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Loss of SPEG Inhibitory Phosphorylation of RyR2 Promotes Atrial Fibrillation

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Abstract

Background: Enhanced diastolic calcium (Ca^{2+}) release via ryanodine receptor type-2 (RyR2) has been implicated in atrial fibrillation (AF) promotion. Diastolic sarcoplasmic reticulum (SR) Ca^{2+} leak is caused by increased RyR2 phosphorylation by protein kinase A (PKA) or Ca^{2+}/calmodulin-dependent kinase-II (CaMKII) phosphorylation, or less dephosphorylation by protein phosphatases. However, considerable controversy remains regarding the molecular mechanisms

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DISCLOSURES

XHTW is a founding partner of Elex Biotech, a start-up company that developed drug molecules to target ryanodine receptors for treatment of cardiac arrhythmias. Other authors have no conflicts related to this study.

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underlying altered RyR2 function in AF. We thus sought to determine the role of 'striated muscle preferentially expressed protein kinase' (SPEG), a novel regulator of RyR2 phosphorylation, in AF pathogenesis.

Methods: Western blotting was performed with right atrial biopsies from paroxysmal (p)AF patients. SPEG atrial knock-out (aKO) mice were generated using adeno-associated virus 9 (AAV9). In mice, AF inducibility was determined using intracardiac programmed electrical stimulation (PES), and diastolic Ca^{2+} leak in atrial cardiomyocytes was assessed using confocal Ca^{2+} imaging. Phospho-proteomics studies and western blotting were used to measure RyR2 phosphorylation. In order to test the effects of RyR2-S2367 phosphorylation, knock-in mice with an inactivated S2367 phosphorylation site (S2367A) and a constitutively activated S2367 residue (S2367D) were generated using CRISPR-Cas9.

Results: Western blotting revealed decreased SPEG protein levels in atrial biopsies from pAF patients in comparison to patients in sinus rhythm. SPEG aKO mice exhibited increased susceptibility to pacing-induced AF by PES and enhanced Ca^{2+} spark frequency in atrial cardiomyocytes with Ca^{2+} imaging, establishing a causal role for decreased SPEG in AF pathogenesis. Phospho-proteomics in hearts from SPEG cardiomyocyte knock-out mice identified RyR2-S2367 as a novel kinase substrate of SPEG. Additionally, western blotting demonstrated that RyR2-S2367 phosphorylation was also decreased in pAF patients. RyR2-S2367A mice exhibited an increased susceptibility to pacing-induced AF as well as aberrant atrial SR Ca^{2+} leak. In contrast, RyR2-S2367D mice were resistant to pacing-induced AF.

Conclusions: Unlike other kinases (PKA, CaMKII) that increase RyR2 activity, SPEG phosphorylation reduces RyR2-mediated SR Ca^{2+}-release. Reduced SPEG levels and RyR2-S2367 phosphorylation typified patients with pAF. Studies in S2367 knock-in mouse models showed a causal relationship between reduced S2367 phosphorylation and AF susceptibility. Thus, modulating SPEG activity and phosphorylation levels of the novel S2367 site on RyR2 may represent a novel target for AF treatment.

Keywords

Atrial fibrillation; excitation-contraction coupling; ion channels; RyR2; SPEG

INTRODUCTION

Atrial fibrillation (AF) is the most common sustained cardiac arrhythmia affecting over 33.5 million individuals worldwide.[1] AF is associated with high morbidity and mortality as it can lead to both ischemic stroke and heart failure.[2] AF progresses from the acute, self-terminating form, known as paroxysmal AF (pAF), to long-standing persistent (chronic) AF (cAF), accompanied by atrial structural and electrical remodeling. Enhanced diastolic sarcoplasmic reticulum (SR) calcium (Ca^{2+}) release through ryanodine receptor type-2 (RyR2) has been implicated in AF pathogenesis.[3] However, the molecular mechanisms driving this process remain incompletely understood and, in some regards, controversial.[4]

Multiple studies have been published regarding the effects of RyR2 phosphorylation on channel activity in cardiovascular disease, including AF.[5, 6] Most studies have shown that hyperphosphorylation of RyR2 at S2808 and S2814, by protein kinase A (PKA) and Ca^{2+}/

calmodulin-dependent kinase-II (CaMKII) respectively, increases diastolic SR Ca^{2+}-leak through RyR2.[7, 8] Increased SR Ca^{2+}-leak increases the activity of the Na^{+}/Ca^{2+}-exchanger (NCX), resulting in delayed after-depolarizations, which may serve as a trigger for arrhythmic activity in the presence of an AF substrate.[3] While the effect of CaMKII on enhanced SR Ca^{2+}-leak is well-validated, experiments to determine the effects of PKA phosphorylation of RyR2 have resulted in inconsistent data regarding its relevance to human disease.[9–12] Additionally, *in vitro* experiments examining single channel RyR2 activity have shown that dephosphorylation of RyR2 by phosphatases might enhance channel activity, suggesting that inhibitory phosphorylation sites may decrease channel opening countering the effects of S2808 and S2814 phosphorylation.[13]

S2808 and S2814 hyperphosphorylation are seen in cAF but not pAF,[14] and it is unclear whether these events are truly driving AF or are the consequence of the rapid atrial rate and the related remodeling during AF. Furthermore, these post-translational modifications cannot explain the increase in RyR2 activity seen in pAF, as enhanced RyR2 activity has been shown in bilayer studies from human pAF samples with unchanged S2808 and S2814 phosphorylation.[14] Identifying regulators of RyR2 activity in pAF could aid in developing novel therapeutics to halt the AF progression.

Our lab has recently identified ‘striated muscle preferentially expressed protein kinase’ (SPEG) as a binding partner of RyR2 implicated in human heart failure progression.[15] SPEG is a member of the myosin light chain kinase family with two tandem serine-threonine kinase domains 1 and 2, known to phosphorylate junctophilin-2 and sarco/endoplasmic reticulum calcium ATPase-2a (SERCA2a), respectively.[16] SPEG is essential for junctional complex formation in the ventricles with loss of SPEG leading to aberrant Ca^{2+}-handling and loss of transverse-tubule structures resulting in dilated cardiomyopathy and heart failure.[15] However, SPEG’s presence in atria and its role in AF has not been explored.

Here, we report for the first time that SPEG protein levels are decreased in human pAF and that loss of SPEG is sufficient to provide a substrate for AF pathogenesis. Further, we show that SPEG phosphorylates RyR2 at a previously uncharacterized serine (S2367) located in the central domain of the channel.[17] Importantly, in contrast to previously studied phosphorylation sites that activate RyR2 (e.g. S2808, S2814), we show that SPEG mediated RyR2-S2367 phosphorylation suppresses pathogenic diastolic SR Ca^{2+}-leak. Moreover, SPEG loss and phospho-ablation of RyR2 S2367 increases diastolic SR Ca^{2+}-leak. Therapeutics that improve SPEG kinase activity may provide an effective approach to halting the onset and progression of AF.

METHODS

Data Availability Statement.

The data that support the findings of this study are available from the corresponding author upon reasonable request, according to TOP guidelines. Please see the Online Supplement for detailed materials and methods.

Human Samples.

Right atrial appendages from patients undergoing open-heart surgery were collected after obtaining informed written consent. The study was approved by the ethics committee (IRB) of the Medical Faculty of the University Duisburg-Essen, Essen, Germany (AZ:12-5268-BO).

Animal Studies.

All mouse studies were performed according to the protocols approved by the Baylor College of Medicine Institutional Animal Care and Use Committee and conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. All experiments using mice were performed in a blinded manner. Number of mice used in each experiment is included in the results section. Generation and breeding of cardiomyocyte-specific SPEG conditional knock-out (SPEG cKO), atrial-specific SPEG conditional knock-out mouse (SPEG aKO), RyR2-S2367A and RyR2-S2367D knock-in mouse models are described further in the Online Supplement.

Western Blot.

Protein and phosphorylation levels in human and mouse samples were measured using SDS-page gel and western blotting. For detailed protocols, see the Online Supplement.

Echocardiography.

Ventricular function in mice was assessed using echocardiography using a Vevo 2000 system as described.[15, 18] Temperature was maintained between 36.5–37.5°C while mice were anesthetized using 1.5% isoflurane.

Programmed Electrical Stimulation (PES).

PES was performed in mice to test for arrhythmia susceptibility as described.[19]

Cell Isolation and Ca^{2+} Imaging.

Atrial and ventricular myocytes were isolated using Langendorff perfusion and imaged using a Zeiss LSM 880 confocal imaging system. To measure Ca^{2+}-spark latency of individual RyR2 clusters, cells were treated with 50 nM ryanodine.[20] Cell isolation, data collection and analysis methods and spark parameter cut-offs (Table I in the Supplement) are in the Online Supplement.

STimulated Emission Depletion (STED) Nanoscopy.

Immunofluorescence confocal imaging and superresolution STED microscopy (nanoscopy) were used as described previously.[21] Detailed staining and imaging protocols can be found in the Online Supplement.

Mass Spectrometry.

RyR2 phosphorylation was examined by immunoprecipitating RyR2 from SPEG cKO and control heart lysates followed by phospho-proteomics[22] as detailed in the Online Supplement.

Statistical Analysis.

All data analysis was performed using Graphpad-Prism or SPSS software. When normality could be assumed, unpaired student t-test or one-way ANOVA followed by Tukey's post-hoc analysis was utilized for nominal data. If assumptions of normality were not met, Mann-Whitney U test between two groups or Kruskall-Wallis followed by Dunn's post hoc-test between more than 2 groups was used. Fisher's exact test was used for categorical data. The ROUT test was used to identify possible outliers (Q=1%). For Ca^{2+}-imaging studies, to account for clustering of the data by mouse and genotype, the generalized estimating equation was used. Alpha was set at 0.05 for all studies.

RESULTS

SPEG protein levels are decreased in patients with pAF

SPEG is a critical mediator of ventricular cardiac function and its protein levels are decreased in patients with HF.[15] However, it is unknown whether SPEG levels are altered in patients with AF. To test this, we performed western blotting using human atrial samples from pAF patients or patients in normal sinus rhythm (NSR). Characteristics of all patients enrolled in our study were comparable between both groups (Table II in the Supplement). Western blotting (Figure 1A) of a subset of samples (Table III in the Supplement) revealed a 30.5±0.07% decrease in SPEGβ (p=0.031, Figure 1B) and a 33.3±0.06% decrease in SPEGα (p=0.003, Figure 1C) in pAF (n=23) compared to NSR controls (n=22). These experiments were conducted on tissue from patients with a history of pAF, but in NSR at time of tissue collection. There was no correlation between atrial SPEG levels and the time between the last documented AF episode and time of tissue collection during cardiac surgery (see Figure IA–B in the Supplement). To test whether a recent AF episode biased the results, we compared SPEG levels in patients who were in AF as recent as 24 hours before surgery (red dots) vs. those were had their last AF episode >24 hours before surgery but detected similar SPEG levels in both groups (Figure IC–D in the Supplement). In addition, SPEG levels were found to be unchanged in patients with cAF (Figure II and Table IV in the Supplement). Therefore, differences in SPEG levels between pAF and NSR patients are unlikely to be a consequence of the high atrial rate during AF but rather represent a molecular change that predisposes to AF development.

Atrial-specific knockout of SPEG increases AF inducibility in mice

SPEG is known to be a crucial regulator of ventricular Ca^{2+}-homeostasis.[15] Therefore, we hypothesized that decreased SPEG would be sufficient to enhance susceptibility of AF since improper SR Ca^{2+}-handling plays a significant role in AF inducibility.[3] We therefore performed PES studies in conditional cardiomyocyte-specific SPEG KO (cKO) mice with preserved left ventricular function. Mice were injected with tamoxifen for three days followed by echocardiography two-weeks post-injection (Figure IIIA in the Supplement). An ejection fraction of >45% was set as a pre-determined cut-off for inclusion in the study to prevent confounding effects of reduced ventricular systolic function (Figure IIIB in the Supplement). With these criteria, 4 cKO and 1 control mouse were excluded from the study. PES studies revealed that 87.5% of SPEG cKO (n=8) mice exhibited AF after rapid atrial

pacing in comparison to none of the mER-Cre-mER controls (n=6) (p=0.005, Figure IIIC–D in the Supplement).

Next, to rule out the possibility that increased AF susceptibility was secondary to early ventricular dysfunction, we developed conditional atrial-specific SPEG knockout (aKO) mice by injecting $SPEG^{fl/fl}$ mice and wild-type (WT) littermates with an adeno-associated virus 9 (AAV9) to express an atrial-specific Cre (AAV9-ANF-Cre). Western blotting revealed a 40.5±0.3% decrease in SPEGβ levels in SPEG aKO mice (n=4) in comparison to WT controls (n=4, p=0.020, Figure 2A–B). Similarly, atrial SPEGα levels were reduced by 30.1±0.04% in SPEG aKO mice compared to WT controls (p=0.029; Figure 2A,C). Importantly, ventricular SPEGβ and SPEGα levels were unaltered in SPEG aKO mice (Figure IV in the Supplement).

To determine whether atrial-specific SPEG loss was sufficient to promote AF, SPEG aKO mice underwent PES studies. Baseline ECG intervals and nodal refractory times were similar between groups (Table V in the Supplement). However, SPEG aKO mice were more susceptible to AF induction after PES (62.5%) compared to WT controls (10%, p=0.043, Figure 2D–E). These data suggest that atrial-specific loss of SPEG is sufficient to provide a substrate for AF pathogenesis and could thus play a key role for the early development of AF.

Loss of SPEG in atrial myocytes enhances SR Ca^{2+}-leak

Previous studies have demonstrated that SPEG regulates ventricular Ca^{2+}-homeostasis.[15] Therefore, to test the hypothesis that atrial SPEG loss could also enhance diastolic Ca^{2+}-leak, we performed confocal Ca^{2+}-imaging studies of atrial myocytes isolated from $SPEG^{fl/fl}$ and WT mice treated with AAV9-ANF-Cre-2A-mCherry (Figure 3A). The use of the mCherry fluorescent marker allowed us to identify cells positive for Cre expression in our SPEG aKO mouse model (Figure 3B). There was no difference in transduction efficiency between SPEG aKO mice and WT controls (Figure 3C). During 1-Hz pacing, atrial myocytes from SPEG aKO mice did not exhibit changes in systolic Ca^{2+}-transient amplitude in comparison to myocytes from WT controls irrespective of mCherry expression (Figure VA–B in the Supplement). Furthermore, SPEG loss had no effect on SR load or NCX and SERCA2a activities (Figure VC–E in the Supplement).

Interestingly, mCherry-positive myocytes from SPEG aKO mice demonstrated an increased Ca^{2+}-spark frequency (CaSpF,1.62 ± 0.28 sparks/100μm/s) compared to both mCherry-positive cells from WT controls (0.47±0.17 sparks/100μm/s, p=0.005) and mCherry-negative cells from SPEG aKO mice (0.36±0.07 sparks/100μm/s, p=0.002) (Figure 3D–E). Results remained significant when the CaSpF was normalized to SR Ca^{2+}-load (Figure VF in the Supplement). However, there were no significant differences in mCherry-negative cells from SPEG aKO mice compared to WT controls (Figure 3E) indicating that SPEG regulates Ca^{2+}release in a cell-autonomous manner. Additionally, since there was no difference between mCherry-negative and mCherry-positive WT cells, it is unlikely that the findings are due to toxic effects of either mCherry or Cre.

SPEG co-localizes with RyR2 clusters in atrial myocytes.

To test whether SPEG co-localizes with RyR2 clusters in mouse atrial myocytes, immunofluorescence imaging was performed using both confocal microscopy and STED superresolution nanoscopy (Figure 4). Whereas confocal imaging suggested co-localized signals throughout atrial myocytes (Figure 4A), STED nanoscopy resolved co-localized signals specifically in transversal striations but not at the lateral surface (Figure 4B), as confirmed by image segmentation (Figure 4C). Signal intensity profiling of individual RyR2 clusters showed a closely co-localized SPEG signal distribution (Figure 4D). Both the percentage of RyR2 and SPEG cluster signals overlapping with each other (Figure 4E) and overlapping area fractions were similarly high in atrial myocytes (Figure 4F), excluding subsurface signals from analysis. Hence, RyR2 clusters in transverse striations are highly co-localized with SPEG. In addition, many SPEG signals could be identified at the cell surface but not co-localized with RyR2 (Figure 4C).

SPEG phosphorylates S2367 on RyR2

Next, we tested whether RyR2 phosphorylation was altered in SPEG cKO mice. RyR2 phospho-peptides were identified using mass spectrometry of RyR2 immunoprecipitates from heart lysates from SPEG cKO mice; tamoxifen-injected mER-Cre-mER and Cre-negative-SPEG$^{fl/fl}$ mice served as controls (Figure 5A). We successfully detected RyR2 peptides containing previously characterized phosphorylation sites, S2808 and S2814 (S2809 and S2815 in humans), as well as peptides containing previously uncharacterized sites (Figure 5B). Phosphorylation levels of the peptides containing S2808 and S2814 were unchanged in agreement with previous studies.[15] By contrast, a significant decrease was found in RyR2-S2367 phosphorylation (S2368 in humans) in SPEG cKO hearts (0.126±0.13 a.u., n=3, vs. 1.00±0.29, n=5; Figure 5C). To avoid confabulation with mouse S2368, we shall use S2367 to denote this site in both mouse and human data. Of note, under these conditions, S2030 phosphorylation (S2031 in humans), another previously studied phosphorylation site,[23] was not detected in either group.

A custom-made phospho-epitope specific polyclonal antibody that recognizes phosphorylated S2367 (Figure VI in the Supplement) was used to verify our mass spectrometry findings. In hearts of SPEG cKO mice, SPEG levels were downregulated by 60.9±3.1% compared to controls (p=0.036; Figure 5D–E). SPEG reduction was not as large as previous studies as mice underwent tamoxifen for 3 days vs. 5.[15] The level of S2367 phosphorylation on RyR2 was proportionally reduced by 55.8±8.4% in SPEG cKO mice compared to controls (p<0.016; Figure 5F–G), confirming that S2367 is phosphorylated by SPEG *in vivo*. Additionally, S2814 and S2808 phosphorylation was unchanged in SPEG cKO hearts as detected by western blots (Figure VII in the Supplement), consistent with the mass spectrometry data. To test the validity of these findings in atria, we examined atrial S2367, S2814, and S2808 phosphorylation in SPEG aKO mice. RyR2-S2367 phosphorylation relative to total RyR2 was decreased by 36.4±11.3 (p=0.02), while S2814 and S2808 phosphorylation levels were unchanged (Figure VIII in the Supplement).

Furthermore, to test for crosstalk between previously characterized phosphorylation sites and RyR2-S2367 phosphorylation, we performed western blotting for pS2367 relative to

total RyR2 in atria from RyR2-S2808A, RyR2-S2814A, and RyR2-S2814D mice, but found no significant differences (Figure IX in the Supplement). These results imply that RyR2-S2367 phosphorylation is independent of these other sites.

RyR2-S2367 phosphorylation is decreased in patients with pAF

To test whether decreased SPEG levels also cause a loss of S2367 phosphorylation in pAF patients, we examined RyR2 phosphorylation via western blot using biopsies from patients in pAF and NSR controls (Table VI in the Supplement; Figure 6A). Consistent with previous work, we found that total RyR2 protein levels were increased by 48.9±6.7% in patients with pAF (n=20) compared to NSR controls (n=21, p=0.01; Figure 6B).[14] RyR2-S2367 phosphorylation was not significantly altered in pAF patients relative to GAPDH expression levels. However, RyR2-S2367 phosphorylation normalized to total RyR2 was decreased by 26.4±0.06% in pAF patients (n=20) in comparison to NSR controls (n=21, p=0.036). On the contrary, phosphorylation of S2808 and S2814 relative to total RyR2 levels were unchanged in pAF patients (Figure X in the Supplement). Like in mice, phosphorylation of S2367 was not correlated with levels of S2814 or S2808 phosphorylation in human atrial samples (Figure XI in the Supplement). These results suggest that a reduction in RyR2-S2367 phosphorylation may be a unique marker of RyR2 dysfunction in pAF patients.

Ablation of S2367 phosphorylation enhances susceptibility to AF

Although S2367 has been reported previously as a RyR2 phosphorylation site in a phospho-proteomics study,[24] the functional consequences of S2367 phosphorylation has not been explored, yet. To determine the physiological significance of S2367 phosphorylation, two RyR2 knock-in mouse models were generated using CRIPSR-Cas9 mediated mutagenesis: RyR2-S2367A mice where the S2367 phosphorylation site is genetically ablated and RyR2-S2367D mice where the S2367 site is constitutively activated. Compared to WT littermates, RyR2-S2367A and S2367D mice exhibited normal ventricular function at 3 months of age with echocardiography (Table VII in the Supplement). RyR2-S2367A and RyR2-S2367D mice also exhibited no changes in RyR2-S2808 or RyR2-S2814 phosphorylation (Figure XII in the Supplement).

Baseline ECG intervals were unchanged in both S2367A and S2367D mice compared to WT littermates (Table VIII in the Supplement). However, PES studies revealed an enhanced susceptibility to AF in S2367A mice in comparison to WT littermates (Figure 7A): 58% (7/12) of S2367A exhibited AF compared to 0% (0/16; p<0.001) of WT (Figure 7B). In contrast, S2367D mice did not exhibit an increased incidence of AF. Thus, loss of S2367 phosphorylation lowered the threshold for AF induction, suggesting that decreased SPEG-mediated phosphorylation of RyR2 could contribute to AF induction in pAF patients.

Finally, to determine whether S2367D mice were protected from AF induction, S2367D mice and WT littermates were subjected to PES after an intraperitoneal injection of carbachol (50 μg/kg) (Figure 7C). While 55.6% (5 of 9) WT mice exhibited pacing-induced AF after carbachol, only 7.7% (1 of 13; p=0.02) of S2367D mice developed AF (Figure 7D). In contrast to previous RyR2 phospho-mimetic mutants,[5] hyperphosphorylation of RyR2-S2367 leads to protection against AF after carbachol injection.

Ablation of RyR2-S2367 phosphorylation enhances diastolic Ca^{2+} leak

Following a 1-Hz pacing train, diastolic SR Ca^{2+}-release events were recorded under resting conditions. The CaSpF was increased (1.66±0.33 sparks/100μM/s) in atrial myocytes from S2367A mice compared to cells from WT mice (0.61±0.10 sparks/100μM/s, p=0.001; Figure 8A–B). The CaSpF in S2367D myocytes (0.80±0.21 sparks/100μM/s) was similar to those in WT cells. In addition, an increased number of spontaneous Ca^{2+} waves were observed in myocytes from S2367A mice (43.8% of myocytes) in comparison to myocytes from WT littermates (13.6%, p=0.04) and S2367D mice (13.0%, p=0.03; Figure 8C–D). Thus, increased diastolic SR Ca^{2+}-leak in atrial myocytes from S2367A mice was sufficient to trigger cell-wide Ca^{2+}-waves. Therefore, a reduction in S2367 phosphorylation on RyR2 could provide a trigger for arrhythmogenic activity through increased Ca^{2+}-leak from RyR2.

To further examine the mechanistic role of S2367 phosphorylation on individual RyR2 channels, we performed Ca^{2+}-imaging studies in WT and S2367A myocytes treated with 50 nM ryanodine. Using a previously published method,[20] we measured the Ca^{2+}-spark latency time of individual RyR2 clusters in WT and S2367A atrial myocytes (Figure XIIIA in the Supplement). With this approach, we found a significant leftward shift in the spark-spark delay time in S2367A vs WT atrial myocytes (Figure XIIIB in the Supplement). These results further validate the increase in channel activity with loss of S2367 phosphorylation.

DISCUSSION

Enhanced RyR2-mediated SR Ca^{2+}-leak has been implicated in the pathogenesis of AF in numerous studies.[3, 4] In patients suffering from early stages of AF (i.e., pAF), RyR2 single channel open probability was shown to be increased despite unaltered phosphorylation levels at the well-characterized S2808 and S2814 sites on RyR2.[14] Our present data provide a potential mechanistic explanation for the abnormal RyR2 channel activity that promotes delayed afterdepolarizations and ectopic activity in pAF patients.[14] We found that SPEG is a critical negative regulator of RyR2 Ca^{2+}-release. Reduced SPEG-mediated phosphorylation of the novel S2367 phosphorylation site on RyR2 promotes SR Ca^{2+}-waves and increases susceptibility to AF in knock-in mouse models. To the best of our knowledge, this represents the first example of a kinase-mediated inhibitory phosphorylation event that reduces RyR2 channel activity. Reduced RyR2 phosphorylation at S2367 plays a critical role in the development of AF since RyR2-S2367A knock-in mice are more susceptible to AF as a result of genetic ablation of this phosphorylation site. Taken together, our data suggest that countering the reduction in SPEG levels and ensuing reduction in S2367 phosphorylation of RyR2 may represent a novel therapeutic approach for the treatment of AF.

Altered RyR2-mediated Ca^{2+} handling in AF

AF is the most prevalent type of cardiac arrhythmia worldwide. The clinical course of AF is progressive in nature: it first occurs as a paroxysmal form with short-lasting AF episodes, but over time it is perpetuated and becomes chronic in nature with long-lasting persistent AF episodes.[25] Numerous factors can promote the development and maintenance of AF, including genetic variants, extracardiac risk factors (i.e., aging, obesity, alcohol abuse), and cardiac remodeling.[26] Experimental studies on atrial biopsies from patients with AF and

various animal models revealed that alterations in intracellular Ca^{2+}-handling within atrial myocytes play a major role in AF pathophysiology.[3, 4] Aberrant diastolic SR Ca^{2+}-releases can cause afterdepolarization-mediated triggered activity, conduction block, and Ca^{2+}-driven subcellular alternans in AF.[27] Abnormal SR Ca^{2+}-release is now believed to be an important source of atrial ectopic activity, and sustained ectopic activity produces a 'driver' that promotes AF.[28]

Several factors contribute to the increased incidence of spontaneous SR Ca^{2+}-release events, including increased SR Ca^{2+}-load and enhanced RyR2-mediated SR Ca^{2+} release. In pAF patients, changes in Ca^{2+}-handling are more likely to be directly linked to disease pathogenesis and less likely to be secondary to atrial remodeling.[14, 27] In these patients, the amplitude of the L-type Ca^{2+}-current and NCX current were unaltered.[14] In contrast, the Ca^{2+}-transient amplitude and SR Ca^{2+}-load are larger in atrial myocytes from pAF patients, [14] likely because of enhanced activity of SERCA2a coupled with an increased number of RyR2 channels and an enhanced open probability of single RyR2 channels. The increase in RyR2 protein expression levels in pAF patients has been attributed to altered post-transcriptional regulation of RyR2 due to reduced microRNA-106b-25 expression.[29] However, the reason for the enhanced open probability of single RyR2 channels was unclear.

Altered phosphorylation of RyR2 in AF

It is well established that the activity of single RyR2 channels is modulated by various post-translational modifications including phosphorylation, oxidation, and S-nitrosylation.[7, 30, 31] Several phosphorylation sites have been characterized on RyR2, including PKA phosphorylation sites S2030 and S2808,[7, 32] and CaMKII site S2814.[8, 19] Most studies have shown that increased phosphorylation of S2808 and S2814 leads to enhanced RyR2 activity. [4] In patients with pAF, phosphorylation levels of the S2808 and S2814 sites remain unaltered,[14] whereas in patients with more advanced disease stages (i.e., cAF) phosphorylation at these residues is increased.[4, 5, 33] Enhanced CaMKII kinase activity has been shown to enhance RyR2 phosphorylation levels at the S2814 site.[34] Moreover, recent studies have shown that reduced RyR2 dephosphorylation by protein phosphatase 1 leads to increased S2808 and S2814 phosphorylation in cAF patients.[21, 22, 35]

In addition to the well-studied S2808 and S2814 phosphorylation sites, other sites (S2797, S2810, S2811, and T2876) were identified by *in vitro* kinase assays with purified PKA or CaMKII. All but the T2876 site were also detected by phospho-proteomics *in vivo* suggesting that RyR2 phosphorylation sites cluster in a hotspot for RyR2 activation.[36, 37]

Identification of the novel S2367 phosphorylation site on RyR2

In this study, we identified the S2367 residue as a novel phosphorylation site targeted by SPEG. Although not located within the above mentioned phosphorylation hotspot domain, the S2367 residue resides within a hotspot domain for cardiac disease-causing inherited mutations.[17] Interestingly, in addition to the S2367 site found in our study, four additional serine residues are located within the ten neighboring amino acids suggesting that this may represent a second RyR2 phosphorylation hotspot. To our knowledge, S2367 is the first inhibitory RyR2 phosphorylation site identified, which introduces a new paradigm into the

field of phosphorylation-mediated RyR2 regulation. Future studies will need to examine how S2367 phosphorylation affects the biophysical properties of the channel, in particular whether the open state is more stable and/or whether the closed state is more favored. It would also be interesting to assess whether the S2367 mutation alters clustering of the channels and/or their subcellular localization.[33]

Our results revealed that RyR2 phosphorylation at the new S2367 phosphorylation site is reduced in patients with pAF, suggesting that the loss of this particular post-translational modification might underlie the enhanced RyR2 activity observed in patients with pAF.[14] Studies in knock-in mouse models in which the S2367 site was genetically ablated by the S2367A mutation support this idea. For example, S2367A knock-in mice were more susceptible to pacing-induced AF (Figure 7). Moreover, the frequency of spontaneous SR Ca^{2+}-release events were increased in atrial myocytes from S2367A mice compared to WT littermates (Figure 8). Additionally, the Ca^{2+}-spark latency period was reduced in ryanodine-treated S2367A atrial myocytes, suggestive of enhanced channel open probability. In contrast, knock-in mice with constitutive pseudo-phosphorylation of S2367 due to the S2367D mutation were protected from AF induction following PES (Figure 7). These findings suggest a critical role of the S2367 phosphorylation site in AF pathogenesis as SPEG phosphorylation of RyR2 was shown to be both necessary and sufficient for disease development. To translate these findings into the clinical setting, future work may need to identify regulators of SPEG kinase activity and/or S2367 phosphorylation to discover drug targets that can be used for the treatment of pAF patients. Additionally, identifying the specific kinase domain that auto-phosphorylates SPEG itself will allow for the development of small molecule activators of SPEG catalytic activity.

Role of SPEG in the atria

Our studies confirm that SPEG is a key regulator of RyR2 in the atria as loss of SPEG expression within atrial myocytes promotes AF and abnormal SR Ca^{2+}-handling. The SPEG gene generates 4 different isoforms through both an alternative promotor and tissue-specific splicing; the SPEGα and SPEGβ isoforms are expressed in cardiomyocytes.[21] Previous studies established that SPEG is a striated-muscle specific kinase required for skeletal and cardiac muscle integrity.[15, 38] Loss-of-function mutations in SPEG can cause centronuclear myopathy with dilated cardiomyopathy in patients.[39] Disruption of the *Speg* gene was shown to cause cardiac dysfunction in mice.[15, 39] Given the important role of SPEG in the ventricle, we used a recently developed atrial-specific gene vector to selectively downregulate SPEG levels within atrial myocytes using AAV9 that expressed Cre using an ANF promotor (Figure 2, Figure IV in the Supplement).[40] In these SPEG aKO mice, we demonstrated that loss of SPEG was sufficient to provide a substrate for AF inducibility.

One of the major targets of SPEG is RyR2, and reduced SPEG phosphorylation of RyR2 increases diastolic SR Ca^{2+}-leak (Figure 3). Additionally, SPEG regulates other molecules involved in Ca^{2+}-handling and cardiac contractility, including junctophilin-2, SERCA2a, and myotubularin.[15, 16, 39] In contrast to studies done by Quan *et al.*[16] that reported that SPEG phosphorylates SERCA2a increasing SR Ca^{2+}-reuptake, we did not detect any changes in SERCA2a activity in SPEG aKO atrial myocytes (Figure V in the Supplement). It is possible

that differences in methods to estimate SERCA2a activity or different mouse backgrounds might account for this.[15, 16] Other reasons that may explain the lack of an effect on SERCA2a activity in atrial myocytes compared to ventricular myocytes is a potential compensatory effect of sarcolipin that is exclusive to atrial myocytes.[41] SPEG was shown to be critical for transverse-tubule stability within ventricular myocytes.[15] Therefore, it is possible that loss of SPEG within atrial myocytes leads to disruption of the integrity of junctional membrane complexes, thereby negatively impacting SR Ca^{2+}-handling. Compared to ventricular myocytes, atrial myocytes have fewer transverse-tubules, although a higher density of axial tubules have been reported.[42] This corresponds with limited local junctophilin-2 expression in atrial as compared to ventricular myocytes.[43] Therefore, it is likely that the impact of reduced SPEG on structural tubule integrity is less pronounced in atrial vs. ventricular myocytes, although the extent and function of junctional membrane complexes on axial tubules requires future investigation.

POTENTIAL LIMITATIONS

For this work, we generated atrial-specific SPEG knock-out mice using AAV9-mediated overexpression of Cre driven by an ANF promoter.[40] This approach led to a 30–40% reduction in atrial SPEG levels (Figure 2). It is possible that further refinement of this approach or a transgenic approach could yield a higher level of SPEG reduction. On the other hand, this 30–40% loss actually mimicked the reduction in SPEG expression in human atrial biopsies (Figure 1). Moreover, our data showed that this level of SPEG downregulation was sufficient clearly increase AF inducibility (Figure 3). We also developed novel RyR2 phosphorylation site knock-in mice carrying mutation S2367A or S2367D, respectively. The substitution of serine for a phosphomimetic aspartic acid residue can be used to model the physiological effects of protein phosphorylation at a specific residue.[19, 44] Such amino acid substitutions may be similar but not identical to a phosphate group. Another limitation is that phosphorylation is typically a transient event that can be modulated by various physiological and pathological factors. This level of modulation is not possible when the residue is permanently mutated to the phosphomimetic state. Another limitation of our study is that our mouse studies were performed in young, 3–4 month-old, otherwise healthy mice. In patients with AF, other risk factors such as age or disease-related remodeling are generally present. Thus, changes in SPEG levels and RyR2-S2367 phosphorylation may have different effects when combined with other pathologies. The variability is higher in humans with both NSR and pAF vs that seen in our inbred mouse lines. Other limitations of using mouse models include differences in electrophysiological parameters including action potential shape and duration and the absence of complex atrial remodeling seen in pAF patients with comorbidities. Future studies will be needed to validate these findings in ex vivo human tissue and large animal models that better mimic human electrophysiology. Also, although we used unbiased approaches to identify what site SPEG phosphorylates on RyR2, we cannot completely rule out effects of currently unknown kinase substrates of SPEG. It will be important for future work on SPEG to acquire a more comprehensive list of kinase targets and their effect to more fully understand the effects of SPEG loss in atrial fibrillation.

CONCLUSIONS

Our findings provide for the first time a potential explanation for enhanced RyR2 single channel activity associated with SR Ca^{2+}-leak in patients with pAF. We discovered that reduced levels of SPEG result in less phosphorylation of the newly identified RyR2 phosphorylation site S2367. This new phosphorylation site regulates RyR2 in a mechanistically novel paradigm as it inhibits RyR2 channel activity, in particular channel openings under low cytosolic Ca^{2+}-concentrations typically seen during diastole. Our data revealed a causal relationship between reduced S2367 phosphorylation and AF susceptibility. Thus, modulating SPEG activity and phosphorylation levels of the novel S2367 site on RyR2 may represent a promising target for the treatment of AF.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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NON-STANDARD ABBREVIATIONS AND ACRONYMS

AAV9	Adeno-associated virus 9
AF	Atrial fibrillation
aKO	Atrial-specific knock-out
Ca^{2+}	Calcium
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase-II
CaSpF	Ca^{2+}-spark frequency
cAF	Chronic atrial fibrillation
cKO	Cardiomyocyte-specific knock-out

CTL	Control
NCX	Na^+/Ca^{2+}-exchanger
NSR	Normal sinus rhythm
pAF	Paroxysmal atrial fibrillation
PES	Programmed electrical stimulation
PKA	Protein kinase A
RyR2	Ryanodine receptor type-2
SERCA2a	Sarco/endoplasmic reticulum calcium ATPase-2[a]
SPEG	Striated muscle-preferentially expressed protein kinase
SR	Sarcoplasmic reticulum
STED	Stimulated emission depletion
WT	Wild-type

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Clinical Perspective

What Is New?

- This study is the first to demonstrate that decreased expression of ‘striated muscled preferentially expressed protein kinase’ (SPEG) in atria is causally linked to altered diastolic calcium handling and human paroxysmal atrial fibrillation (pAF).
- Phospho-proteomics studies identified serine 2367 (S2367) on ryanodine receptor type-2 (RyR2) as a novel kinase substrate of SPEG.
- Through the study of novel RyR2 phospho-mutant mouse models, it was revealed that in contrast to previously characterized phosphorylation sites on RyR2, S2367 phosphorylation inhibits diastolic Ca^{2+}-release from RyR2 while loss of phosphorylation of this site increases AF susceptibility.

What Are the Clinical Implications?

- Loss of S2367 phosphorylation on RyR2 by reduced SPEG levels contributes to abnormal Ca^{2+}-handling and enhances susceptibility to AF.
- Normalizing S2367 phosphorylation and SPEG activity may provide novel therapeutic opportunities for the treatment of AF.

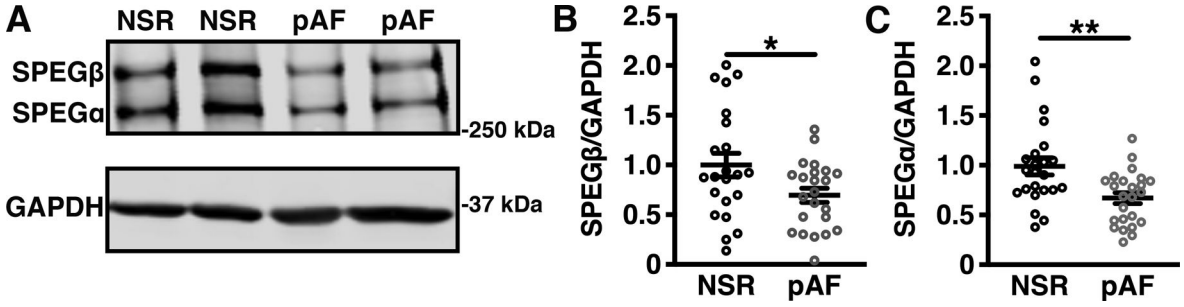

Figure 1. SPEG protein levels are decreased in patients with pAF.

Representative western blots (**A**) and bar graph quantifications of (**B**) SPEGβ and (**C**) SPEGα protein levels relative to GAPDH loading control in right atrial appendage biopsies from patients in paroxysmal AF (pAF; n=23) or normal sinus rhythm (NSR; n=22). Significance determined using Student's t-test. *P<0.05. **P<0.01.

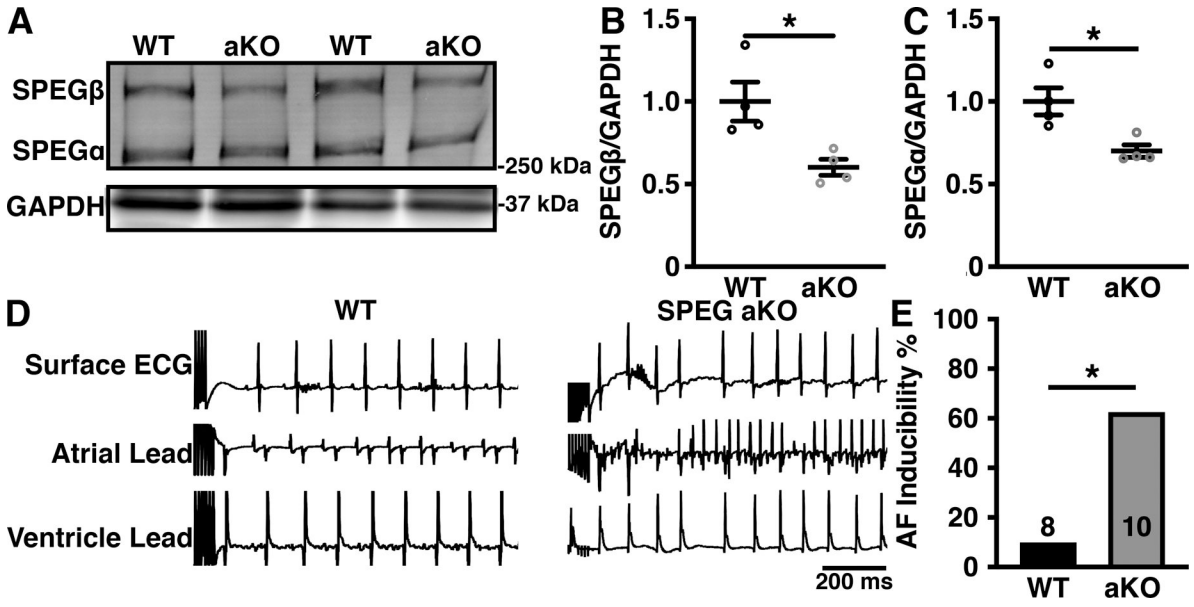

Figure 2. Atrial-specific knockout of SPEG increases AF inducibility in mice.

(**A**) Western blots of atrial SPEG levels in SPEG aKO mice and WT controls three weeks after injection of AAV9-ANF-Cre. (**B-C**) Bar graphs showing quantification of atrial SPEGβ and SPEGα levels in SPEG aKO mice and WT controls, normalized to GAPDH levels. Analysis using Mann-Whitney U test. (**D**) Representative surface ECG and intracardiac traces from SPEG aKO mice and WT controls showing AF in SPEG aKO. (**E**) Bar graph showing increased incidence of AF inducibility in SPEG aKO vs control mice analyzed using Fisher's Exact test. *P<0.05.

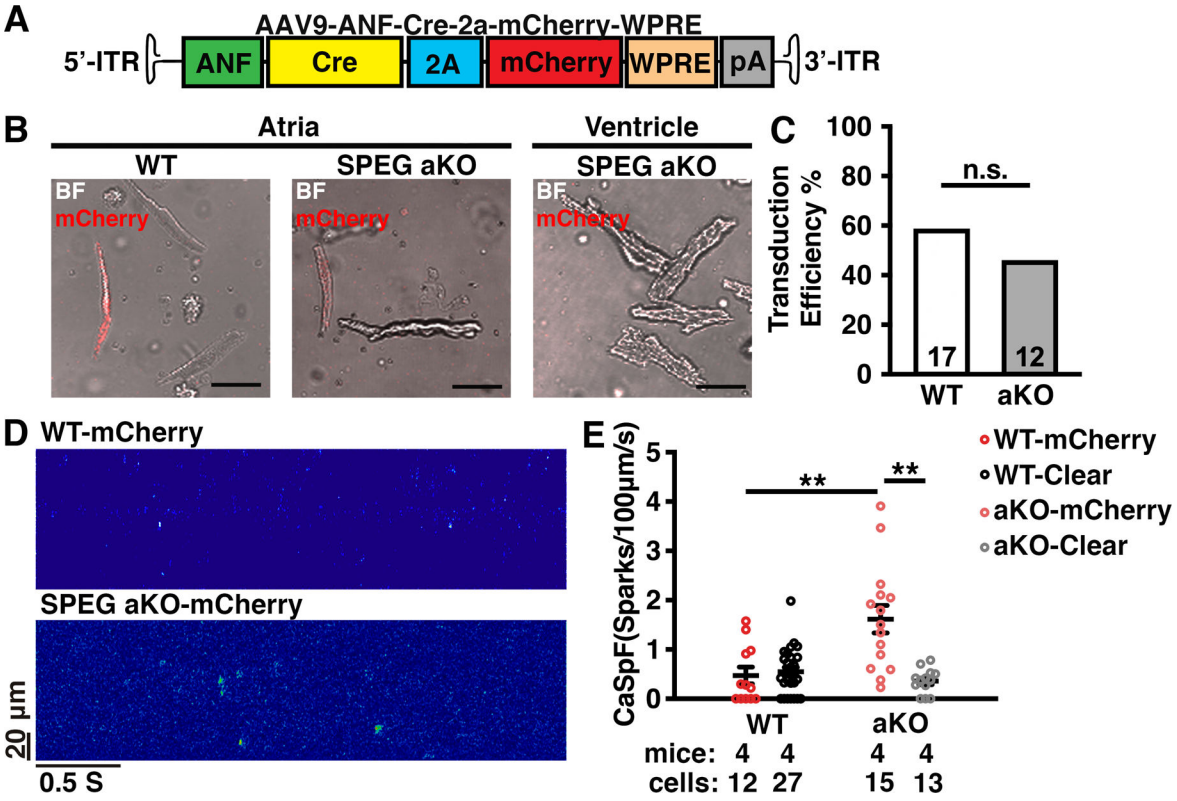

Figure 3. Loss of SPEG in atrial myocytes enhances SR Ca^{2+} leak.

(**A**) Diagram of AAV9-ANF-Cre-2a-mCherry viral vector. (**B**) Representative confocal images showing mCherry expression in atrial but not ventricular myocytes isolated from SPEG aKO mice and WT controls, injected with AAV9-ANF-Cre-2a-mCherry. Scale bar is 50 µm. (**C**) Bar graph showing transduction efficiency of atrial myocytes isolated from SPEG aKO mice and WT controls; data analyzed using Fisher's Exact. (**D**) Representative confocal images showing Ca^{2+} sparks in mCherry-positive atrial myocytes isolated from SPEG aKO mice and WT controls. (**E**) Quantification of Ca^{2+} spark frequencies analyzed using the generalized estimating equation. **P<0.01; n.s., non-significant.

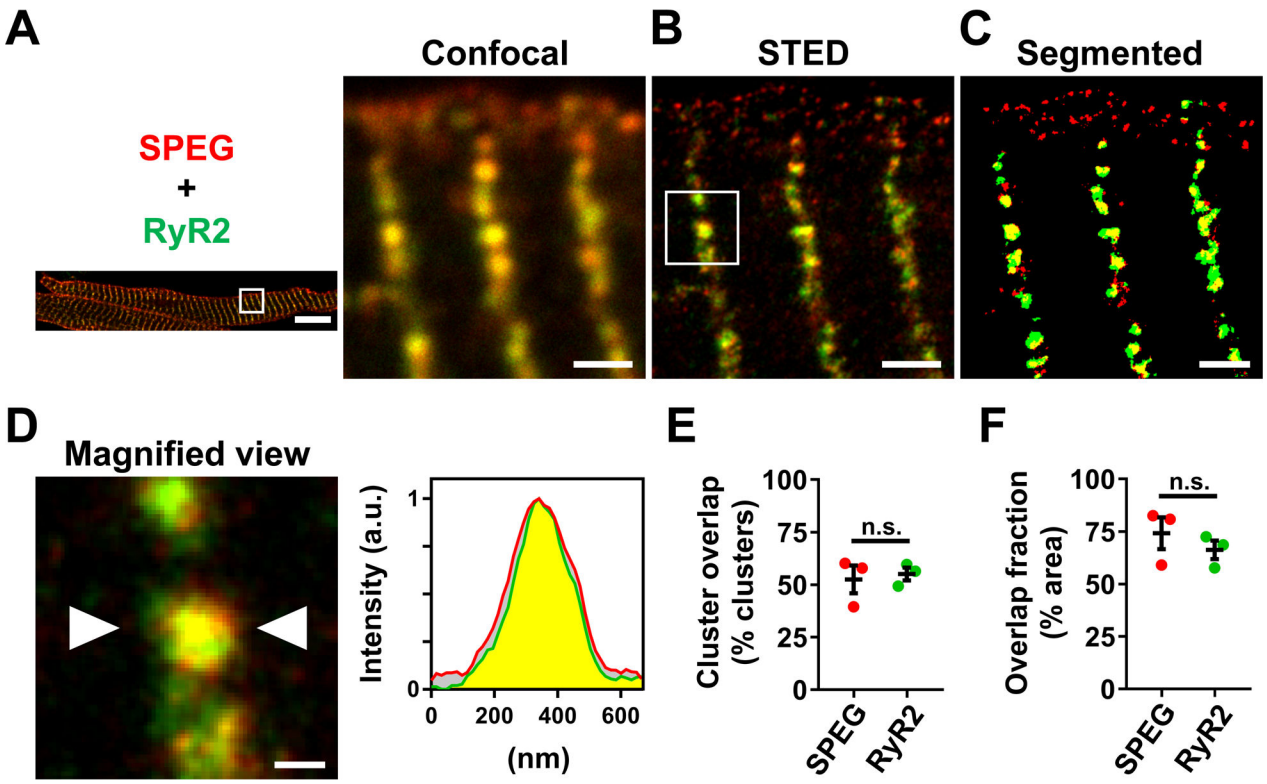

Figure 4. SPEG co-localizes with RyR2 clusters.

Co-immunofluorescence imaging of SPEG (red) and RyR2 (green) in isolated mouse atrial myocytes using both **(A)** confocal microscopy and **(B)** STED nanoscopy. **(C)** STED image segmentation showed that SPEG and RyR2 signals are closely co-localized in transverse striations, but additional SPEG clusters are found at the surface sarcolemma without RyR2. **(D)** Left: Zoom-in demonstrating the local association of SPEG with individual RyR2 clusters. White triangles indicate the orientation of the signal intensity profiling (right). Co-localized signals are indicated in yellow. **(E-F)** Co-localization analysis of segmented STED images revealed both **(E)** a high percentage of overlapping SPEG and RyR2 clusters, and (**F**) abundant overlapping area fractions. Analysis excluded subsurface signals. White boxes in **(A)** and **(B)** indicate magnified regions. Scale bars 5 μm (cell overview), 1 μm (confocal, STED, segmented), and 200 nm (magnified view). n = 3 individual hearts including 41 atrial myocytes. n.s., not significant, Student's t-test.

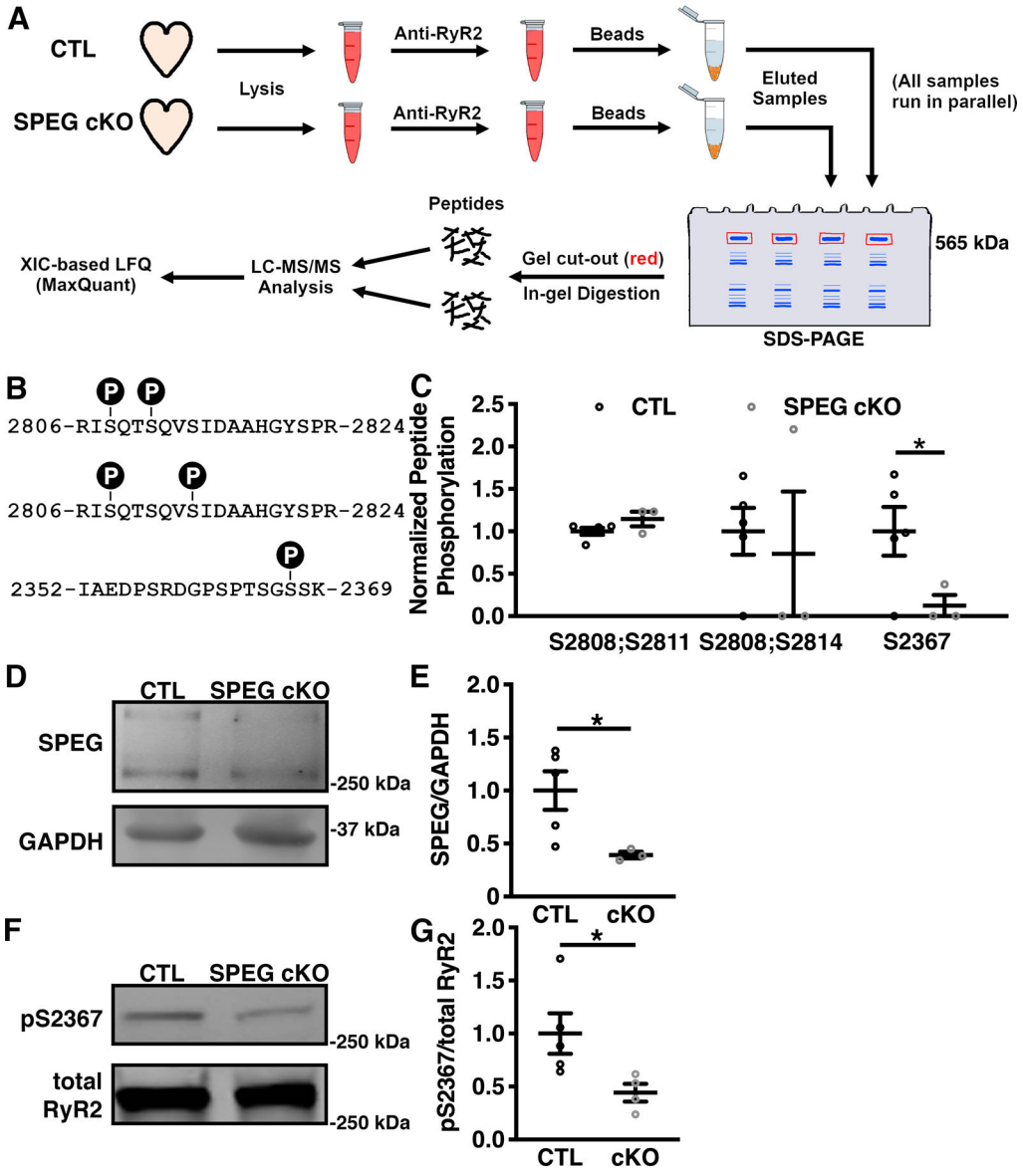

Figure 5. SPEG phosphorylates S2367 on RyR2.

(**A**) Phospho-proteomics work flow showing that following preparation of heart lysates from SPEG cKO mice and controls (CTL), RyR2 was immunoprecipitated, typsinized within an SDS-PAGE gel, subjected to liquid chromatography tandem mass spectrometry (LC-MS/MS), and analyzed using ion chromatogram (XIC)-based label free quantification (LFQ) with MaxQuant software. (**B**) The three most common RyR2 phospho-peptides detected by mass spectrometry. (**C**) Quantification of peptide phosphorylation levels normalized to total peptide levels; analysis using the Welch's t-test. (**D**) Representative western blot image of SPEG levels in hearts from SPEG cKO and CTL mice. (**E**) Quantification of SPEG levels on western blots relative to GAPDH (**F**) Representative western blot image of S2367 phosphorylation in hearts from SPEG cKO and CTL mice. (**G**) Quantification of S2367 phosphorylation levels on western blots; analysis using Mann Whitney U test. *P<0.05.

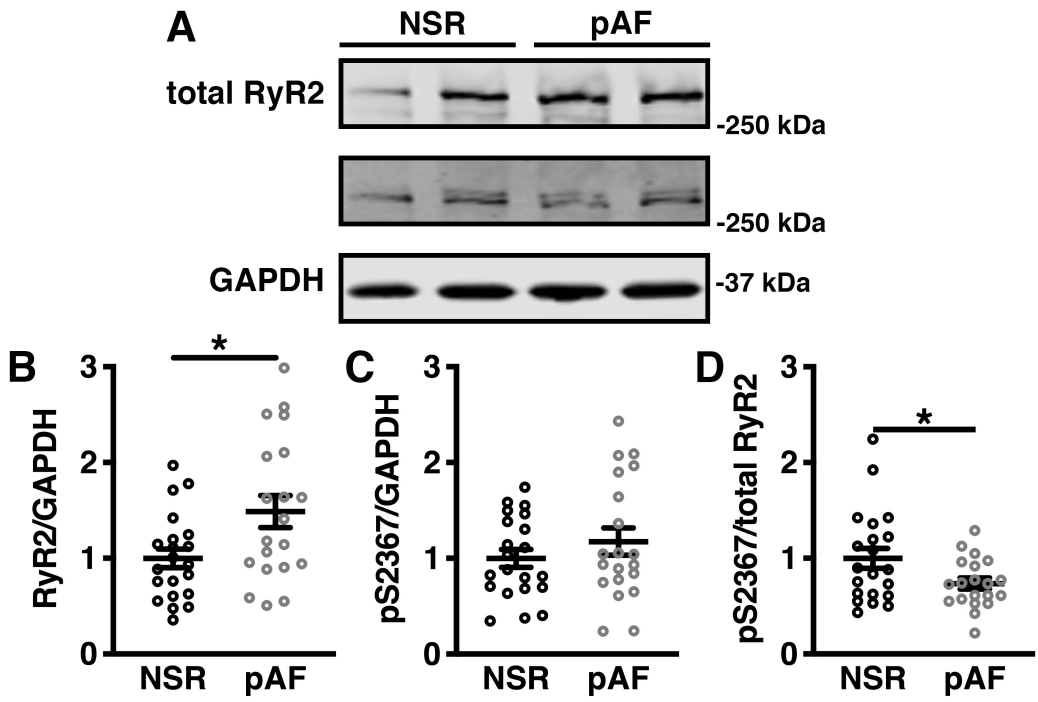

Figure 6. RyR2-S2367 phosphorylation is decreased in patients with pAF.

(**A**) Representative western blot images of total RyR2, RyR2-pS2367, and GAPDH loading control in right atrial appendage biopsies from pAF patients and controls in normal sinus rhythm (NSR). (**B**) Quantification of total RyR2 levels normalized to GAPDH. (**C**) Quantification of pS2367 phosphorylation levels normalized to GAPDH. (**D**) Quantification of pS2367 levels normalized to total RyR2 levels. Analyses were performed using Student's t-tests. *P<0.05.

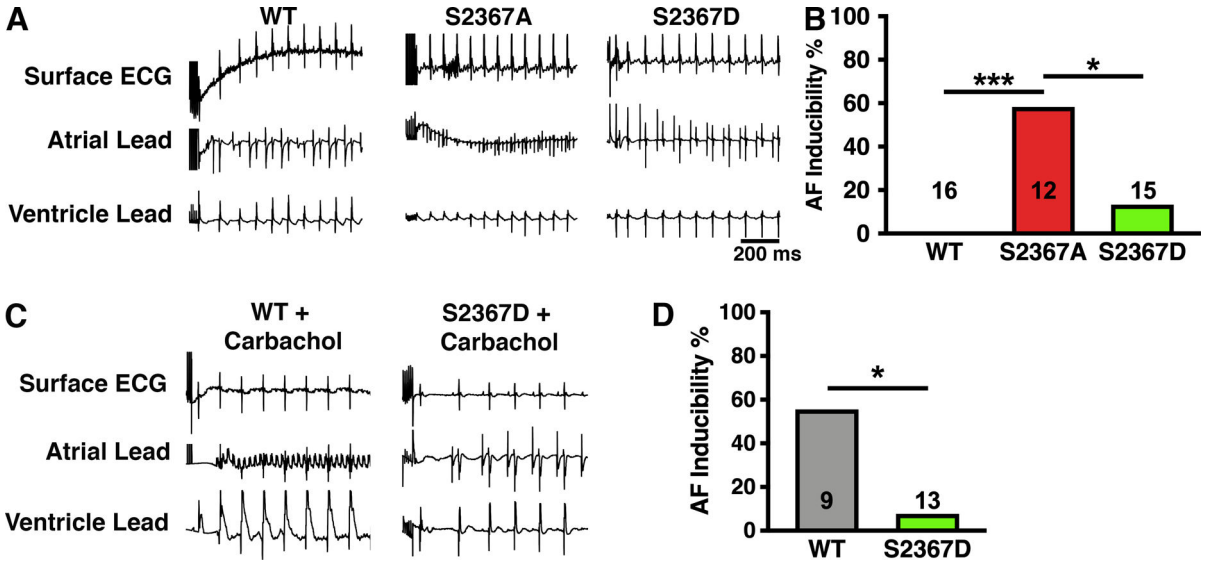

Figure 7. Ablation of S2367 phosphorylation enhances susceptibility to AF.

(**A**) Representative surface ECGs and intracardiac electrogram tracings from WT, S2367A, and S2367D mice during and after rapid atrial burst pacing. (**B**) Bar graph showing percent AF inducibility in WT, S2367A, and S2367D mice. (**C**) Representative surface ECGs and intracardiac electrogram tracings directly after rapid atrial pacing of WT and S2367D mice following a carbachol injection. (**D**) Bar graph showing percent AF inducibility in S2367D mice vs WT controls after carbachol injection. Analysis performed using Fisher's exact. *P<0.05. ***P<0.001.

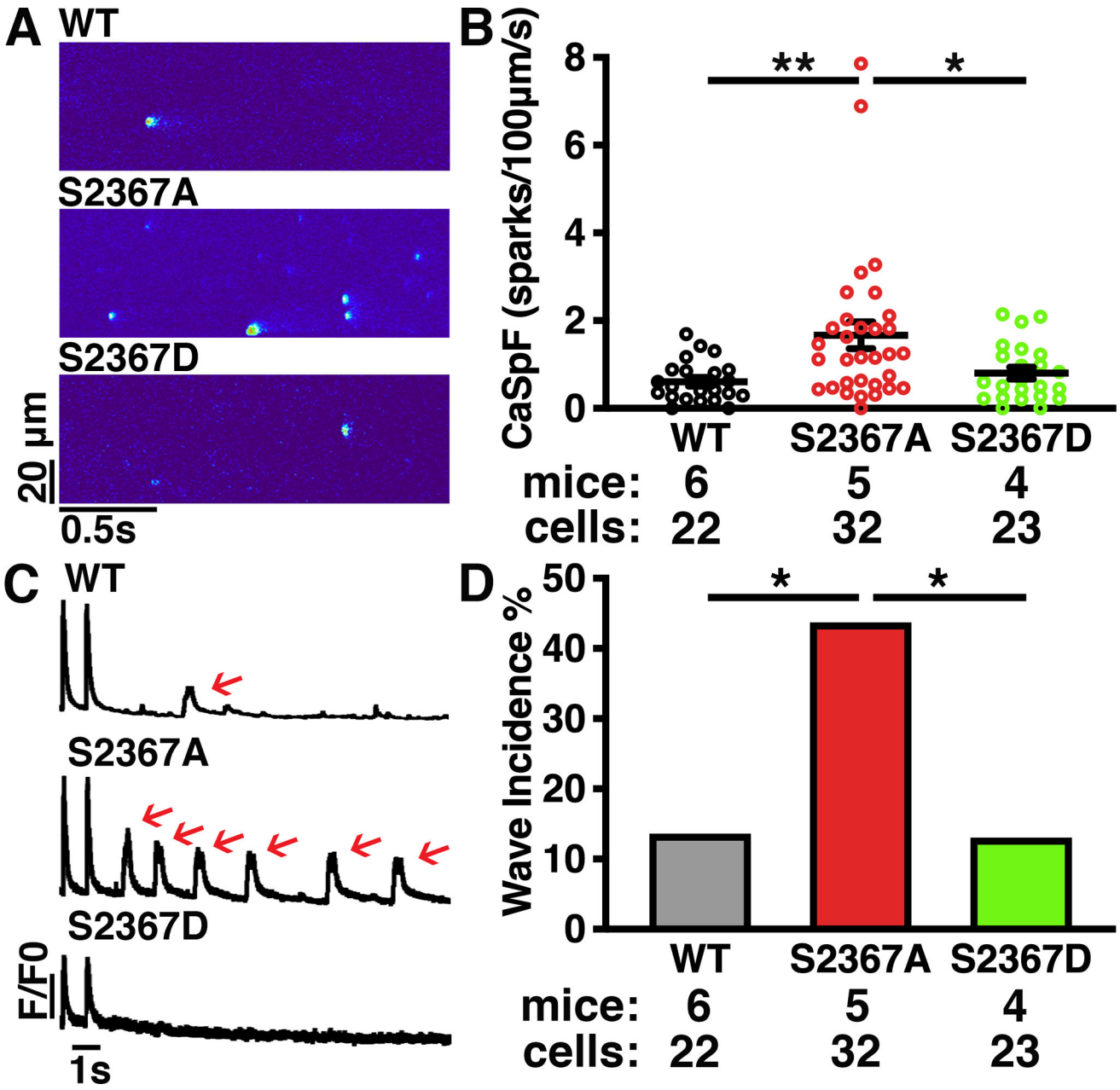

Figure 8. Ablation of RyR2-S2367 phosphorylation enhances diastolic Ca^{2+} leak.

(**A**) Representative confocal line scan tracings showing Ca^{2+} sparks in atrial myocytes from WT, S2367A, and S2367D mice. (**B**) Quantification of Ca^{2+}-spark frequency (CaSpF, sparks /100 µM/ s) in atrial myocytes from WT, S2367A, and S2367D mice. (**C**) Representative cytosolic Ca^{2+} concentration traces showing SR Ca^{2+} waves (red arrows) after 1-Hz pacing in atrial myocytes from WT, S2367A, and S2367D mice. (**D**) Quantification of Ca^{2+} wave incidence in atrial myocytes from WT, S2367A, and S2367D mice. Analysis performed using generalized estimating equation. *P<0.05. **P<0.01. Number of mice and cells from each genotype listed below bar graph.