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Loss of SPEG Inhibitory Phosphorylation of Ryanodine Receptor Type-2 Promotes Atrial Fibrillation

Editorial, see p 1173

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BACKGROUND: Enhanced diastolic calcium (Ca^{2+}) release through ryanodine receptor type-2 (RyR2) has been implicated in atrial fibrillation (AF) promotion. Diastolic sarcoplasmic reticulum Ca^{2+} leak is caused by increased RyR2 phosphorylation by PKA (protein kinase A) or CaMKII (Ca^{2+}/calmodulin-dependent kinase-II) phosphorylation, or less dephosphorylation by protein phosphatases. However, considerable controversy remains regarding the molecular mechanisms underlying altered RyR2 function in AF. We thus aimed to determine the role of SPEG (striated muscle preferentially expressed protein kinase), a novel regulator of RyR2 phosphorylation, in AF pathogenesis.

METHODS: Western blotting was performed with right atrial biopsies from patients with paroxysmal AF. SPEG atrial knockout mice were generated using adeno-associated virus 9. In mice, AF inducibility was determined using intracardiac programmed electric stimulation, and diastolic Ca^{2+} leak in atrial cardiomyocytes was assessed using confocal Ca^{2+} imaging. Phosphoproteomics studies and Western blotting were used to measure RyR2 phosphorylation. To test the effects of RyR2-S2367 phosphorylation, knockin mice with an inactivated S2367 phosphorylation site (S2367A) and a constitutively activated S2367 residue (S2367D) were generated by using CRISPR-Cas9.

RESULTS: Western blotting revealed decreased SPEG protein levels in atrial biopsies from patients with paroxysmal AF in comparison with patients in sinus rhythm. SPEG atrial-specific knockout mice exhibited increased susceptibility to pacing-induced AF by programmed electric stimulation and enhanced Ca^{2+} spark frequency in atrial cardiomyocytes with Ca^{2+} imaging, establishing a causal role for decreased SPEG in AF pathogenesis. Phosphoproteomics in hearts from SPEG cardiomyocyte knockout mice identified RyR2-S2367 as a novel kinase substrate of SPEG. Western blotting demonstrated that RyR2-S2367 phosphorylation was also decreased in patients with paroxysmal AF. RyR2-S2367A mice exhibited an increased susceptibility to pacing-induced AF, and aberrant atrial sarcoplasmic reticulum Ca^{2+} leak, as well. 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 sarcoplasmic reticulum Ca^{2+} release. Reduced SPEG levels and RyR2-S2367 phosphorylation typified patients with paroxysmal AF. Studies in S2367 knockin 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.

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Clinical Perspective

What Is New?

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

What Are the Clinical Implications?

- Loss of serine 2367 phosphorylation on RyR2 by reduced SPEG levels contributes to abnormal Ca^{2+} handling and enhances susceptibility to atrial fibrillation.
- Normalizing serine 2367 phosphorylation and SPEG activity may provide novel therapeutic opportunities for the treatment of atrial fibrillation.

Atrial fibrillation (AF) is the most common sustained cardiac arrhythmia affecting >33.5 million individuals worldwide.[1] AF is associated with high morbidity and mortality because 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 PKA (protein kinase A) and CaMKII (Ca^{2+}/calmodulin-dependent kinase-II), 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, resulting in delayed afterdepolarizations, which may serve as a trigger for arrhythmic activity in the presence of an AF substrate.[3] Although 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] 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 posttranslational modifications cannot explain the increase in RyR2 activity seen in pAF, because 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 laboratory has recently identified SPEG (striated muscle preferentially expressed protein kinase) 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 2 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, the presence of SPEG in atria and its role in AF have 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. Furthermore, we show that SPEG phosphorylates RyR2 at a previously uncharacterized serine (S2367) located in the central domain of the channel.[17] In contrast with previously studied phosphorylation sites that activate RyR2 (eg, S2808, S2814), we show that SPEG-mediated RyR2-S2367 phosphorylation suppresses pathogenic diastolic SR Ca^{2+} leak. Moreover, SPEG loss and phosphoablation 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 on reasonable request, according to Transparency and Openness Promotion guidelines. Please see the Data 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 of the Medical Faculty of the University Duisburg-Essen, Essen, Germany (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. The number of mice used in each experiment is included in the Results section. Generation and breeding of cardiomyocyte-specific SPEG conditional knockout (SPEG cKO), atrial-specific SPEG conditional knockout mouse (SPEG aKO), RyR2-S2367A, and RyR2-S2367D knockin mouse models are described further in the Data Supplement.

Western Blot

Protein and phosphorylation levels in human and mouse samples were measured using sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blotting. For detailed protocols, see the Data Supplement.

Echocardiography

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

Programmed Electric Stimulation

Programmed electric stimulation (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 nmol/L ryanodine.[20] Detailed cell isolation, data collection, and analysis methods are in the Data Supplement. Spark parameter cutoffs are in Table I in the Data Supplement.

Stimulated Emission Depletion Nanoscopy

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

Mass Spectrometry

RyR2 phosphorylation was examined by immunoprecipitating RyR2 from SPEG cKO and control heart lysates followed by phosphoproteomics[22] as detailed in the Data Supplement.

Statistical Analysis

All data analysis was performed using Graphpad-Prism or SPSS software. When normality could be assumed, unpaired Student *t* test or 1-way ANOVA followed by Tukey post hoc analysis was used for nominal data. If assumptions of normality were not met, the Mann-Whitney *U* test between 2 groups or Kruskal-Wallis followed by the Dunn post hoc test between >2 groups was used. The Fisher 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 heart failure.[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 patients with pAF or patients in normal sinus rhythm (NSR). Characteristics of all patients enrolled in our study were comparable between both groups (Table II in the Data Supplement). Western blotting (Figure 1A) of a subset of samples (Table III in the Data 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) in comparison with NSR controls (n=22). These experiments were conducted on tissue from patients with a history of pAF, but were in NSR, at the time of tissue collection. There was no correlation between atrial SPEG levels and the time between the last documented AF episode and the time of tissue collection during cardiac surgery (see Figure IA and IB in the Data Supplement). To test whether a recent AF episode biased the results, we compared SPEG levels in patients who were in AF as recently as 24 hours before surgery (red dots) versus those who had their last AF episode >24 hours before surgery but detected similar SPEG levels in both groups (Figure IC and ID in the Data Supplement). SPEG levels were found to be unchanged in patients with cAF (Figure II and Table IV in the Data Supplement). Therefore, differences in SPEG levels between patients with pAF and NSR are unlikely to be a consequence of the high atrial rate during AF; rather, these differences 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

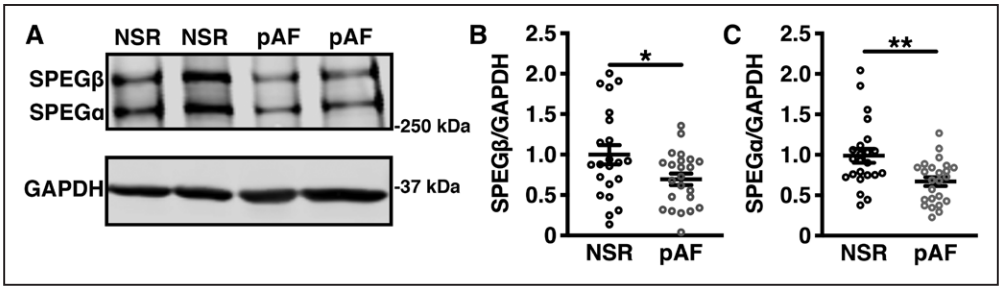

Figure 1. SPEG protein levels are decreased in patients with paroxysmal AF.
Representative Western blots (**A**) and bar graph quantifications of SPEGβ (**B**) and SPEGα (**C**) protein levels relative to GAPDH-loading control in right atrial appendage biopsies from patients in paroxysmal AF (pAF; n=23) or normal sinus rhythm (NSR; n=22). Significance was determined using the Student *t* test. **P*<0.05. ***P*<0.01. AF indicates atrial fibrillation; and SPEG, striated muscle preferentially expressed protein kinase.

decreased SPEG would be sufficient to enhance the susceptibility of AF, because 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 3 days followed by echocardiography 2 weeks after injection (Figure IIIA in the Data Supplement). An ejection fraction of >45% was set as a predetermined cutoff for inclusion in the study to prevent confounding effects of reduced ventricular systolic function (Figure IIIB in the Data 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 with none of the mER-Cre-mER controls (n=6) (*P*=0.005; Figure IIIC and IIID in the Data 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-atrial natriuretic factor [ANF]-Cre). Western blotting revealed a 40.5±0.3% decrease in SPEGβ levels in SPEG aKO mice (n=4) in comparison with WT controls (n=4, *P*=0.020; Figure 2A and 2B). Similarly, atrial SPEGα levels were reduced by 30.1±0.04% in SPEG aKO mice in comparison with WT controls (*P*=0.029; Figure 2A and 2C). It is important to note that ventricular SPEGβ and SPEGα levels were unaltered in SPEG aKO mice (Figure IV in the Data 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 Data Supplement). However, SPEG aKO mice were more susceptible to AF induction after PES (62.5%) than WT controls (10%, *P*=0.043; Figure 2D and 2E). 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 with myocytes from WT controls irrespective of mCherry expression (Figure VA and VB in the Data Supplement). Furthermore, SPEG loss had no effect on SR load or Na^{+}/Ca^{2+}-exchanger and SERCA2a activities (Figure VC and VE in the Data Supplement).

mCherry-positive myocytes from SPEG aKO mice demonstrated an increased Ca^{2+}-spark frequency (1.62±0.28 sparks per 100 μm·s^{-1}) in comparison with both mCherry-positive cells from WT controls (0.47±0.17 sparks per 100 μm·s^{-1}; *P*=0.005) and mCherry-negative cells from SPEG aKO mice (0.36±0.07 sparks per 100 μm·s^{-1}; *P*=0.002) (Figure 3D and 3E). Results remained significant when the Ca^{2+}-spark frequency was normalized to SR Ca^{2+} load (Figure VF in the Data Supplement). However, there were no significant differences in mCherry-negative cells from SPEG aKO mice in comparison with WT controls (Figure 3E) indicating that SPEG regulates Ca^{2+} release in a cell-autonomous manner. Because there was no difference between mCherry-negative and mCherry-positive WT cells, it is unlikely that the findings are attributable to the toxic effects of either mCherry or Cre.

SPEG Colocalizes With RyR2 Clusters in Atrial Myocytes

To test whether SPEG colocalizes with RyR2 clusters in mouse atrial myocytes, immunofluorescence imaging

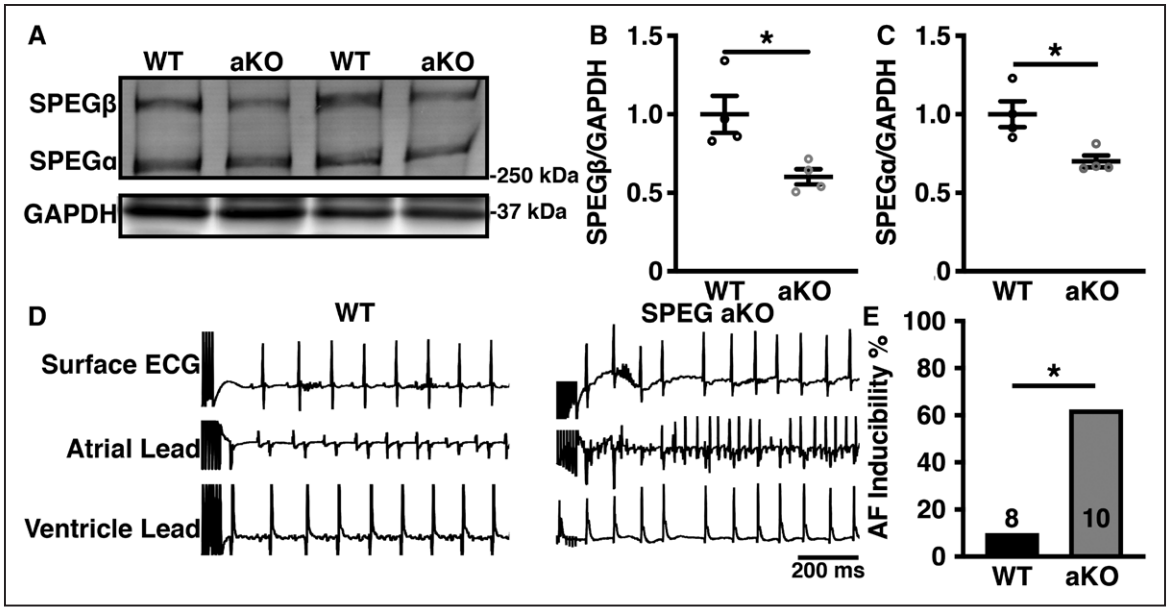

Figure 2. Atrial-specific knockout of SPEG increases AF inducibility in mice.
A, Western blots of atrial SPEG levels in SPEG aKO mice and WT controls 3 weeks after injection of AAV9-ANF-Cre. **B** and **C**, Bar graphs showing quantification of atrial SPEGβ and SPEGα levels in SPEG aKO mice and WT controls, normalized to GAPDH levels. Data were analyzed using the 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 versus control mice analyzed using the Fisher exact test. **P*<0.05. AF indicates atrial fibrillation; aKO, atrial-specific knockout; SPEG, striated muscle preferentially expressed protein kinase; and WT, wild type.

was performed using both confocal microscopy and stimulated emission depletion superresolution nanoscopy (Figure 4). Whereas confocal imaging suggested colocalized signals throughout atrial myocytes (Figure 4A), stimulated emission depletion nanoscopy resolved colocalized 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 colocalized 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 colocalized with SPEG. In addition, many SPEG signals could be identified at the cell surface but not colocalized with RyR2 (Figure 4C).

SPEG Phosphorylates S2367 on RyR2

Next, we tested whether RyR2 phosphorylation was altered in SPEG cKO mice. RyR2 phosphopeptides 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), and peptides containing previously uncharacterized sites, as well (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, versus 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 phosphoepitope-specific polyclonal antibody that recognizes phosphorylated S2367 (Figure VI in the Data Supplement) was used to verify our mass spectrometry findings. In hearts of SPEG cKO mice, SPEG levels were downregulated by 60.9±3.1% in comparison with controls (*P*=0.036; Figure 5D and 5E). SPEG reduction was not as large as previous studies because mice underwent tamoxifen for 3 days versus 5.[15] The level of S2367 phosphorylation on RyR2 was proportionally reduced by 55.8±8.4% in SPEG cKO mice in comparison with controls (*P*<0.016; Figure 5F and 5G), confirming that S2367 is phosphorylated by SPEG in vivo. In addition, S2814 and S2808 phosphorylation was unchanged in SPEG cKO hearts as detected by Western blots (Figure VII in the Data 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),

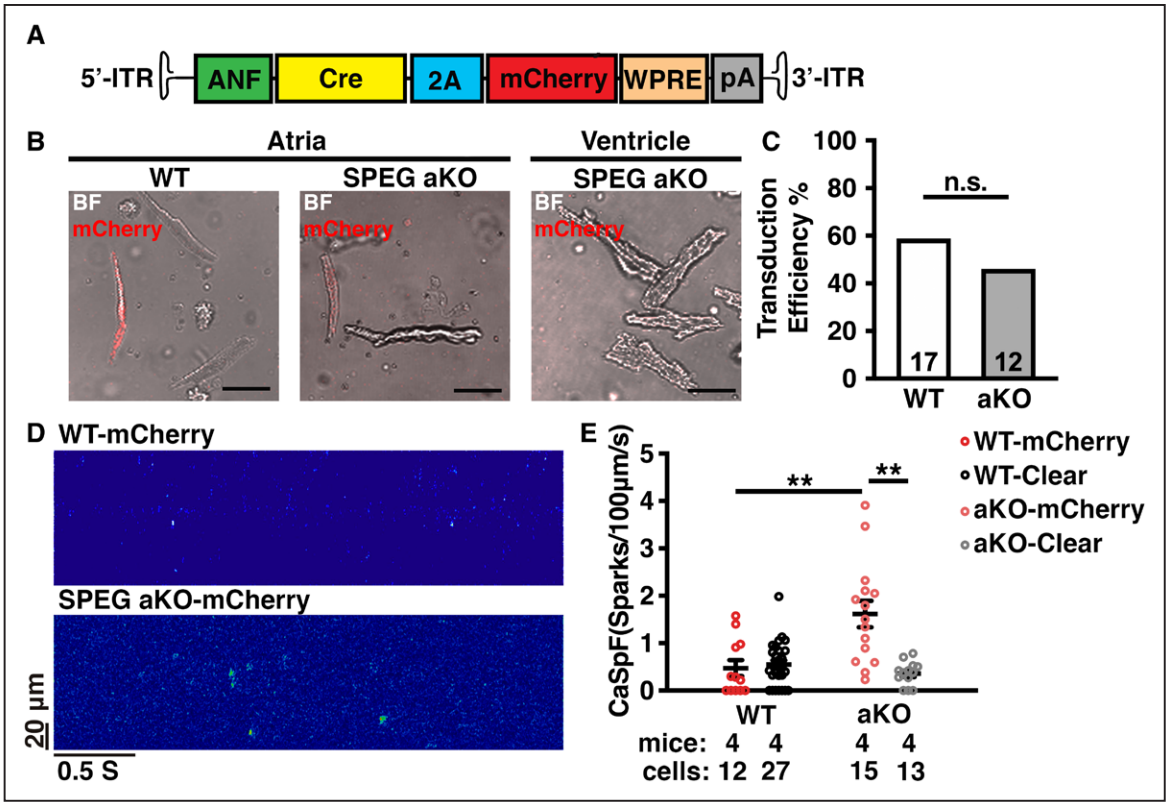

Figure 3. Loss of SPEG in atrial myocytes enhances SR Ca^{2+} leak.
A, Diagram of AAV9-ANF-Cre-2a-mCherry viral vector. **B**, Representative confocal images showing mCherry expression in atrial but not ventricular myocytes isolated from SPEG aKO mice and WT controls, injected with AAV9-ANF-Cre-2a-mCherry. Scale bar is 50 µm. **C**, Bar graph showing transduction efficiency of atrial myocytes isolated from SPEG aKO mice and WT controls; data were analyzed using the Fisher exact test. **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. aKO indicates atrial-specific knockout; ANF, atrial natriuretic factor; BF, bright field; CaSpF, Ca^{2+}-spark frequency; ITR, inverted terminal repeat; n.s., nonsignificant; SPEG, striated muscle preferentially expressed protein kinase; SR, sarcoplasmic reticulum; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element; and WT, wild type.

whereas S2814 and S2808 phosphorylation levels were unchanged (Figure VIII in the Data 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 Data 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 patients with pAF, we examined RyR2 phosphorylation through Western blot using biopsies from patients with pAF and NSR controls (Table VI in the Data 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) in comparison with NSR controls (n=21, *P*=0.01; Figure 6B).[14] RyR2-S2367 phosphorylation was not significantly altered in patients with pAF relative to GAPDH expression levels. However, RyR2-S2367 phosphorylation normalized to total RyR2 was decreased by 26.4±0.06% in patients with pAF (n=20) in comparison with NSR controls (n=21, *P*=0.036). On the contrary, phosphorylation of S2808 and S2814 relative to total RyR2 levels was unchanged in patients with pAF (Figure X in the Data Supplement). As in mice, phosphorylation of S2367 was not correlated with levels of S2814 or S2808 phosphorylation in human atrial samples (Figure XI in the Data Supplement). These results suggest that a reduction in RyR2-S2367 phosphorylation may be a unique marker of RyR2 dysfunction in patients with pAF.

Ablation of S2367 Phosphorylation Enhances Susceptibility to AF

Although S2367 has been reported previously as a RyR2 phosphorylation site in a phosphoproteomics study,[24] the functional consequences of S2367 phosphorylation have not yet been explored. To determine the physiological significance of S2367 phosphorylation,

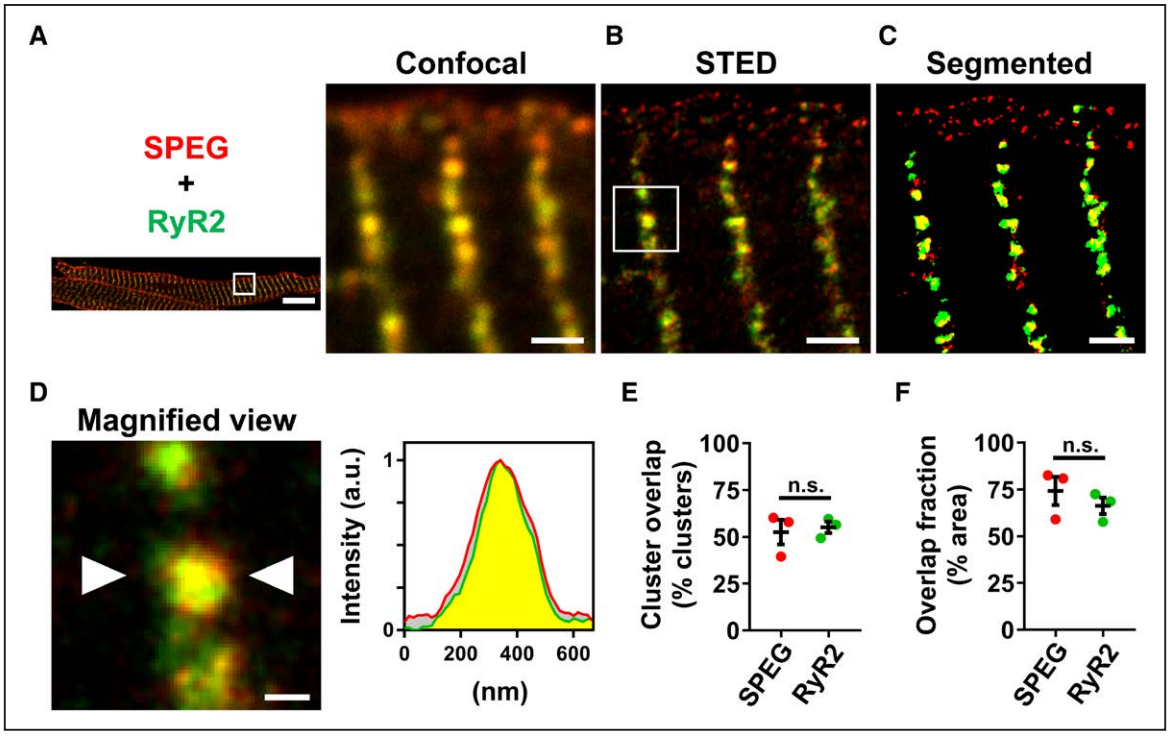

Figure 4. SPEG colocalizes with RyR2 clusters.
Coimmunofluorescence imaging of SPEG (red) and RyR2 (green) in isolated mouse atrial myocytes using both confocal microscopy (**A**) and STED nanoscopy (**B**). **C**, STED image segmentation showed that SPEG and RyR2 signals are closely colocalized in transverse 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**). Colocalized signals are indicated in yellow. **E** and **F**, Colocalization analysis of segmented STED images revealed both a high percentage of overlapping SPEG and RyR2 clusters (**E**) and abundant overlapping area fractions (**F**). 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. indicates not significant, Student *t* test; RyR2, ryanodine receptor type-2; SPEG, striated muscle preferentially expressed protein kinase; and STED, stimulated emission depletion.

2 RyR2 knockin mouse models were generated using CRISPR-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. In comparison with WT littermates, RyR2-S2367A and S2367D mice exhibited normal ventricular function at 3 months of age with echocardiography (Table VII in the Data Supplement). RyR2-S2367A and RyR2-S2367D mice also exhibited no changes in RyR2-S2808 or RyR2-S2814 phosphorylation (Figure XII in the Data Supplement).

Baseline ECG intervals were unchanged in both S2367A and S2367D mice in comparison with WT littermates (Table VIII in the Data Supplement). However, PES studies revealed an enhanced susceptibility to AF in S2367A mice in comparison with WT littermates (Figure 7A): 58% (7/12) of S2367A exhibited AF in comparison with 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 patients with pAF.

Last, 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). Although 55.6% (5/9) WT mice exhibited pacing-induced AF after carbachol, only 7.7% (1/13; $P=0.02$) of S2367D mice developed AF (Figure 7D). In contrast with previous RyR2 phosphomimetic 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 in isolated atrial myocytes under resting conditions. The Ca^{2+}-spark frequency was increased (1.66±0.33 sparks per 100 µm·s^{-1}) in atrial myocytes from S2367A mice in comparison with cells from WT mice (0.61±0.10 sparks per 100 µm·s^{-1}, $P=0.001$; Figure 8A and 8B). The Ca^{2+}-spark frequency in S2367D myocytes (0.80±0.21 sparks per 100 µm·s^{-1}) 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 with myocytes from WT littermates (13.6%, $P=0.04$) and S2367D mice (13.0%,

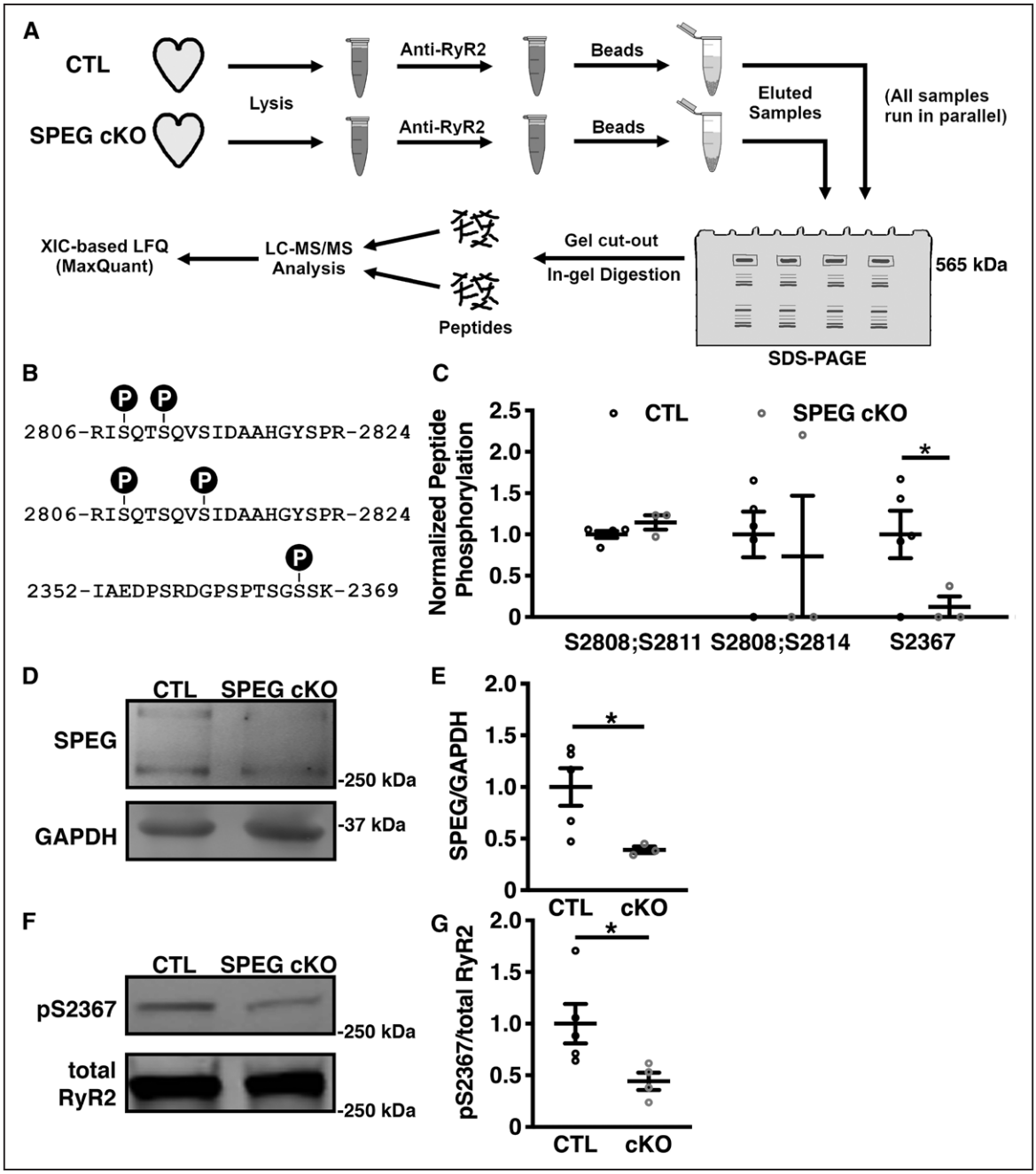

Figure 5. SPEG phosphorylates S2367 on RyR2.
A, Phosphoproteomics workflow showing that after the preparation of heart lysates from SPEG cKO mice and controls (CTL), RyR2 was immunoprecipitated, typsinized within an SDS-PAGE gel, subjected to liquid chromatography tandem mass spectrometry (LC-MS/MS), and analyzed using ion chromatogram (XIC)–based label free quantification (LFQ) with MaxQuant software. **B**, The 3 most common RyR2 phosphopeptides detected by mass spectrometry. **C**, Quantification of peptide phosphorylation levels normalized to total peptide levels; analysis using the Welch *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; data were analyzed using the Mann-Whitney *U* test. **P*<0.05. cKO indicates cardiomyocyte-specific knockout; RyR2, ryanodine receptor type-2; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; and SPEG, striated muscle preferentially expressed protein kinase.

P=0.03; Figure 8C and 8D). Thus, increased diastolic SR Ca^{2+} leak in atrial myocytes from S2367A mice was sufficient to trigger cellwide 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 nmol/L ryanodine. Using a previously published method,[20] we measured the Ca^{2+}-spark latency time of individual RyR2 clusters in WT and

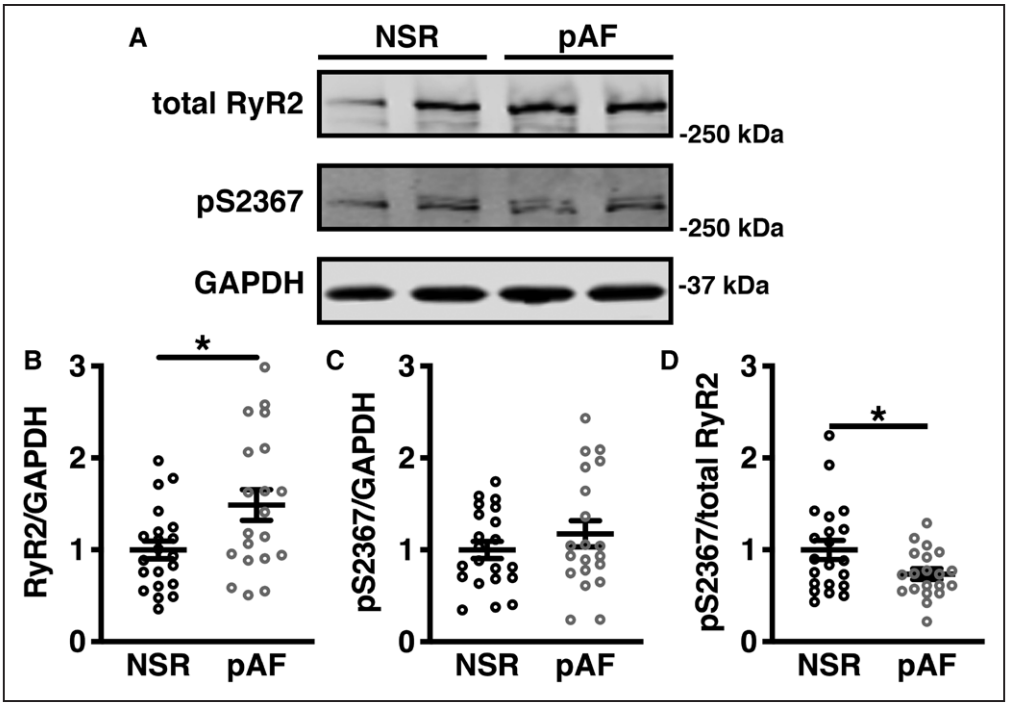

Figure 6. RyR2-S2367 phosphorylation is decreased in patients with pAF.
A, Representative Western blot images of total RyR2, RyR2-pS2367, and GAPDH loading control in right atrial appendage biopsies from patients with pAF and controls in normal sinus rhythm (NSR). **B**, Quantification of total RyR2 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 *t* tests. **P*<0.05. pAF indicates paroxysmal atrial fibrillation; and RyR2, ryanodine receptor type-2.

S2367A atrial myocytes (Figure XIIIA in the Data Supplement). With this approach, we found a significant leftward shift in the spark-spark delay time in S2367A versus WT atrial myocytes (Figure XIIIB in the Data 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 experiencing early stages of AF (ie, 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 patients with pAF.[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 knockin 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, because RyR2-S2367A knockin 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 (ie, 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]

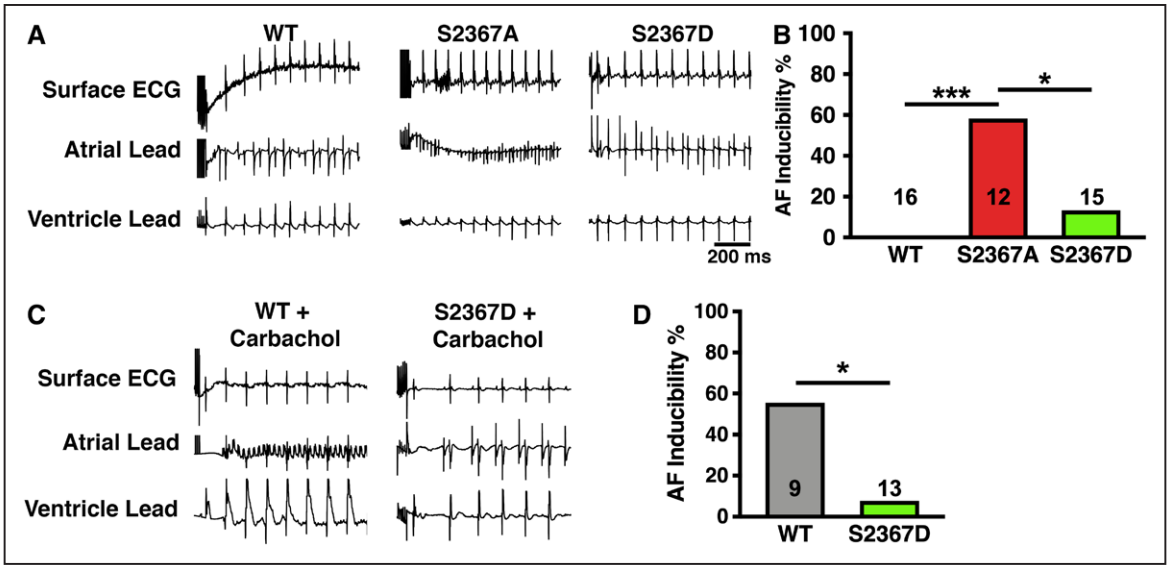

Figure 7. Ablation of S2367 phosphorylation enhances susceptibility to AF.
A, Representative surface ECGs and intracardiac electrogram tracings from WT, S2367A, and S2367D mice during and after rapid atrial burst pacing. **B**, Bar graph showing percent AF inducibility in WT, S2367A, and S2367D mice. **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 versus WT controls after carbachol injection. Analysis performed using the Fisher exact test. **P*<0.05. ****P*<0.001. AF indicates atrial fibrillation; and WT, wild type.

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 patients with pAF, 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 Na^{+}/Ca^{2+}-exchanger current were unaltered.[14] In contrast, the Ca^{2+}-transient amplitude and SR Ca^{2+} load are larger in atrial myocytes from patients with pAF, [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 patients with pAF has been attributed to altered posttranscriptional regulation of RyR2 attributable 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 posttranslational 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 (ie, 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 patients with cAF.[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 phosphoproteomics 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] In addition to the S2367 site found in our study, 4 additional serine residues are located within the 10 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 whether the closed state

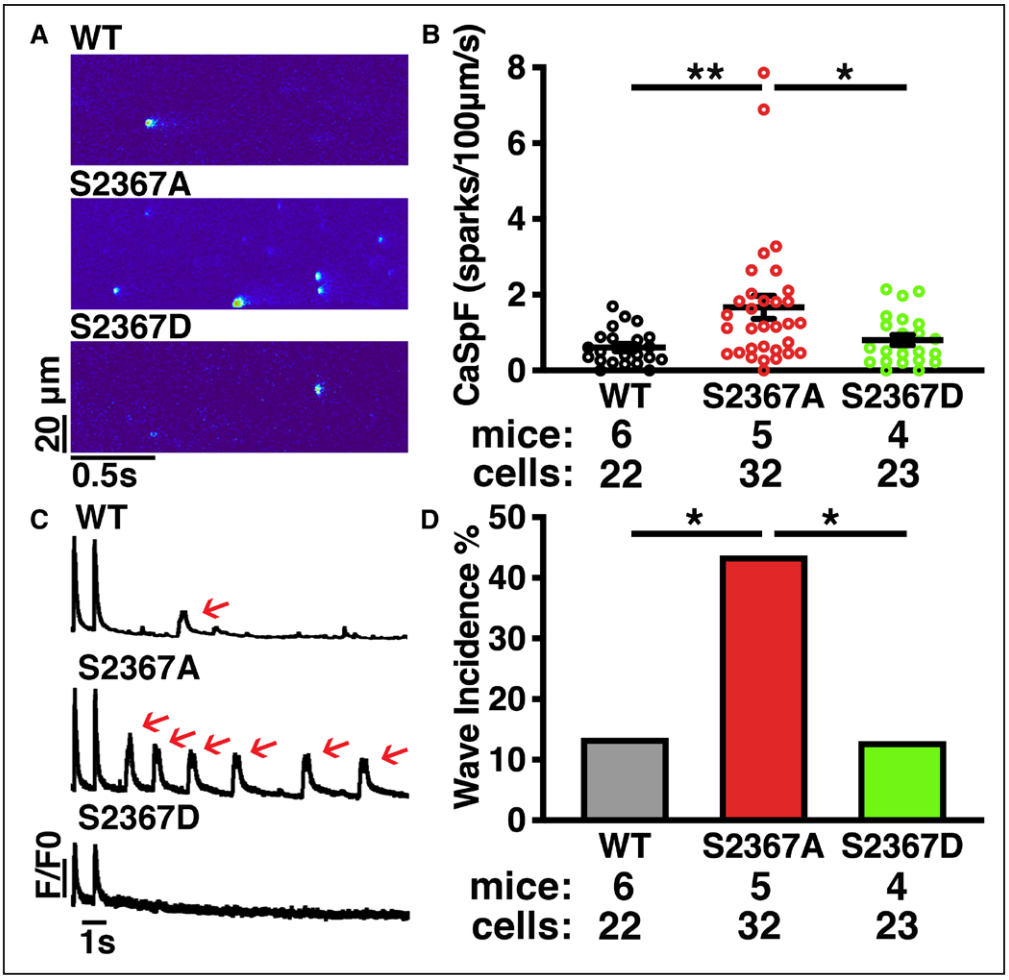

Figure 8. Ablation of RyR2-S2367 phosphorylation enhances diastolic Ca^{2+} leak.
A, Representative confocal line scan tracings showing Ca^{2+} sparks in atrial myocytes from WT, S2367A, and S2367D mice. **B**, Quantification of Ca^{2+}-spark frequency (CaSpF, sparks per 100 µmol·L^{-1}·s^{-1}) in atrial 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 was performed using the generalized estimating equation. *P<0.05. **P<0.01. Number of mice and cells from each genotype listed below bar graph. RyR2 indicates ryanodine receptor type-2; SR, sarcoplasmic reticulum; and WT, wild type.

is more favored. It would also be interesting to assess whether the S2367 mutation alters clustering of the channels and 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 posttranslational modification might underlie the enhanced RyR2 activity observed in patients with pAF.[14] Studies in knockin mouse models in which the S2367 site was genetically ablated by the S2367A mutation support this idea. For example, S2367A knockin 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 in comparison with WT littermates (Figure 8). In addition, the Ca^{2+}-spark latency period was reduced in ryanodine-treated S2367A atrial myocytes, suggestive of enhanced channel open probability. In contrast, knockin mice with constitutive pseudophosphorylation of S2367 attributable 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, because 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 S2367 phosphorylation to discover drug targets that can be used for the treatment of patients with pAF. Identifying the specific kinase domain that autophosphorylates 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, because the 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 Data Supplement).[40] In these SPEG aKO mice, we demonstrated that the 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). In addition, SPEG regulates other molecules involved in Ca^{2+} handling and cardiac contractility, including junctophilin-2, SERCA2a, and myotubularin.[15,16,39] In contrast with 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 Data Supplement). It is possible that differences in the 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 in comparison with 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 impairing SR Ca^{2+} handling. In comparison with ventricular myocytes, atrial myocytes have fewer transverse tubules, although a higher density of axial tubules has been reported.[42] This corresponds with limited local junctophilin-2 expression in atrial in comparison with ventricular myocytes.[43] Therefore, it is likely that the effect of reduced SPEG on structural tubule integrity is less pronounced in atrial versus 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 knockout mice by using AAV9-mediated overexpression of Cre driven by an ANF promoter.[40] This approach led to a 30% to 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. However, this 30% to 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 clearly sufficient to increase AF inducibility (Figure 3). We also developed novel RyR2 phosphorylation site knockin 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- to 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 in SPEG levels and RyR2-S2367 phosphorylation is higher in humans with both NSR and pAF versus 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 patients who have pAF 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 the 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 because 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.

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Disclosures

Dr Wehrens is a founding partner of Elex Biotech, a startup company that developed drug molecules to target ryanodine receptors for treatment of cardiac arrhythmias. The other authors have no disclosures.

Supplemental Materials

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