# Aging increases pulmonary veins arrhythmogenesis and susceptibility to calcium regulation agents

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**BACKGROUND** Aging and pulmonary veins (PVs) play a critical role in the pathophysiology of atrial fibrillation. Abnormal $Ca^{2+}$ regulation and ryanodine receptors are known to contribute to PV arrhythmogenesis.

**OBJECTIVE** The purpose of this study was to investigate whether aging alters PV electrophysiology, $Ca^{2+}$ regulation proteins, and responses to rapamycin, FK-506, ryanodine, and ouabain.

**METHODS** Conventional microelectrodes were used to record action potential and contractility in isolated PV tissue samples in 15 young (age 3 months) and 16 aged (age 3 years) rabbits before and after drug administration. Expression of sarcoplasmic reticulum $Ca^{2+}$ ATPase (SERCA2a), ryanodine receptor, and $Na^+/Ca^{2+}$ exchanger was evaluated by western blot.

**RESULTS** Aged PVs had larger amplitude of delayed afterdepolarizations, greater depolarized resting membrane potential, longer action potential duration, and higher incidence of action potential alternans and contractile alternans with increased expression of $Na^+/Ca^{2+}$ exchanger and ryanodine receptor and decreased expression of SERCA2a. Rapamycin (1,10,100 nM), FK-506 (0.01, 0.1, 1 $\mu$M), ryanodine (0.1, 1 $\mu$M), and ouabain (0.1, 1 $\mu$M) concentration-dependently increased PV spontaneous rates and the incidence of delayed afterdepolarizations in young and aged PVs. Compared with results in young PVs, rapamycin and FK-506 in aged PVs increased PV spontaneous rates to a greater extent and exhibited a larger delayed afterdepolarization amplitude. In PVs without spontaneous activity, rapamycin and FK-506 induced spontaneous activity only in aged PVs, but ryanodine and ouabain induced spontaneous activity in both young and aged PVs.

**CONCLUSION** Aging increases PV arrhythmogenesis via abnormal $Ca^{2+}$ regulation. These findings support the concept that ryanodine receptor dysfunction may result in high PV arrhythmogenesis and aging-related arrhythmogenic vulnerability.

**KEYWORDS** Atrial fibrillation; Aging; Calcium; Ryanodine; Pulmonary Veins

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## Introduction

Atrial fibrillation (AF) induces cardiac dysfunction and strokes and is the most common cardiac arrhythmia seen in clinical practice.[1,2] Aging plays an important role in AF genesis.[1] However, the mechanism of aging-induced AF is not fully elucidated. Aging has been shown to decrease conduction velocity, change action potential characteristics, increase atrial dispersion,[3-5] and alter calcium regulation in cardiomyocytes.[6–10] All of these effects may facilitate AF occurrence. Studies have shown that abnormal ryanodine receptors (RyRs) may induce AF.[11–14] Calcium release through $Ca^{2+}$-induced $Ca^{2+}$ release via RyRs is essential for cardiac function. Dysfunction of RyRs induces diastolic $Ca^{2+}$ leak and activates the transient inward current, in concert with an increase in the $Na^+/Ca^{2+}$ exchanger (NCX) currents, causing membrane depolarization and generating delayed afterdepolarizations (DADs).[15] These findings suggest that aging induces abnormal $Ca^{2+}$ homeostasis in cardiomyocytes, causing AF.

The pulmonary veins (PVs) are important sources of AF initiation[16,17] and have a role in AF maintenance.[18] Studies have indicated that abnormal $Ca^{2+}$ regulation may underlie PV arrhythmogenic activity.[12,19,20] Honjo et al[12] reported that low-dose ryanodine induced PV firing, which suggests that abnormal RyR contributes to PV arrhythmogenic activity. Because aging is important in the genesis of AF, it is

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reasonable to believe that aging has effects on PV arrhythmogenesis. A recent study showed that glycolytic inhibition, which is known to alter $Ca^{2+}$ handling, increased spontaneous AF in aged rats.[21] Accordingly, aging may potentiate PV arrhythmogenesis and increase AF inducibility through abnormal $Ca^{2+}$ regulation.

FKBP12.6-deficient mice have increased susceptibility to AF.[22] Rapamycin and FK-506 induce RyR dysfunction by dissociating the RyR–FKBP12.6 complex and enhancing sarcoplasmic reticulum (SR) $Ca^{2+}$ leak.[23–25] We hypothesized that these effects would increase PV electrical activity and result in the different arrhythmogenesis observed between young and aged PVs. The purposes of this study were to investigate the effects of aging on electrophysiologic characteristics and the $Ca^{2+}$ regulatory proteins consisting of SR $Ca^{2+}$ adenosine triphosphatase (SERCA2a), RyR, and NCX, and to compare the pharmacologic responses of young and aged PVs to the $Ca^{2+}$ regulation agents rapamycin, FK-506, ryanodine, and ouabain.

## Methods

### Rabbit PV tissue preparations

The investigation conformed to the institutional *Guide for the Care and Use of Laboratory Animals.* Fifteen male young rabbits (age 3 months; weight 1.5–2.0 kg) and 16 male aged rabbits (age 3 years; weight 4.0–5.0 kg) were anesthetized with intraperitoneal injection of sodium pentobarbital (40 mg/kg). PV isolation was performed in Tyrode's solution of the following composition (in mM): 137 NaCl, 4 KCl, 15 $NaHCO_3$, 0.5 $NaH_2PO_4$, 0.5 $MgCl_2$, 2.7 $CaCl_2$, and 11 dextrose.[20,26] The right superior PV was separated from the atrium at the level of the left atrium–PV junction and separated from the lungs at the ending of the PV myocardial sleeves (Figure 1). The PV myocardial sleeves were ~5 × 5 × 0.5 mm in the young group and ~8 × 8 × 0.7 mm in the aged group. One end of the preparation, consisting of the PV and atrium–PV junction (3.0 × 1.0 × 0.3 cm), was pinned to the bottom of a tissue bath using needles. The other end of the preparation was connected to a Grass (RI, USA) FT03C force transducer using silk thread. The adventitia of the PVs faced upward. PVs from aged and young rabbits were superfused at a constant rate (3 mL/min) with Tyrode's solution, which was saturated with a 97% $O_2$–3% $CO_2$ gas mixture. Temperature and pH were maintained constant at 37°C and 7.4, respectively, throughout the entire experiment. Preparations were allowed to equilibrate for 1 hour before electrophysiologic study.

### Electrophysiologic and pharmacologic studies

The transmembrane action potential (AP) of the PVs was recorded using machine-pulled glass capillary microelectrodes filled with 3 M KCl. The PV preparation was connected to a World Precision Instruments (FL, USA) electrometer (model FD223) under tension with 150 mg. Electrical and mechanical events were displayed simultaneously on a Gould (OH, USA) 4072 oscilloscope and a Gould TA11 recorder. Signals were recorded with DC coupling and 10-kHz low-pass filter cutoff frequency using a data acquisition system. Signals were recorded digitally with 16-bit resolution at a rate of 125 kHz. APs were recorded from the distal part of the right superior PV, within 3 mm of the end of the PV myocardial sleeves in all

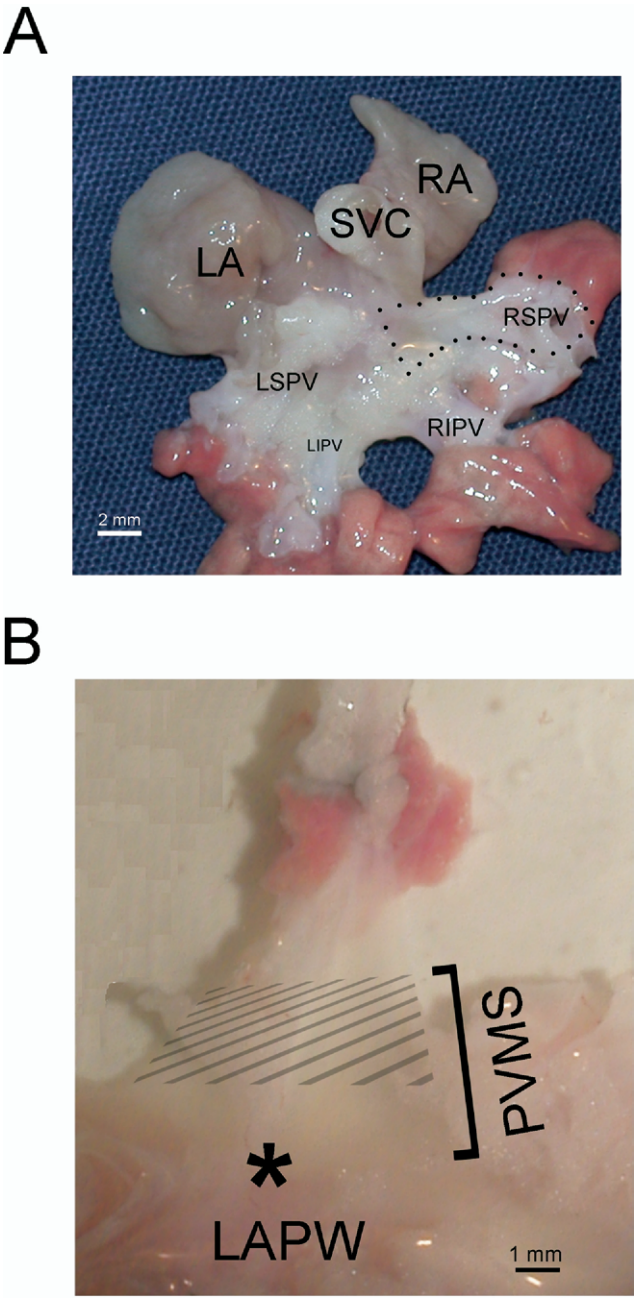


**Figure 1** Pulmonary vein (PV) preparations. **A:** Four PVs, including right superior PV (RSPV), left superior PV (LSPV), right inferior PV (RIPV), left inferior PV (LIPV), and left atrium (LA), right atrium (RA), and superior vena cava (SVC). The tissue preparation was isolated from the right superior PV (*dotted line*). **B:** *Hatched area* indicates recording zone at the distal part of PV myocardial sleeve (PVMS) for all isolated RSPV preparations studied. One end of the tissue preparation, the left atrial posterior wall (LAPW), was fixed with the pins; the other end (lung and distal PV junction) was connected to a force transducer using a silk thread. Note that the electrical stimulus was applied at the left atrium–PV junction (*asterisk*).

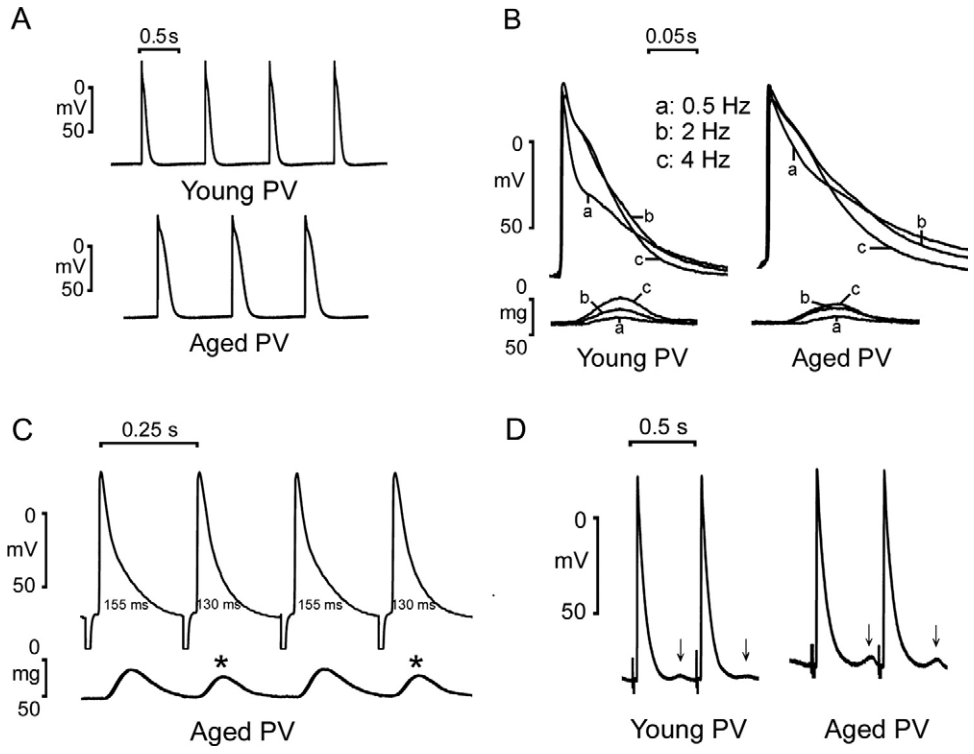


**Figure 2** Comparison of baseline action potential (AP) parameters in young and aged pulmonary veins (PVs). **A, B:** Tracings reveal slower spontaneous rates, longer AP duration, and less contractile force in aged PVs than in young PVs. **C:** Alternans of AP duration and contractile force were observed in aged PVs with a 4-Hz electrical stimulus. $APD_{90}$ alternation is depicted here (155 ms vs 130 ms). Shorter $APD_{90}$ is concordant with the smaller contractile force (*asterisk*). **D:** Larger delayed afterdepolarizations were found in aged PVs compared with young PVs.

preparations. The detailed map of recording sites for different preparations is shown in Figure 1B. The electrical stimulus was applied at the PV–LA junction, and the pacing threshold was 5 to 10 V. An electrical stimulus with 10-ms duration and suprathreshold strength (30% above threshold) was provided by a Grass S88 stimulator through a Grass SIU5B stimulus isolation unit. In order to reduce the effects of tissue injury or ischemia during the dissection procedures on PV spontaneous activity in the experiments, only data from well-prepared specimens were collected (resting membrane potential $<-60$ mV, action potential amplitude $>70$ mV, contractile force $>10$ mg).

Different concentrations of rapamycin (1, 10, 100 nM), FK-506 (0.01, 0.1, 1 $\mu$M), ouabain (0.1, 1 $\mu$M), or ryanodine (0.1, 1 $\mu$M) were sequentially superfused to test the pharmacologic responses of each drug. To avoid contamination with previously used drugs, APs and contractile force were compared between baseline and after the washoff period for each drug. Among the four drugs used in this study, only the effects of ryanodine were not reversed; the effects of the other three drugs (rapamycin, FK-506, ouabain) were completely reversed after washoff (Table 2–5). The pharmacologic effects of at most three drugs at every dose in each preparation were tested; ryanodine was always the last drug tested. The PV preparations were treated with each drug for at least 20 minutes, and stable AP parameters were recorded for at least 15 minutes for each concentration. After all of the concentrations of one drug

**Table 1** Baseline electrophysiologic characteristics of young and aged pulmonary veins at different frequencies of electrical stimuli

| Electrophysiologic property | Young (n = 8) | Aged (n = 10) | *P* value |
|---|---|---|---|
| APA (mV) | | | |
| 0.5 Hz | 96 ± 2 | 94 ± 4 | .68 |
| 2 Hz | 104 ± 1* | 98 ± 3† | .16 |
| 4 Hz | 90 ± 5 | 88 ± 3† | .69 |
| RMP (−mV) | | | |
| 0.5 Hz | 79 ± 2 | 74 ± 1 | <.05 |
| 2 Hz | 77 ± 1* | 72 ± 1† | <.05 |
| 4 Hz | 73 ± 2* | 68 ± 1† | <.05 |
| $V_{max}$ (m/s) | | | |
| 0.5 Hz | 134 ± 14 | 104 ± 12 | .15 |
| 2 Hz | 142 ± 16 | 99 ± 12 | <.05 |
| 4 Hz | 100 ± 15* | 70 ± 12† | .12 |
| $APD_{50}$ (ms) | | | |
| 0.5 Hz | 20 ± 3 | 26 ± 4 | .14 |
| 2 Hz | 37 ± 4* | 50 ± 3† | <.05 |
| 4 Hz | 38 ± 5* | 48 ± 4† | .20 |
| $APD_{90}$ (ms) | | | |
| 0.5 Hz | 105 ± 6 | 139 ± 8 | <.05 |
| 2 Hz | 106 ± 5 | 130 ± 6† | <.05 |
| 4 Hz | 102 ± 6 | 115 ± 6† | .13 |
| Contractile force (mg) | | | |
| 0.5 Hz | 21 ± 5 | 22 ± 6 | .90 |
| 2 Hz | 35 ± 11 | 40 ± 9† | .72 |
| 4 Hz | 55 ± 11* | 48 ± 10† | .66 |

*$P$ <0.05, vs 0.5 Hz in the young PV group.
†$P$ <.05, vs 0.5 Hz in the aged PV group.

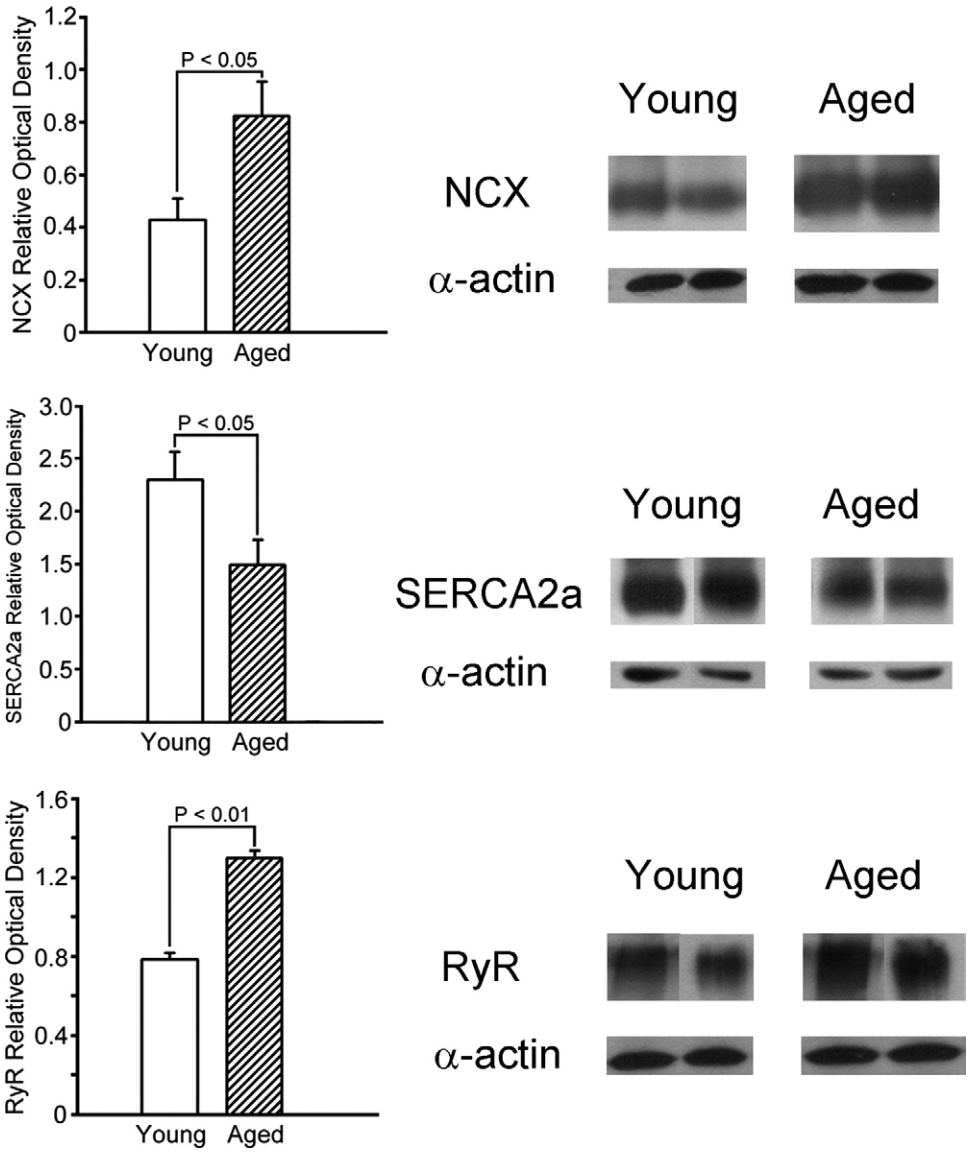


**Figure 3** Western blot of expression of $Na^+/Ca^{2+}$ exchanger (NCX), sarcoplasmic reticulum $Ca^{2+}$ adenosine triphosphatase (SERCA2a), and ryanodine receptor (RyR) from young pulmonary veins (n = 4) and aged pulmonary veins (n = 4). Two samples of young and aged pulmonary vein tissues are shown.

were tested, the preparation was washed off with Tyrode's solution for at least 1 hour. If differences or "rundown" of contractile force measurements and action potentials between baseline and washoff periods was observed, the tissue was not used for further experiments.

The 90% and 50% AP durations ($APD_{90}$ and $APD_{50}$, respectively), AP amplitude (APA), resting membrane potential (RMP), maximum upstroke velocity ($V_{max}$), and contractile force were measured during 2-Hz electrical stimuli before and after drug administration. $V_{max}$ was acquired by the maximum positive value of the first derivative of the AP. The amplitude of the DADs was measured with 2-Hz electrical stimuli.

## Western blot of RyR, NCX, and SERCA2a expression

Homogenates of young or aged rabbit proximal PV tissues (containing mainly cardiomyocytes) were suspended in lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 20 mM NaF, 2 mM $Na_3VO_4$, and protease inhibitor cocktails (Sigma-Aldrich Corp., Missouri, USA). Bradford assay was used to determine the protein concentration in homogenates and load equivalent amounts of total protein for each sample. Proteins were separated in 5% or 8% SDS–PAGE under reducing conditions and electrophoretically transferred into an equilibrated polyvinylidene difluoride membrane (Amersham Biosciences, Buckinghamshire, UK). Blots were probed with a mouse monoclonal antibody against SERCA2a (1:12,000 dilution; Affinity Bioreagents), NCX (1:250 dilution; Affinity Bioreagents, CO, USA), RyR (1:1,000 dilution; Affinity Bioreagents), and a secondary antibody conjugated with horseradish peroxidase. Bound antibodies were detected with the ECL detection system (SantaCruz Biotechnology, CA, USA) and analyzed with Image-Pro Plus software. Targeted bands were normalized to cardiac α-sarcomeric actin (Sigma-Aldrich Corp.) to confirm equal protein loading.

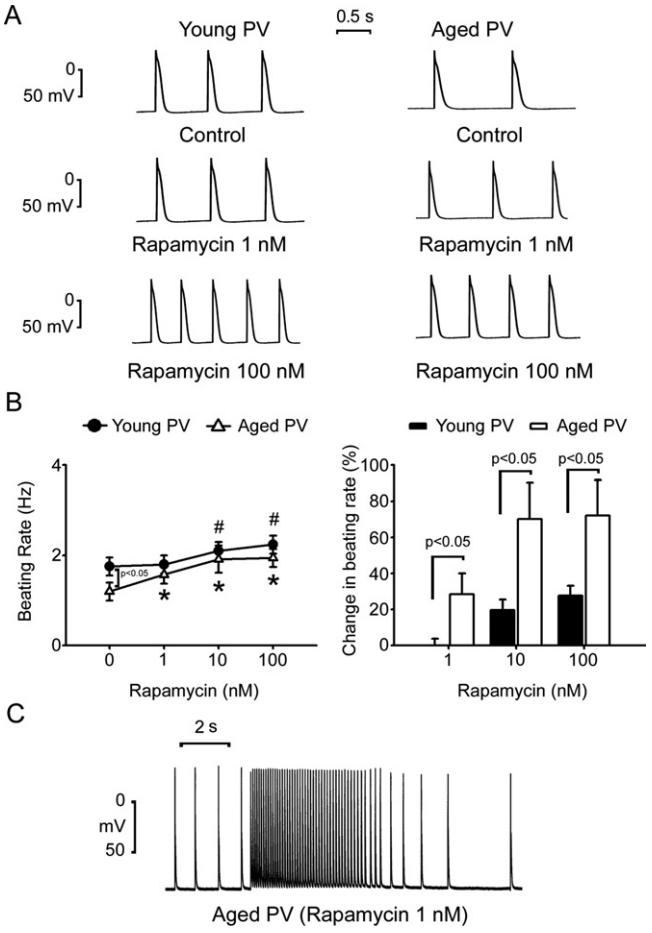


**Figure 4** Effects of rapamycin on young and aged pulmonary veins (PVs) with spontaneous activity. **A:** Rapamycin 1 nM increased the firing rates in aged PVs but not in young PVs. However, rapamycin 100 nM increased the firing rates in both groups. **B:** Concentration– response curve of the effects of rapamycin on PV firing rate **(left)** and percent increase in firing rates **(right)** in young PVs (n = 7) and aged PVs (n = 6). *$P$ <.05 vs before rapamycin administration in aged PVs. #$P$ <.05 vs before rapamycin administration in young PVs. **C:** Example of PV burst firing induced by rapamycin (1 nM) in an aged PV with spontaneous activity.

## Statistical analysis

All quantitative data are expressed as mean ± SEM. Repeated-measures analysis of variance (ANOVA) with Fisher least significant difference was used to compare differences before and after drug administration. Unpaired t-test and two-way ANOVA were used to compare differences between aged and young PVs at baseline and after drug administration, respectively. Nominal variables were compared by Chi-square analysis with Yates correction or Fisher's exact test. $P$ <.05 was considered significant.

## Results

### Electrophysiologic characteristics of young and aged PVs

Seven (47%) of 15 young rabbits and 6 (38%) of 16 aged rabbits ($P$ >.05) had PV spontaneous activity. Aged PVs had lower spontaneous rates (1.2 ± 0.2 Hz vs 1.8 ± 0.2 Hz, $P$ <.05) than young PVs (Figure 2A).

In PVs without spontaneous activity, greater depolarized RMP, smaller $V_{max}$, and longer $APD_{90}$ and $APD_{50}$ occurred in aged PVs than in young PVs. However, APA and contractile force did not differ between the two groups (Figure 2B and Table 1). Compared with pacing at 0.5 Hz, greater depolarization of RMP at 2 and 4 Hz, smaller $V_{max}$ at 4 Hz, shortened $APD_{90}$, prolonged $APD_{50}$, and increased contractility occurred in the young and aged PVs at 2 and 4 Hz. However, $APD_{90}$ shortening from 2 to 4 Hz was greater in aged PVs than in young PVs (12% ± 2% vs 4% ± 3%, $P$ <.05). In contrast, the rate-dependent increase in contractile force from 2 to 4 Hz was less in aged PVs than in young PVs (40% ± 19% vs 109% ± 27%, $P$ <.05). Moreover, during pacing at 4 Hz, $APD_{90}$ alternans (43%) and contractile alternans (57%) were observed in aged PVs (n = 16) but not in young PVs ($P$ <.05; Figure 2C). The differences in $APD_{90}$ (12 ± 3 ms vs 3 ± 1 ms, $P$ <.05) and contractile force (8 ± 2 mg vs 2 ± 1 mg, $P$ <.05) between beats were significantly larger in aged PVs than in young PVs.

DADs were observed in 33% of young PVs (n = 15) and 56% of aged PVs (n = 16) with and without spontaneous activity ($P$ >.05). However, aged PVs had a larger amplitude of DADs than did the young PVs (2.2 ± 0.2 mV vs 1.6 ± 0.3 mV, $P$ <.05; Figure 2D).

Figure 3 shows the protein level of NCX, SERCA2a, and RyR from young and aged PVs. Compared with young PVs, expression of NCX and RyR was increased but expression of SERCA2a was decreased in aged PVs.

### Effects of rapamycin on electrical activity in young and aged PVs

In PVs with spontaneous activity, rapamycin (1, 10, 100 nM) concentration-dependently increased the spontaneous rates (Figure 4A and B) and induced nonsustained burst firing (rate >4 Hz) in aged PVs (Figure 4C), but did so only at the higher concentrations (10,100 nM) in young PVs. Rapamycin increased PV spontaneous rates to a greater extent in aged PVs than in young PVs at concentrations of 1, 10, and 100 nM ($P$ <.05; Figure 4A).

In PVs without spontaneous activity, rapamycin concentration-dependently depolarized RMP and shortened $APD_{90}$ and $APD_{50}$ in aged PVs at concentrations of 1, 10, and 100 nM but did so in young PVs only at concentrations of 10 and 100 nM (not 1 nM; Figure 5A and Table 2). Rapamycin (10, 100 nM) decreased contractile force significantly in aged PVs but not in young PVs (Figure 5A). In addition, rapamycin did not affect APA in either aged or young PVs.

Rapamycin (1, 10, 100 nM) increased the incidence of DADs from 33% to 40%, 73%, and 73% in young PVs (n = 15, $P$ <.05) and from 56% to 69%, 81%, and 81% in aged PVs (n = 16, $P$ <.05). However, rapamycin exhibited significantly larger amplitude of DADs in aged PVs

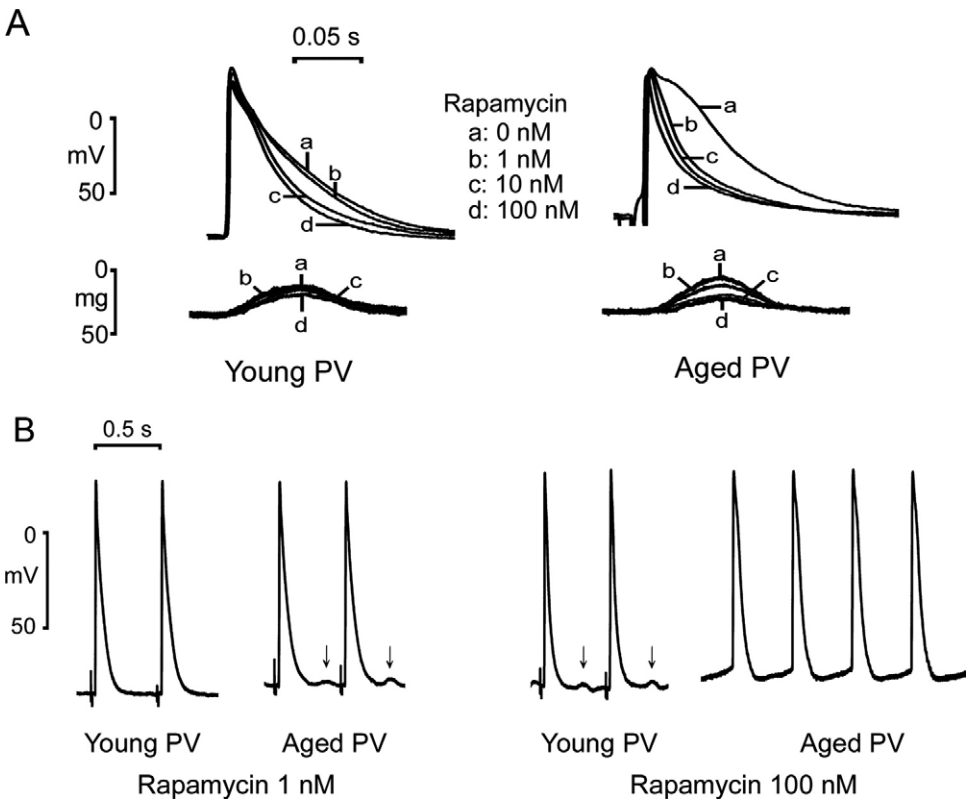


**Figure 5** Effects of rapamycin on young and aged pulmonary veins (PVs) without spontaneous activity. **A:** Superimposed tracings show the effects of rapamycin (1, 10, 100 nM) on action potential configuration and contractile force in PV without spontaneous activity. **B:** Examples of rapamycin (1 nM)–induced delayed afterdepolarizations (DADs) in aged PVs but not in young PVs. Note that 100 nM rapamycin induced spontaneous activity in aged PVs but only induced DAD without spontaneous activity in young PVs.

than in young PVs (Figure 5B and Table 2). Moreover, rapamycin (10, 100 nM) induced nonsustained spontaneous activity in 5 (50%) of 10 aged PVs but not in any young PVs (n = 8, $P < .05$). These effects were completely reversed in both young and aged PVs after washoff of rapamycin (Table 2).

**Table 2** Electrophysiologic characteristics of young and aged pulmonary veins at 2-Hz electrical stimuli before and after rapamycin administration

| Electrophysiologic property | Rapamycin (nM) | | | | |
|---|---|---|---|---|---|
| | 0 | 1 | 10 | 100 | Washoff |
| APA (mV) | | | | | |
| Young | 104 ± 2 | 101 ± 2 | 103 ± 3 | 96 ± 6 | 103 ± 2 |
| Aged | 97 ± 5 | 96 ± 5 | 103 ± 4 | 96 ± 6 | 97 ± 4 |
| RMP (−mV) | | | | | |
| Young | 77 ± 1 | 77 ± 1 | 74 ± 2† | 72 ± 2† | 77 ± 2 |
| Aged | 72 ± 1* | 71 ± 1*† | 68 ± 1*† | 67 ± 1*† | 72 ± 1* |
| $V_{max}$ (m/s) | | | | | |
| Young | 145 ± 17 | 152 ± 13 | 143 ± 11 | 144 ± 14 | 139 ± 11 |
| Aged | 106 ± 20* | 100 ± 20* | 101 ± 15* | 111 ± 20* | 101 ± 10* |
| $APD_{50}$ (ms) | | | | | |
| Young | 37 ± 4 | 34 ± 4 | 27 ± 5† | 27 ± 4† | 36 ± 4 |
| Aged | 51 ± 5* | 40 ± 5† | 40 ± 4† | 37 ± 4† | 51 ± 6* |
| $APD_{90}$ (ms) | | | | | |
| Young | 104 ± 5 | 95 ± 5 | 82 ± 8† | 80 ± 9† | 105 ± 4 |
| Aged | 133 ± 8* | 118 ± 9*† | 118 ± 9*† | 115 ± 7*† | 131 ± 7* |
| Contractile force (mg) | | | | | |
| Young | 34 ± 9 | 35 ± 13 | 32 ± 13 | 30 ± 12 | 34 ± 7 |
| Aged | 40 ± 11 | 40 ± 14 | 33 ± 11† | 32 ± 13† | 39 ± 11 |
| DAD amplitude (mV) | | | | | |
| Young | 1.6 ± 0.2 | 1.8 ± 0.2 | 3.2 ± 0.2† | 3.2 ± 0.2† | 1.6 ± 0.2 |
| Aged | 2.2 ± 0.1* | 3.0 ± 0.2*† | 4.2 ± 0.3*† | 4.4 ± 0.2*† | 2.1 ± 0.2* |

*$P < .05$, young (n = 8) vs aged (n = 10) PV groups with same concentration of rapamycin.
†$P < .05$ vs baseline within same age group.

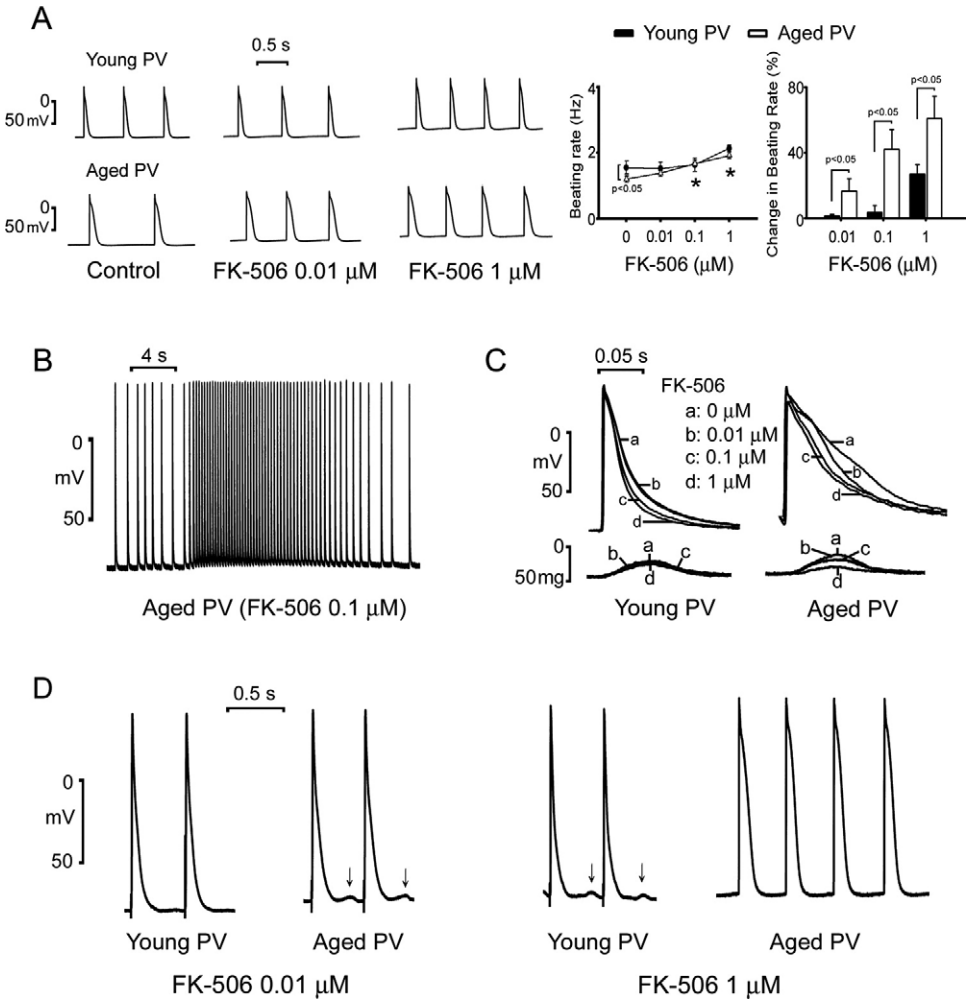


**Figure 6** Effects of FK-506 on pulmonary vein (PV) electrical activity. **A:** Effects of different concentrations of FK-506 on spontaneous rates in young and aged PVs with spontaneous activity. **Right:** Concentration–response curve of the effects of FK-506 on PV spontaneous rate and percent increase in young PVs (n = 7) and aged PVs (n = 6) before and after FK-506 (0.01, 0.1, 1 $\mu$M) administration. **B:** Example of FK-506 (0.01 $\mu$M)–induced burst PV firing in an aged PV with spontaneous activity. **C:** Superimposed tracings show the effects of FK-506 (0.01, 0.1, 1 $\mu$M) on action potential configuration and contractile force. **D:** Examples of FK-506 (0.01 $\mu$M)–induced delayed afterdepolarizations (DADs) in aged PVs but not in young PVs. Note that 1 $\mu$M FK-506 induced spontaneous activity in aged PVs but only DADs in young PVs. *$P$ <.05, before vs after FK-506 (0.01, 0.1, 1 $\mu$M) administration in aged PVs. #$P$ <.05, before vs after FK-506 (0.01, 0.1, 1 $\mu$M) administration in young PVs.

**Table 3** Electrophysiologic (EP) characteristics of young and aged pulmonary veins at 2-Hz electrical stimuli before and after FK-506 administration

| Electrophysiologic property | FK-506 ($\mu$M) | | | | |
|---|---|---|---|---|---|
| | 0 | 0.01 | 0.1 | 1 | Washoff |
| APA (mV) | | | | | |
| Young | 104 ± 2 | 102 ± 4 | 97 ± 8 | 96 ± 9 | 103 ± 2 |
| Aged | 98 ± 2 | 98 ± 4 | 98 ± 3 | 96 ± 3 | 97 ± 4 |
| RMP(−mV) | | | | | |
| Young | 77 ± 1 | 77 ± 1 | 75 ± 2 | 72 ± 2† | 77 ± 2 |
| Aged | 72 ± 1* | 71 ± 1*† | 70 ± 1*† | 68 ± 1*† | 72 ± 1* |
| $V_{max}$ (m/s) | | | | | |
| Young | 139 ± 11 | 140 ± 7 | 141 ± 11 | 137 ± 12 | 140 ± 6 |
| Aged | 101 ± 10* | 108 ± 15 | 107 ± 14* | 102 ± 11* | 105 ± 14* |
| $APD_{50}$ (ms) | | | | | |
| Young | 35 ± 4 | 32 ± 4 | 27 ± 6† | 26 ± 6† | 36 ± 4 |
| Aged | 51 ± 6* | 46 ± 7† | 46 ± 7*† | 44 ± 8† | 52 ± 6* |
| $APD_{90}$ (ms) | | | | | |
| Young | 105 ± 4 | 100 ± 5 | 89 ± 6† | 81 ± 9† | 105 ± 6 |
| Aged | 129 ± 7* | 121 ± 7† | 118 ± 8*† | 112 ± 8*† | 129 ± 10* |
| Contractile force (mg) | | | | | |
| Young | 34 ± 13 | 33 ± 13 | 31 ± 13 | 30 ± 15 | 35 ± 11 |
| Aged | 41 ± 12 | 40 ± 10 | 38 ± 10† | 33 ± 13† | 40 ± 14 |
| DAD amplitude (mV) | | | | | |
| Young | 1.6 ± 0.2 | 1.7 ± 0.2 | 2.2 ± 0.6 | 3.1 ± 0.4† | 1.6 ± 0.2 |
| Aged | 2.2 ± 0.1* | 2.6 ± 0.2 | 2.9 ± 0.3† | 4.1 ± 0.5*† | 2.1 ± 0.2* |

*$P$ <.05, young (n = 7) vs aged (n = 10) PV groups with same concentration of FK-506.
†$P$ < .05 vs baseline within same age group.

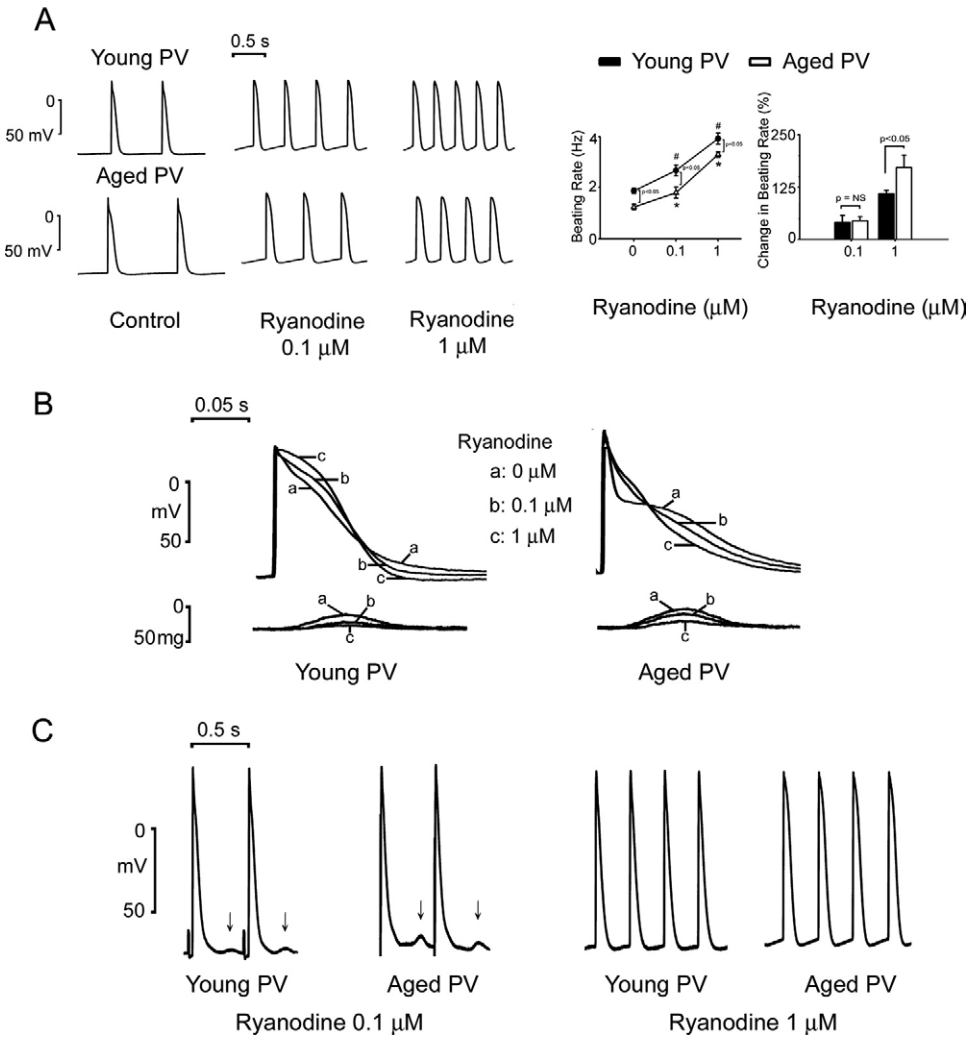


**Figure 7** Effects of ryanodine on pulmonary vein (PV) electrical activity. **A:** Tracings and concentration–response curve of the effects of ryanodine (0.1, 1$\mu$M) on firing rate and percent increase in young PVs (n = 7) and aged PVs (n = 6). **B:** Superimposed tracings show the effects of ryanodine (0.1, 1 $\mu$M) on action potential configuration and contractile force in PVs without spontaneous activity. **C:** Ryanodine (0.1 $\mu$M) induced a larger amplitude of delayed afterdepolarizations in aged PVs than in young PVs. Ryanodine (1 $\mu$M) induced spontaneous activity in both young and aged PVs. *$P$ <.05, before vs after ryanodine (0.1, 1 $\mu$M) administration in aged PVs. #$P$ <.05, before vs after ryanodine (0.1, 1 $\mu$M) administration in young PVs.

## Effects of FK-506 on electrical activity in young and aged PVs

Similar to rapamycin, FK-506 (0.01, 0.1, 1 $\mu$M) concentration-dependently induced nonsustained burst firing and increased the spontaneous rates to a greater extent in aged PVs (Figure 6). FK506 only at higher concentrations (0.1, 1 $\mu$M) increased spontaneous rates and induced nonsustained burst firing in young PVs. FK-506 (0.01, 0.1, 1 $\mu$M) also had more significant effects on RMP, $APD_{90}$, and $APD_{50}$ in aged PVs. FK-506 (0.1, 1 $\mu$M) decreased contractile force significantly in aged PVs but not in young PVs (Figure 6C and Table 3).

FK-506 (0.01, 0.1, 1 $\mu$M) increased the incidence of DADs from 56% to 62%, 75%, and 81% in aged PVs (n = 16, $P$ <.05). FK-506 (only 0.1, 1 $\mu$M) increased the incidence of DADs from 31% to 38% and 62% in young PVs (n = 13 $P$ <.05). FK-506 (0.01, 0.1, 1 $\mu$M) exhibited significantly larger amplitude of DADs in aged PVs than in young PVs (Figure 6D and Table 3). The effects of FK-506 were completely reversed after washoff (Table 3).

## Effects of ryanodine on electrical activity in young and aged PVs

In PVs with spontaneous activity, ryanodine (0.1, 1 $\mu$M) concentration-dependently increased spontaneous rates in both young and aged PVs (Figure 7A). However, the magnitude of percent increase in spontaneous rates caused by ryanodine (1 $\mu$M) was larger in aged PVs than in young PVs ($P$ <.05; Figure 7A). PV spontaneous rates in the presence of ryanodine were faster than in the presence of rapamycin or FK-506 in young and aged PVs. However, ryanodine did not generate any PV burst firing.

In PVs without spontaneous activity, ryanodine (0.1, 1 $\mu$M) concentration-dependently depolarized RMP, decreased APA, shortened $APD_{90}$, prolonged $APD_{50}$, and decreased contractile force in both young and aged PVs (Figure 7B and Table 4). The magnitude of change in AP parameters was similar in both groups. In addition, ryanodine did not affect $V_{max}$ or diastolic tension in either aged or young PVs.

Ryanodine (0.1, 1 $\mu$M) increased the incidence of DADs from 36% to 50% and 86% in young PVs (n = 14, $P$ <.05) and from 53% to 73% and 87% in aged PVs (n = 15,

**Table 4** Electrophysiologic characteristics of young and aged pulmonary veins at 2-Hz electrical stimuli before and after ryanodine administration

| Electrophysiologic property | Ryanodine ($\mu$M) | | | |
|---|---|---|---|---|
| | 0 | 0.1 | 1 | Washoff |
| APA (mV) | | | | |
| Young | 104 ± 3 | 98 ± 2 | 96 ± 3† | 95 ± 3† |
| Aged | 98 ± 2 | 93 ± 2† | 92 ± 2† | 92 ± 3† |
| RMP (−mV) | | | | |
| Young | 77 ± 1 | 72 ± 1† | 69 ± 1† | 72 ± 2† |
| Aged | 72 ± 1* | 70 ± 1† | 67 ± 1† | 67 ± 1*† |
| $V_{max}$ (m/s) | | | | |
| Young | 146 ± 20 | 140 ± 16 | 146 ± 22 | 144 ± 14 |
| Aged | 103 ± 19* | 103 ± 10* | 109 ± 11* | 101 ± 20* |
| $APD_{50}$ (ms) | | | | |
| Young | 36 ± 4 | 43 ± 6† | 50 ± 6† | 47 ± 4† |
| Aged | 49 ± 7* | 49 ± 8 | 54 ± 7 | 54 ± 4 |
| $APD_{90}$ (ms) | | | | |
| Young | 106 ± 6 | 94 ± 8† | 92 ± 7† | 90 ± 9† |
| Aged | 130 ± 8* | 112 ± 10† | 101 ± 9† | 105 ± 7† |
| Contractile force (mg) | | | | |
| Young | 33 ± 9 | 16 ± 5† | 6 ± 1† | 6 ± 2† |
| Aged | 43 ± 15 | 31 ± 13† | 15 ± 8† | 18 ± 3† |
| DAD amplitude (mV) | | | | |
| Young | 1.6 ± 0.1 | 2.0 ± 0.1† | 3.4 ± 0.2† | 3.2 ± 0.2† |
| Aged | 2.3 ± 0.1* | 3.1 ± 0.3*† | 4.5 ± 0.5*† | 4.4 ± 0.2† |

*$P$ <.05, young (n = 8) vs aged (n = 10) PV groups with same concentration of ryanodine.
†$P$ <.05 vs baseline within same age group.

$P$ <.05). Ryanodine (0.1, 1 $\mu$M) exhibited significantly larger amplitude of DADs in aged PVs than in young PVs (Figure 7C and Table 4). However, ryanodine (1 $\mu$M) induced a similar incidence of nonsustained spontaneous activity in 7 (70%) of 10 aged PVs and in 6 (75%) of 8 young PVs ($P$ >.05). The effects of ryanodine were not reversed after washoff (Table 4).

### Effects of ouabain on electrical activity in young and aged PVs

Ouabain (0.1, 1 $\mu$M) concentration-dependently increased spontaneous rates in both young and aged PVs (Figure 8A). However, ouabain (0.1 $\mu$M) exhibited higher spontaneous rates in young PVs than in aged PVs (Figure 8A). PV spontaneous rates in the presence of ouabain were faster than those induced by rapamycin and FK-506. However, ouabain did not generate any PV burst firing in either young or aged PVs.

In PVs without spontaneous activity, ouabain (0.1, 1 $\mu$M) shortened $APD_{90}$ and $APD_{50}$ in both young and aged PVs but significantly depolarized RMP only in aged PVs. Ouabain at 1$\mu$M (but not 0.1$\mu$M) decreased APA and increased contractile force and diastolic tension in both groups (Figure 8B and Table 5). Moreover, ouabain (1$\mu$M) increased contractility (119% ± 29% vs 50% ± 18%, $P$ <.05) and diastolic tension (33% ± 8% vs 11% ± 5%, $P$ <.05) to a greater extent in young PVs than in aged PVs.

Ouabain (0.1, 1 $\mu$M) increased the incidence of DADs from 31% to 54% and 92% in young PVs (n = 13, $P$ <.05) and from 50% to 62% and 75% in aged PVs (n = 16, $P$ <.05). Ouabain (0.1 $\mu$M) exhibited significantly larger amplitude of DADs in aged PVs than in young PVs (Figure 8C and Table 5). However, ouabain (1 $\mu$M) induced a similar incidence of nonsustained spontaneous activity in aged PVs (70%) and young PVs (86%; $P$ >.05). These effects were completely reversed after washoff (Table 5).

## Discussion

### Electrophysiology and $Ca^{2+}$ regulatory proteins in young and aged PVs

Aging has significant effects on the cardiac electrophysiology and genesis of AF. In this study, we demonstrated that aging also changed PV electrical characteristics seen as larger amplitude of DADs and lesser negative RMP, which would facilitate the genesis of trigged activity. In addition, greater magnitude of AP duration adaptation and decrease in $V_{max}$ were observed in aged PVs. These effects may predispose aged PVs to greater arrhythmogenesis by facilitating microreentry in the PVs,[27] which is one mechanism of PV arrhythmogenesis. Moreover, aging significantly decreased the magnitude of the rate-dependent increase in contractile force in PVs. This result may be caused by abnormal $Ca^{2+}$ regulation in aged PVs. Studies have indicated that mechanical alternans and

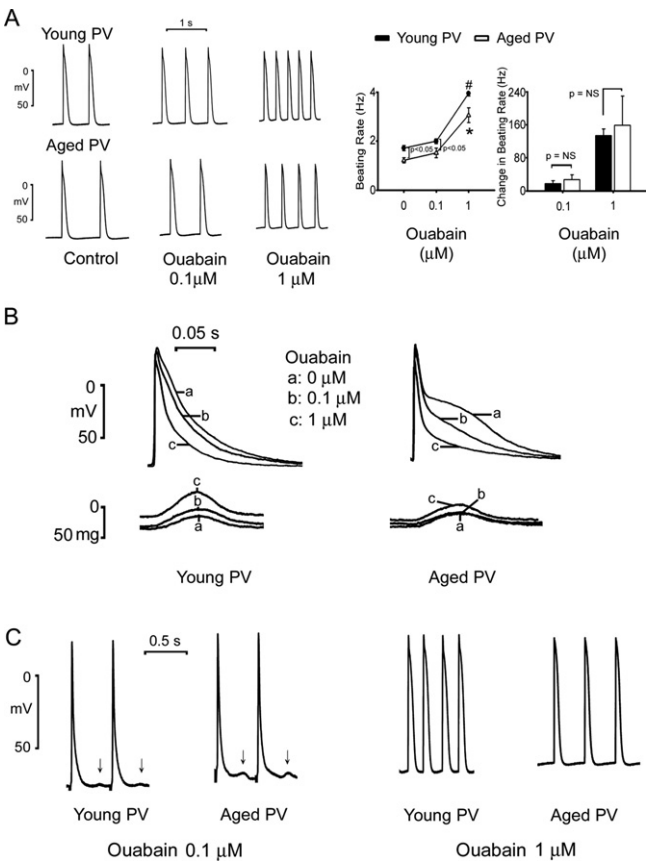


**Figure 8** Effects of ouabain on electrical activity of young and aged pulmonary vein s (PVs). **A:** Tracings and concentration–response curve of ouabain (0.1, 1 $\mu$M) on firing rates and percent increase in young PVs (n = 5) and aged PVs (n = 5) with spontaneous activity. **B:** Superimposed tracings show the effects of ouabain (0.1, 1 $\mu$M) on action potential configuration and contractile force in PVs without spontaneous activity. **C:** Examples of ouabain-induced delayed afterdepolarizations (0.1 $\mu$M) and spontaneous activity (1 $\mu$M) in young and aged PVs. *$P$ <.05, before vs after ouabain (0.1, 1 $\mu$M) administration in aged PVs. #$P$ <.05, before vs after ouabain (0.1, 1 $\mu$M) administration in young PVs.

APD alternans may arise from oscillations in SR $Ca^{2+}$ release and decrease in SERCA2a.[28,29] Our study found that aging may enhance the occurrence of mechanical alternans and APD alternans. Taken together, these findings suggest the existence of abnormal $Ca^{2+}$ regulation in aged PVs and its contribution to aging-related arrhythmogenesis.

Similar to previous studies,[9,10] SERCA2a expression was decreased in aged PVs compared with young PVs. Additionally, we found an increase in NCX expression in aged PVs. Removal of intracellular $Ca^{2+}$ occurs only via function of the NCX and SERCA2a. Therefore, under conditions of decreased SERCA2a, NCX activity increases $Ca^{2+}$ removal within the cells. Moreover, aged PVs had an increased level of RyR, which may potentiate SR $Ca^{2+}$ leak. These changes, in addition to the increase in NCX and greater depolarized RMP, enhance the genesis of DADs and subsequently triggered arrhythmias. These findings resemble the arrhythmogenic mechanism of heart failure.[30] Studies have shown that increasing phosphorylated RyR may increase diastolic calcium leak and induce triggered activity.[31] However, the specific phosphorylated RyRs in aged and young PVs were not evaluated in this study.

## Role of RyR/dysfunction and abnormal $Ca^{2+}$ regulation in PV electrophysiology

Rapamycin and FK-506 both dissociate the RyR–FKBP12.6 complex, but only FK-506 inhibits calcineurin activity.[32] Similar pharmacologic responses to rapamycin and FK-506 highly suggest that RyR dysfunction has an arrhythmogenic potential in the PVs, and inhibition of calcineurin seems not to play a role in PV arrhythmogenesis. Our results consistently showed that rapamycin and FK-506 decreased AP duration and increased the amplitude of DADs and the incidence of drug-induced spontaneous activity to a greater extent in aged PVs than in young PVs. Based on these findings, we suggest that aged PVs are more susceptible to SR $Ca^{2+}$ leakage than are young PVs. These effects may arise from more depolarized RMP and increases of RyR or NCX in aged PVs. Low-dose ryanodine is known to lock the RyR receptors into a subconductance state, which may cause $Ca^{2+}$-independent $Ca^{2+}$ release from the SR.[32] In aged PVs, ryanodine caused a larger amplitude of DADs and induced a larger percent increase in PV spontaneous rates. However, in contrast to FK-506 and rapamycin, ryanodine did not generate PV burst firing but induced faster PV spontaneous rates in both young and aged PVs. This finding may result from ryanodine's known effects of leaving RyR channels continuously open and possibly inducing a large amount of SR $Ca^{2+}$ leakage.[15] In contrast, rapamycin and FK-506 only increased the frequency of RyR channel opening and mean open lifetime.[23,25] The larger amount of SR $Ca^{2+}$ leakage caused by ryanodine, leading to greater depletion of SR $Ca^{2+}$ stores, also would produce a far greater decrease in contractile force as demonstrated in the present study.

Our previous study showed that ouabain had significant arrhythmogenic potential via $Ca^{2+}$ overload in the PVs.[20] Ouabain (1 $\mu$M) greatly increased contractile force and induced a slightly higher incidence of spontaneous activity in young PVs than in aged PVs, suggesting a smaller SR $Ca^{2+}$ store in aged cardiomyocytes.[9,10]

## Study limitations

The data from this study should be interpreted with caution. First, excitation of PV activity was not mapped, and the mechanisms of PV arrhythmogenesis were not fully elucidated. However, the recording of APs with slow diastolic depolarization and DADs in the PVs suggests that automaticity and triggered activity play a role in PV electrical activity. These findings are similar to those reported by Chou et al.[33] In addition, without studying caffeine-induced calcium transients, SR $Ca^{2+}$ content in PVs cannot be directly evaluated. However, the higher incidence of mechanical alternans and the smaller magnitude of rate-dependent

**Table 5** Electrophysiologic characteristics of young and aged pulmonary veins at 2-Hz electrical stimuli before and after ouabain administration

| Electrophysiologic property | Ouabain ($\mu$M) | | | |
|---|---|---|---|---|
| | 0 | 0.1 | 1 | Washoff |
| APA (mV) | | | | |
| Young | 104 ± 5 | 96 ± 4 | 89 ± 7† | 104 ± 3 |
| Aged | 98 ± 6 | 98 ± 5 | 89 ± 5† | 98 ± 2 |
| RMP (−mV) | | | | |
| Young | 77 ± 1 | 75 ± 2 | 67 ± 3† | 77 ± 1 |
| Aged | 72 ± 1* | 70 ± 1*† | 67 ± 1† | 72 ± 1* |
| $V_{max}$ (m/s) | | | | |
| Young | 140 ± 6 | 146 ± 10 | 137 ± 13 | 146 ± 20 |
| Aged | 105 ± 14* | 102 ± 11* | 100 ± 10* | 103 ± 19* |
| $APD_{50}$ (ms) | | | | |
| Young | 36 ± 4 | 33 ± 3† | 30 ± 3† | 36 ± 4 |
| Aged | 52 ± 6* | 40 ± 6† | 31 ± 6† | 49 ± 5* |
| $APD_{90}$ (ms) | | | | |
| Young | 105 ± 6 | 100 ± 6† | 94 ± 6† | 106 ± 6 |
| Aged | 129 ± 10* | 117 ± 9† | 110 ± 9† | 130 ± 8* |
| Contractile force (mg) | | | | |
| Young | 35 ± 11 | 41 ± 11 | 71 ± 15† | 33 ± 9 |
| Aged | 40 ± 14 | 39 ± 12 | 48 ± 13 | 43 ± 15 |
| DAD amplitude (mV) | | | | |
| Young | 1.6 ± 0.2 | 2.0 ± 0.2 | 3.9 ± 0.3† | 1.5 ± 0.1 |
| Aged | 2.3 ± 0.2* | 2.7 ± 0.1*† | 4.2 ± 0.2† | 2.2 ± 0.2* |

*$P$ <.05, young (n = 7) vs aged (n = 10) PV groups with same concentration of ouabain.
†$P$ <.05 vs baseline within same age group.

increase in contractile force in aged PVs highly suggest that aging reduces SR $Ca^{2+}$ in PVs, similar to results from previous studies.[9,10] Second, mechanoelectrical feedback plays an important role in PV arrhythmogenesis.[26] Under the same amount of tension applied to PV preparations, the smaller-sized preparations of young PVs would have stretched more than the larger-sized preparations of aged PVs. Therefore, arrhythmogenesis in aged PV may have been underestimated. Third, whether aging has the same effects on the PV and the atrium is not clear because calcium handling proteins in the atrium were not investigated. However, a previous study showed that SERCA2a decreased in human aged atria,[9] similar to our observation in aged PVs.

## Conclusion

We have demonstrated for the first time a significant aging-associated alteration in PV electrophysiology and $Ca^{2+}$ regulatory proteins. The greater degree of arrhythmogenic effects of rapamycin, FK-506, and ryanodine in aged PVs suggests that RyR abnormality plays an important role in PV arrhythmogenesis and aging-related arrhythmogenic vulnerability.

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