adding 1 mM BaCl₂ to the bath solution. In HEK293 cells stably expressing Kir3.1 and Kir3.4, SiRNA was used to silence PKCe (PKCe siRNA, Santa Cruz Biotechnology) according to the manufacturer’s protocol.

In-vivo electrophysiology

Mice were anesthetized with 1.8% isoflurane. The ECG was recorded in Lead II configuration, and a 1.1 F octapolar Millar electrophysiology catheter, with an injection port for intracardiac delivery (Mikro-Tip catheter, ERP-801, Millar, USA), was placed in the right atrium through jugular access. The ECG and the intracardiac electrograms were simultaneously recorded using the Advanced Instruments platform. The cardiac signals were recorded via Animal Bio Amp (AD Instruments, USA) and digitized via the PowerLab data acquisition system (AD Instruments, USA). LabChart Pro 7.2 software (AD Instruments, USA) was used for acquisition and analysis of the cardiac electrical signals. To study AF inducibility and duration, intracardiac programmed electrical stimulation of the right atrium was performed via the catheter. The pacing protocol consisted of a 2-s burst, 2 x diastolic threshold, 2.5 ms pulse duration, increasing from 28 to 60 Hz in 2 Hz increments. AF was defined as a period of rapid irregular atrial rhythm lasting at least 2 s. Animals showing AF episodes longer than 2 s were considered as inducible for AF. To test the effects of I_{KACH} block on AF, saline (50 μL) or TPQ (5 μM, in 50 μL saline) was injected through the catheter port.

Optical mapping studies

Optical mapping in the isolated Langendorff-perfused mouse heart was performed as described before. Briefly, the excised hearts were rapidly cannulated and retrogradely perfused with normal Tyrode solution at 37°C. The heart was then placed in the well of a custom-made chamber maintained at 37°C. Optical mapping of the right atrial epicardial surface was carried out using a high-resolution CCD camera (1000 frames/s) and
Di-4-ANEPPS (Sigma, St. Louis, MO, USA). The preparation was monitored using volume-conducted ECG in lead II configuration, recorded continuously with a Biopac System amplifier (DA100C; Biopac Systems, Inc., Goleta, CA, USA). Motion uncoupling was achieved with 7 μM blebbistatin (Abcam, USA). Action potential duration maps were generated as previously reported.86–88

**Total internal reflection fluorescence microscopy**

HEK293T cells were seeded on coverslips. 24 h later, cells were transfected with mCherry-CRY2-PKCεCAT-HA using polyethyleneimine (0.75 μg DNA: 3 μL PEI for 2 h). Total internal reflection fluorescence (TIRF) microscopy experiments were conducted the following day. mCherry was used as a proxy to detect the translocation of mCherry-CRY2-PKCεCAT-HA to the cell membrane. Cells were incubated with 100 μM H2O2 in a solution comprised of (in mM): NaCl 130, KCl 4, MgCl2 1.2, CaCl2 2, HEPES 10, pH was adjusted to 7.4 with NaOH. mCherry was excited with a 561 nm laser (Thor Labs) at 5, 15 and 30 min after treatment with H2O2. Images were captured at 5 s intervals to prevent bleaching and data were saved as separated stacks; the background was subtracted. Data were collected with Micromanager software and analyzed with ImageJ.

**RNAseq**

Atria from 3 WT old and 3 KO old male and female mice were isolated, flash-frozen then homogenized and lysed. mRNA was extracted from the tissue samples using the RNeasy kit from QIAGEN. Libraries were generated with TruSeq RNA Library Prep Kit v2 and sequencing was then performed on the USF Genomics Core NextSeq550 using 150 bp paired-end reads (75 bp each way). Sequencing-quality was measured using fastqc v0.11.5, and quality reports were aggregated using Multiqc v1.7. Resulting sequences were aligned against the *Mus musculus* reference genome GRCm38 (obtained from Ensembl; RefSeq ID: 5,034,988) using hisat2 v2.1.099 with RNA-strandedness set to paired-end. The gene expression-counts were estimated using FeatureCounts v1.5.0-p.90 Transcript-alignments were made against gene features annotated in *Mus musculus* GRCm38.95.gtf from Ensembl.

Differentially expressed genes in old KO versus old WT samples were determined using EdgeR v3.4.1.91 Trimmed mean of M-values (TMM) normalization92 was performed on all samples, and lowly expressed genes (having fewer than 10 counts-per-million reads (CPM) in any of the samples) were filtered out before analysis. Genes having a false discovery rate (FDR)-adjusted p value of less than or equal to 0.05 were considered differentially expressed.

Associated gene-ontology (GO) terms for all genes detected above threshold in the RNAseq data were extracted using the AnnotationDbi93 and org.Mm.eg.db Bioconductor packages for R v3.5.94 Differentially-expressed genes between KO and WT mice were then evaluated for GO-term enrichment against all detected genes using the weighted Fisher elimination hybrid test from topGO.95

**Proteomics**

Atrial samples were obtained from 3 young WT male and 3 young WT female mice. Samples were lysed in RIPA buffer and 1X protease inhibitor cocktail (Roche). SDS (5% final concentration) was added to 50 μg of the protein samples. All samples were prepped with S-Trap micro columns (Protifi) according to the manufacturer’s protocol. For LC-MS analysis, the peptides were characterized using a Thermo Q-exactive-HF-X mass spectrometer coupled to a Thermo Easy nLC 1200. Samples were separated at 300 nL/min on an Acclaim Pepmap 100 trap (75 μm, 2 cm, c18 3um, 100A) and a Thermo easyspray Column (75 μm, 25 cm, c18, 100A) using a standard 120-min gradient with an initial starting condition of 2% buffer B (0.1% formic acid in 90% Acetonitrile) and 98% buffer A (0.1% formic acid in water). The mass spectrometer was outfitted with a Thermo nanospray easy source with the following parameters: Spray voltage: 1.8, Capillary temperature: 250°C, Funnel RF level = 40. Parameters for data acquisition were as follows: for MS data the resolution was 60,000 with an AGC target of 3 × 105 and a max IT time of 50 ms, the range was set to 400–1600 m/z. MS/MS data was acquired with a resolution of 15,000, an AGC of 1 × 105, max IT of 50 ms, and the top 30 peaks were picked with an isolation window of 1.6 m/z with a dynamic execution of 25 s. Data were processed using Max quant 2.0.3.1 free software. A reviewed mouse database was downloaded from Uniprot and searched with the following parameters: a precursor mass tolerance of 10 ppm, and a fragment mass tolerance of 0.02 Da. FDR rate was set at 0.01, LFQ intensities were compared between samples for ID. LFQ intensities of proteins detected above threshold for at least two out of three samples for each sample-group were then assessed for differential abundance. Ratios comparing female versus males were generated and assessed for statistical significance using Welch’s t-test. Absolute differences between female
and male abundances for each protein were calculated to determine direction of difference (positive numbers = higher in females, negative numbers = lower in females) and range over which to normalize for comparison between proteins (Z score). Abundance ratios were then normalized across all proteins such that 0 indicated no difference between the sexes, and the largest number indicated greatest difference. Proteins having a Z score > 1 and Welch’s t-test p value < 0.05 were considered significant. The differentially abundant proteins were assessed for gene ontology enrichment against a background of all proteins detected above threshold for which the female/male ratio could be determined. Gene ontology annotations were obtained from the AnnotationDBI and org.Mm.eg.db R packages (version 1.56.2 and 3.14.0, respectively). The R package topGO version 2.48.0 was used to calculate gene ontology enrichment using the weight01 algorithm and the Fisher exact test. Gene ontology categories with a p value of less than or equal to 0.05 were considered enriched.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

The data are expressed as mean ± SEM, or as boxplots (Median, 25th and 75th percentiles, minimum and maximum). Data normality was assessed with the Shapiro-Wilk test. For statistical analysis, Chi square, Student’s t test, Mann Whitney Wilcoxon test, one-way ANOVA and linear mixed model were used as appropriate. Statistical significance was taken as p < 0.05.