PDE5 Inhibition Suppresses Ventricular Arrhythmias by Reducing SR Ca$^{2+}$ Content

David C. Hutchings, Charles M. Pearman, George W.P. Madders, Lori S. Woods, David A. Eisner, Katharine M. Dibb, Andrew W. Trafford

RATIONALE: PDE5 (phosphodiesterase 5) inhibition reduces the occurrence of ventricular arrhythmias following myocardial ischemia. However, the mechanisms of the antiarrhythmic effects of PDE5 inhibition are unknown. Diastolic calcium (Ca$^{2+}$) waves lead to arrhythmias by inducing delayed afterdepolarizations (DADs). Ca$^{2+}$ waves are initiated when sarcoplasmic reticulum (SR) Ca$^{2+}$ content reaches a threshold level and the SR releases Ca$^{2+}$ spontaneously and generates a depolarizing inward sodium-calcium exchange current.

OBJECTIVE: To determine the effects of PDE5 inhibition on the propensity for ventricular arrhythmias in a proarrhythmic large animal model and establish the role of alterations of intracellular Ca$^{2+}$ cycling/SR Ca$^{2+}$ content.

METHODS AND RESULTS: Arrhythmia burden, monophasic action potentials, and beat-to-beat variability of repolarization were measured in a sheep model using the $I_{Kr}$ inhibitor dofetilide to induce QT prolongation and arrhythmia. Ca$^{2+}$ transients, Ca$^{2+}$ waves, and SR Ca$^{2+}$ content were measured in isolated ventricular myocytes. PDE5 inhibition was achieved using acute application of sildenafil, and PKG (protein kinase G) was inhibited with KT5823. PDE5 inhibition reduced beat-to-beat variability of repolarization and suppressed afterdepolarizations, premature ventricular complexes, and torsade de pointes in vivo. In single cells, dofetilide-induced delayed afterdepolarizations and triggered action potentials were suppressed by PDE5 inhibition. PDE5 inhibition decreased Ca$^{2+}$ wave frequency in all cells and abolished waves in 12 of 22 cells. A decrease in SR Ca$^{2+}$ uptake, increased trans-sarcolemmal Ca$^{2+}$ efflux, and reduced trans-sarcolemmal Ca$^{2+}$ influx led to a reduction of SR Ca$^{2+}$ content and Ca$^{2+}$ wave abolition. These effects were dependent on PKG activation.

CONCLUSIONS: PDE5 inhibition acutely suppresses triggered ventricular arrhythmias in vivo, and cellular data suggests this occurs via suppression of cellular Ca$^{2+}$ waves. These novel antiarrhythmic properties of PDE5 inhibition are mediated by a reduction of SR Ca$^{2+}$ content and are PKG dependent.

GRAPHIC ABSTRACT: An online graphic abstract is available for this article.

Key Words: calcium ■ long QT syndrome ■ sildenafil citrate

Prolongation of action potential (AP) duration (APD) and thence QT interval are established causes of ventricular arrhythmias. Perturbations to cellular Ca$^{2+}$ cycling may contribute to arrhythmias in long QT syndromes by 2 mechanisms: (i) reactivation of L-type Ca$^{2+}$ channel and (ii) spontaneous Ca$^{2+}$ release from the sarcoplasmic reticulum (SR). During the AP, Ca$^{2+}$ is normally released from the SR in response to trans-sarcolemmal Ca$^{2+}$ entry on the L-type Ca$^{2+}$ channel (L-type Ca$^{2+}$ current [$I_{Ca-L}$]). However, under conditions of SR Ca$^{2+}$ overload, Ca$^{2+}$ release from the SR can occur independently from the AP thereby generating propagating Ca$^{2+}$ waves. During these Ca$^{2+}$ waves, some Ca$^{2+}$...
Hutchings et al PDE5 Inhibition Suppresses Calcium Waves

Novelty and Significance

What Is Known?
- Perturbations to Ca^{2+} cycling contribute to arrhythmias in long QT syndromes.
- Incidental use of PDE5 (phosphodiesterase 5) inhibitors is associated with a reduction in post-myocardial infarction mortality in type II diabetes and potential antiarrhythmic effects are unknown.
- PDE5 inhibitors activate cGMP-PKG (protein kinase G) signaling, which can affect Ca^{2+} cycling; however, their effects on Ca^{2+}-dependent arrhythmias have not been investigated.

What New Information Does This Article Contribute?
- The PDE5 inhibitor sildenafil suppresses triggered ventricular arrhythmias in a sheep model of drug-induced QT prolongation.
- In single cardiac myocytes, sildenafil decreased the Ca^{2+} content of the intracellular store, the sarcoplasmic reticulum (SR), thus preventing diastolic Ca^{2+} release and Ca^{2+} waves.
- The decrease in SR Ca^{2+} was achieved via a combination of reduced SR Ca^{2+} uptake and reduced Ca^{2+} sarcolemmal influx.

Nonstandard Abbreviations and Acronyms

- β-AR: beta adrenergic receptor
- AP: action potential
- APD: action potential duration
- BP: blood pressure
- DAD: delayed afterdepolarization
- EAD: early afterdepolarization
- I_{Ca-L}: L-type Ca^{2+} current
- NCX: Na^{+}-Ca^{2+} exchanger
- PDE5: phosphodiesterase 5
- PKG: protein kinase G
- PVC: premature ventricular complex
- SERCA: sarcoplasmic endoplasmic reticulum Ca^{2+} ATPase
- SR: sarcoplasmic reticulum
- TdP: Torsades de Pointes

Prolongation of action potential duration and thence QT interval are established causes of ventricular arrhythmias. Spontaneous Ca^{2+} release from an intracellular store, the SR, may contribute to arrhythmias following myocardial infarction and in long QT syndromes. Given the association between incidental PDE5 inhibitor use and a reduction in cardiovascular mortality following myocardial ischemia in type II diabetics, we sought to determine whether activation of the PKG pathway, using the PDE5 inhibitor sildenafil to increase cGMP, was protective against Ca^{2+}-dependent arrhythmias. Using a drug-induced model of QT prolongation and arrhythmia in the sheep, we found that sildenafil dramatically suppressed the occurrence of afterdepolarizations and ventricular arrhythmias in vivo, and these effects were attributable to a PKG-dependent effect on Ca^{2+} waves and reduced SR Ca^{2+} content. The decrease in SR Ca^{2+} was achieved via a combination of reduced SR Ca^{2+} uptake and reduced Ca^{2+} influx into the cell. We propose that PDE5 inhibition offers a novel paradigm in the management of Ca^{2+}-dependent arrhythmia.
METHODS

Data Availability

The data that support the findings of this study are available from D.C.H. upon reasonable request. An expanded Methods section is available in the Data Supplement.

All procedures involving the use of animals were performed in accordance with The United Kingdom Animals (Scientific Procedures) Act, 1986 and European Union Directive 2010/63. Institutional approval was obtained from The University of Manchester Animal Welfare and Ethical Review Board. The reporting of animals in experimental studies accords with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.25

In Vivo Studies

A model of dofetilide-induced QT prolongation was used in 11 female Welsh mountain sheep (±18 months of age; 33.2±1.1 kg). All procedures were performed under general anesthesia (2%–3% isoflurane in oxygen) and preoperative analgesia (meloxicam, 0.5 mg/kg, s.c.) and antibiotics (enrofloxacin, 2.5 mg/kg, subcutaneously) provided. An endocardial DF-1 defibrillation lead with superior vena cava coil was positioned at the right ventricular apex and connected to a Medtronic implantable defibrillator to allow cardioversion of sustained arrhythmias. Arterial blood pressure (BP) was recorded every 2 to 3 minutes via forelimb plethysmography. Under continuous ECG monitoring, ventricular arrhythmias were induced by intravenous administration of the \( I_{\text{Kr}} \) blocker dofetilide (Stratech Scientific, Ltd, United Kingdom) in increments of 3 µg/kg until episodes of nonsustained ventricular tachycardia/TdP occurred (maximum total dose of 15 µg/kg). Animals were randomized (using a coin toss) to receive either 10 mg sildenafil bolus (Pfizer, United States) or saline control of the same volume (12.5 mL) and thapsigargin (5 µmol/L; Sigma, United Kingdom). Sildenafil, thapsigargin, dofetilde, and KT5823 were prepared in DMSO (dimethylsulfoxide; final concentration not exceeding 0.1% v/v). In all experiments, a vehicle control was used with the same concentration of DMSO. For all research materials, please see the Major Resources Table in the Data Supplement. All cellular experiments were performed at 37°C.

Statistics

Data are presented as mean±SEM for n cells for the myocyte experiments and N animals for in vivo studies. All analysis was performed using GraphPad Prism, version 7.00. Normality of data distribution (raw or following logarithmic transformation) was tested with a D’Agostino-Pearson or Shapiro-Wilk test. For normally distributed data, differences between treatment groups were determined using paired or unpaired t tests as indicated in the figure legends. In some figures where data were ratioed to control values, significance was assessed with a 1-sample t test. Categorical variables were compared between groups using the Fisher exact or \( \chi^2 \) tests as appropriate (Figures 2B, 3C, 5A, and 8C). Nonparametric tests were used when data were not normally distributed (see figure legends). Where sample size was too small to determine normality (<6), a nonparametric test was used. No correction has been made for multiple testing. Exact \( P \) values are presented at \( P<0.001 \), and differences were considered statistically significant at \( P<0.05 \).

RESULTS

Mechanism of Triggered Arrhythmia in a Dofetilide Model of QT Prolongation

Administration of the \( I_{\text{Kr}} \) blocker dofetilide resulted in dose-dependent prolongation of the QT interval (Figure 1A),30 an increase in T wave duration and altered T-wave morphology, and small reductions in heart rate. Systolic arterial pressure was reduced by dofetilide, whereas diastolic pressure was unchanged (Table I in the Data Supplement). Accompanying the effect on the QT interval was a dose-dependent prolongation of the endocardial monophasic APD (Figure 1B), associated with the occurrence of afterdepolarizations (Figure 1C) in 5 of the 6 animals where monophasic APs were recorded (\( P=0.028 \); Figure 1C). In all these 5 animals, afterdepolarizations preceded the onset of arrhythmias on the surface ECG. PVCs developed in 8 of 11 animals, with TdP occurring in 5 animals (Figure 1D). Each episode of TdP was

tolerate multiple caffeine applications in the presence of this high Ca\(^{2+}\) concentration; therefore, the majority of comparisons of SR content were made between different cells (ie, unpaired). PDE5 inhibition was achieved using sildenafil (1 µmol/L; Sigma, United Kingdom). In some experiments, a lower concentration of sildenafil (20 nmol/L) was also tested, where indicated in the text (Figure 4D through 4F; Figure IX in the Data Supplement). PKG was inhibited by preincubation for at least 30 minutes using KT5823 (1 µmol/L; Abcam, United Kingdom). SERCA (sarcoplasmic reticulum Ca\(^{2+}\) ATPase) was inhibited with thapsigargin (5 µmol/L; Sigma, United Kingdom). Sildenafil, thapsigargin, dofetilide, and KT5823 were prepared in DMSO (dimethylsulfoxide; final concentration not exceeding 0.1% v/v). In all experiments, a vehicle control was used with the same concentration of DMSO. For all research materials, please see the Major Resources Table in the Data Supplement. All cellular experiments were performed at 37°C.
preceded by a PVC falling on the preceding T wave (R on T phenomenon). However, 56.3% of PVCs falling on a T wave did not cause TdP. The importance of PVC timing in relation to the preceding T wave and the occurrence of TdP is depicted graphically in Figure 1E. Compared with non-TdP causing PVCs, those causing TdP arose earlier and were more closely clustered in relation to the T-wave apex (Figure 1E; 33.7±9.7 ms after T apex versus 111.5±5.6 ms; \( P<0.0001 \)). Thus, in agreement with previous work, the interval between the T wave and subsequent PVC plays a key role in determining likelihood of triggering TdP.31–34

**PDE5 Inhibitor Sildenafil Suppresses Ventricular Arrhythmias In Vivo**

In all the 5 animals showing TdP, intravenous sildenafil abolished dofetilide-induced TdP within 90 s of administration (Figure 2A and 2Bi; \( P=0.008 \)). In all 8 animals where dofetilide induced PVCs, sildenafil reduced PVC
Hutchings et al. PDE5 Inhibition Suppresses Calcium Waves

Hutchings et al. PDE5 Inhibition Suppresses Calcium Waves

The antiarrhythmic effect of sildenafil persisted for the duration of the experiment (up to 15 minutes) with PVC frequency decreasing by $72.5\pm18.3\%$ between 120 and 180 s ($P=0.017$) and by $90.2\pm6.5\%$ at 5 minutes (Figure 2Bii; $P<0.0005$). Saline alone, as a vehicle control for sildenafil, had no effect on PVC frequency or TdP incidence. Sildenafil also reduced the beat-to-beat variability in QT interval (Figure I in the Data Supplement). Other ECG parameters were unaffected by sildenafil (Table II in the Data Supplement). Sildenafil suppression of TdP appeared to have 2 key components: (1) a reduced frequency of PVCs (Figure 2Bii) and (2) a decrease in the probability that a PVC causes TdP (Figure 2Biii).

To understand why PVCs in sildenafil are less likely to induce TdP, we next investigated the effect of sildenafil on PVC timing in relation to the T wave (Figure 3). Sildenafil delayed the timing of PVCs such that the fraction occurring in the vulnerable window around the apex of the T wave was reduced. The reduction of R on T behavior was not due to any sildenafil-induced changes in QT duration (Figure II in the Data Supplement). Additionally, sildenafil had no effect on left ventricular endocardial monophasic APD (Figure 4A and 4B). These experiments did, however, demonstrate the importance of sildenafil reducing the occurrence of DADs and early afterdepolarizations (EADs; Figure 4C). Because sildenafil decreased heart rate, we investigated the effect of sildenafil on ECG parameters and monophasic APD in 3 atrially paced animals (RR interval, 500 ms). Again, sildenafil had no statistically significant effect on QT interval or monophasic APD (QT: control, 416±12 ms; sildenafil, 415±17 ms; APD$_{90}$: control, 341±18 ms; sildenafil, 310±9 ms).

To determine whether the antiarrhythmic action of sildenafil was related to hemodynamic effects, BP was recorded throughout the study period (every 2–3 minutes). While sildenafil reduced arterial BP (Figure III in the Data Supplement; $P=0.02$), the hypotensive effects of sildenafil were maximal at a dose of 2.5 mg—a level at which there was no significant effect on DAD or EAD frequency. Furthermore, additional doses of sildenafil decreased afterdepolarization frequency but had no additive effect on BP. Thus, we conclude that sildenafil (1) acts independently of the QT interval and BP in suppressing arrhythmias; (2) suppresses afterdepolarizations,
Hutchings et al PDE5 Inhibition Suppresses Calcium Waves

reducing PVC frequency, and (3) reduces R on T through the later timing of PVCs.

**Cellular Mechanisms Responsible for Suppression of Dofetilide-Induced Arrhythmias**

We next sought to establish whether the antiarrhythmic effects of sildenafil on dofetilide-induced arrhythmia were present at the cellular level. Experiments were performed under current clamp control in left ventricular myocytes. Preliminary studies found that only a minority of cells had EADs and DADs when exposed to dofetilide alone (5 µmol/L). In contrast, dofetilide in combination with low external K+ (2 mmol/L) resulted in EADs and DADs in the majority of cells. Under these conditions, both EADs and DADs gave rise to triggered APs. The effects of sildenafil are shown in Figure 4D through 4F, where sildenafil (at both 20 nmol/L and 1 µmol/L concentrations) was seen to abolish DADs (P=0.03; Figure 4Diii and 4Fii). Under the conditions of these experiments,

---

**Figure 3. Sildenafil delays premature ventricular complexes (PVCs) relative to T-wave apex.**

**A.** Representative surface ECG recordings of a PVC in control with R on T effect (left) and in the presence of sildenafil (right). **B.** Effect of sildenafil (10 mg IV bolus) on PVC timing in relation to the preceding T wave. Sildenafil delays PVCs such that they occur further from the T-wave apex and beyond the vulnerable period. **C. Left.** Mean data on proportion of PVCs occurring on T wave in sildenafil (control, n=84 PVCs from 8 animals; sildenafil, n=33 PVCs from 5 animals; χ² test). **C. Right.** Mean data summarizing effect of sildenafil on timing of PVCs after the apex of the preceding T wave. Open circles indicate mean data for each animal, and closed circles indicate individual PVCs. Linear mixed modeling t test from n=5 animals.
Figure 4. Sildenafil suppresses afterdepolarizations in vivo and isolated myocytes without modifying monophasic action potential duration.

A, Representative paired in vivo monophasic action potential (MAP) recordings in sinus rhythm in control (left) and sildenafil (right). Early afterdepolarizations (EADs) and delayed afterdepolarizations (DADs) are indicated by dashed and continuous arrows, respectively. B, Effect of sildenafil on monophasic action potential duration. Representative MAP traces from the same animal (left) and summary data (right). Paired data on APD90 from 4 animals, Wilcoxon signed-rank test. C, Effect of sildenafil on afterdepolarizations. Mean data for DADs (left) and EADs (right). Unpaired data on afterdepolarizations from 5 to 6 animals, Mann-Whitney U test. D, Example paired current clamp (0.25 Hz stimulation) recordings of a myocyte showing DADs when exposed to dofetilide and low K+. DADs are indicated by arrows. Di, After a 10 s exposure to sildenafil (20 nmol/L). Dii, After a 40 s exposure to sildenafil. Diw, Washout of sildenafil. E, Example paired current clamp recordings of a myocyte displaying EADs in dofetilide and low K+. Dashed arrows indicate EADs. Ei, Following a 10 s exposure to sildenafil. Eii, Following a 40 s exposure to sildenafil. Eiv, Following washout of sildenafil. F, Summary data for cells in dofetilide and low K+. Action potential duration, recorded from cells not showing EADs (Fi), DADs (Fii), and EADs (Fiii). For APD90, paired data from 6 cells/3 animals, t test. For DADs and EADs, paired data from 7 cells/3 animals, Wilcoxon signed-rank test. APD indicates action potential duration.
there was no significant effect on EAD occurrence (Figure 4E and 4Fii). Furthermore, in 3 cells that showed DADs but not EADs, sildenafil (20 nmol/L) abolished all DADs. Washout of sildenafil led to the return of DADs (Figure 4Div). Because APD is prolonged by the presence of EADs, we have only examined the effect of sildenafil on APD in those cells that did not display EADs. In these 6 cells, consistent with the in vivo findings, there was no effect on APD (Figure 4Fi). Given the effect of sildenafil on DADS, the next series of experiments were directed at understanding its effects on Ca\(^{2+}\) waves and Ca\(^{2+}\) handling.

**Effect of Sildenafil on Ca\(^{2+}\) Cycling**

In view of the role of Ca\(^{2+}\) in the generation of DADs,\(^4\) \(\ldots\) \(\ldots\) \(\ldots\) \(\ldots\) \(\ldots\) \(\ldots\) subsequent experiments were designed to determine whether the antiarrhythmic effects of sildenafil were mediated by changes in intracellular Ca\(^{2+}\) cycling. Cellular calcium waves were induced in voltage-clamped left ventricular myocytes by raising the external Ca\(^{2+}\) concentration to 10 to 15 mmol/L to increase SR Ca\(^{2+}\) content (control, 44.3±12.6 µmol/L; high Ca\(^{2+}\), 104.4±14.6 µmol/L; \(P=0.0044\); Figure IV in the Data Supplement). An analysis of the effects of raising external Ca\(^{2+}\) on cellular Ca\(^{2+}\) handling is presented in the Data Supplement (Table III in the Data Supplement).

As shown in Figure 5Ai, sildenafil reduced Ca\(^{2+}\) transient amplitude by 25.6±7.3% (\(P=0.0046\); \(n=22\) cells/13 animals). In 12 of 22 cells, sildenafil abolished Ca\(^{2+}\) waves (Figure 5Ai; \(P<0.001\)). In separate, 90 s duration, time control experiments (not shown), waves persisted in all cells studied, and there was no statistical difference in wave properties (\(n=6\) cells/4 animals). In those cells where Ca\(^{2+}\) waves remained, sildenafil had 2 effects: (i) delaying the onset of the Ca\(^{2+}\) wave relative to the preceding Ca\(^{2+}\) transient and (ii) reducing the amplitude of the wave (Figure VC in the Data Supplement) and the resulting peak inward NCX current (Figure 5Ciii). In combination, these effects resulted in a decrease in Ca\(^{2+}\) efflux per wave (to 55.2±10.7% of control; \(P=0.003\)) and per cycle (12.6±4.8 µmol/L per 2-s cycle in control versus 6.5±3.2 sildenafil; \(P=0.016\)).

**Sildenafil Suppresses Ca\(^{2+}\) Waves by Reducing SR Content Without Altering the Threshold SR Ca\(^{2+}\) Content for Ca\(^{2+}\) Waves**

The next experiments were designed to investigate how sildenafil decreased the occurrence of Ca\(^{2+}\) waves. Two possibilities were considered: (i) a reduction of SR Ca\(^{2+}\) content and (ii) an increase in the threshold SR Ca\(^{2+}\) content for Ca\(^{2+}\) wave initiation. In those cells where sildenafil abolished Ca\(^{2+}\) waves, there was a decrease of SR Ca\(^{2+}\), content (Figure 5Ci and 5Cii, right hand column). Conversely, in those cells continuing to wave in sildenafil, SR Ca\(^{2+}\) content (and, therefore, the threshold) was not statistically altered (Figure 5C); however, wave frequency was reduced. A minority of cells (3 cells from 3 animals) did not show Ca\(^{2+}\) waves in elevated external Ca\(^{2+}\), and in these cells, sildenafil decreased SR Ca\(^{2+}\) content (from 58.3±7.1 to 34.0±5.4 µmol/L; \(P=0.033\)). Thus, we conclude that sildenafil reduces Ca\(^{2+}\) wave occurrence by decreasing SR Ca\(^{2+}\) content.

**Mechanisms Underpinning the Decrease of SR Ca\(^{2+}\) Content and Maintenance of Ca\(^{2+}\) Flux Balance in Sildenafil**

The above results raise 2 questions: (i) what is the mechanism of the reduction in SR Ca\(^{2+}\) content and (ii) how does the cell compensate for the loss of Ca\(^{2+}\) efflux during Ca\(^{2+}\) waves to preserve Ca\(^{2+}\) flux balance in sildenafil? We have investigated the following Ca\(^{2+}\) handling pathways.

**L-Type Ca\(^{2+}\) Current**

Sildenafil decreased peak \(I_{\text{Ca-L}}\) (from 7.76±0.97 to 3.87±0.64 pA/pF; \(P<0.000001\); \(n=28\) cells/16 animals; 10–15 mmol/L external Ca\(^{2+}\)), increased the 90% to 10% inactivation time (from 28.7±1.8 to 34.9±1.9 ms; \(P=0.004\)), and decreased the integral of \(I_{\text{Ca-L}}\) (from 2.88±0.31 to 2.11±0.34 µmol/L; \(P<0.0001\); Figure 6A). However, this (≈0.8 µmol/L) decrease of integrated Ca\(^{2+}\) entry is much smaller than the decrease of Ca\(^{2+}\) efflux (over each stimulus cycle) caused by the abolition of Ca\(^{2+}\) waves (≈7 µmol/L per wave), and additional factors must contribute to maintenance of cellular Ca\(^{2+}\) flux balance. In addition, the decreased Ca\(^{2+}\) entry is unlikely to explain the reduction in SR Ca\(^{2+}\) content since a decrease of L-type Ca\(^{2+}\) current does not reduce SR Ca content.\(^3\) \(\ldots\) \(\ldots\) \(\ldots\) \(\ldots\) \(\ldots\) \(\ldots\) \(\ldots\) \(\ldots\) \(\ldots\) \(\ldots\)

**Reduced SERCA Function**

As illustrated in Figure 6B, sildenafil decreased the rate constant of decay of the systolic Ca\(^{2+}\) transient \(k_{\text{SYS}}\) and the calculated contribution due to SERCA \((k_{\text{SERCA}})\): 6.5±0.7 s\(^{-1}\) in control versus 3.7±0.7 s\(^{-1}\) in sildenafil, \(P=0.0012\); \(n=17\) cells/11 animals; \(k_{\text{SERCA}}\): 5.9±0.8 s\(^{-1}\) in control versus 3.0±0.6 s\(^{-1}\) in sildenafil, \(P=0.004\). In subsequent experiments (Figure VI in the Data Supplement), we examined whether the sildenafil reduction in SERCA activity could quantitatively account for the effects on Ca\(^{2+}\) waves; the irreversible SERCA inhibitor thapsigargin was used to reduce SERCA function. Initial experiments showed that a 1-minute exposure to thapsigargin (5 µmol/L) decreased \(k_{\text{SYS}}\) from 6.4±0.9 to 3.8±0.6 s\(^{-1}\), a reduction comparable to that produced by sildenafil (11 cells/7 animals; \(P=0.017\)). There was no statistically significant effect of thapsigargin on \(I_{\text{Ca-L}}\). Accompanying the reduction in SERCA function, waves
were abolished in 6 of 13 cells ($P=0.007$; Figure VI in the Data Supplement, by comparison, waves were abolished in 12 of 22 in sildenafil). In these cells SR Ca\(^{2+}\) content was reduced below threshold (to 34.2±7.4 µmol/L; $P=0.015$; $n=6$–16 cells/4–12 animals). In the remaining 7 cells, which continued to show waves, thapsigargin mimicked sildenafil in reducing the amplitude and delaying the peak of the waves. The mechanism by which the reduced SERCA activity contributes to the reduction of SR Ca\(^{2+}\) content is indicated in Figure 6C. Here, the slowed decay of the systolic Ca\(^{2+}\) transient promotes increased Ca\(^{2+}\) removal from the cell by the NCX. Thus, we conclude that a decrease in SERCA function is a major factor underlying the suppression of Ca\(^{2+}\) waves and reduction of SR Ca\(^{2+}\) content in sildenafil.

**Reduced Background Influx**

Ca\(^{2+}\) entry also occurs under resting conditions via a mechanism distinct from $I_{\text{Ca-L}}$ or $I_{\text{NCX}}$.\(^{37}\) To understand whether sildenafil suppresses waves by modifying this background influx, unstimulated cells were held at the same holding potential as under paced conditions (−40 mV) and spontaneous waves induced with high Ca\(^{2+}\) solution (10–15 mmol/L; Figure 7). Under these conditions, background Ca\(^{2+}\) influx must equal efflux, and efflux can be estimated as the integral of wave $I_{\text{NCX}}$ per unit time (analyzed over 10 s). After applying sildenafil, cells were again allowed to return to steady state before a further calculation of background influx was made. Sildenafil decreased wave frequency (Figure 7A and C) and decreased $I_{\text{NCX}}$ on each wave (Figure 7Cii). Together, these results in a reduction of...
Ca\(^{2+}\) efflux per unit time (Figure 7Ciii) and, therefore, background influx from 8.3±2.5 µmol/L/s in control to 2.6±0.5 µmol/L/s in sildenafil (\(P=0.008\); 12 cells from 7 animals).

In some cells, we examined whether the effects of sildenafil were reversible. Removal of sildenafil led to the reappearance of waves in 4 of 5 cells, and 2 cells that continued to display Ca\(^{2+}\) waves in sildenafil, washout increased the size of waves. Accompanying the return of waves was a reversal of the sildenafil effects on Ca\(^{2+}\) cycling, namely an increase in \(k_{\text{SYS}}\) (6.21±1.29 s\(^{-1}\) in washout versus 3.10±0.96 sildenafil; \(P=0.003\); n=4 cells/3 animals), and an increase in sarcolemmal influx via \(I_{\text{Ca-L}}\) (2.69±0.38 in washout versus 1.79±0.33 µmol/L in sildenafil; \(P=0.047\); n=7 cells/4 animals).

**Sildenafil Prevents DAD-Evoked APs**

We next addressed whether the effect of sildenafil on Ca\(^{2+}\) waves could reduce afterdepolarizations sufficiently to prevent triggering of arrhythmogenic APs. Cells were paced at 0.25 Hz in current clamp mode using the perforated patch technique. While solutions containing 10 to 15 mmol/L Ca\(^{2+}\) were effective at inducing Ca\(^{2+}\) waves, we found that the accompanying afterdepolarizations failed to trigger APs (possibly via membrane stabilization).\(^{38}\) When external K\(^{+}\) was decreased to 2 mmol/L, Ca\(^{2+}\) waves were initiated at a lower external Ca\(^{2+}\) (5–7.5 mmol/L); the resulting afterdepolarizations now triggered APs (Figure 8A). Under these conditions, sildenafil decreased both the frequency of Ca\(^{2+}\) waves and the
resulting triggered APs (Figure 8B) but had no significant effect on APD (Figure 8Biii). Thus, we conclude that the sildenafil effect on waves has clinical relevance in preventing triggering of APs both in single cells and in vivo.

**PKG Inhibition Abolishes the Protective Effect of Sildenafil on Ca²⁺ Waves**

To determine whether the sildenafil reduction in wave propensity depends on PKG, control cells were preincubated for >30 minutes with the PKG inhibitor KT5823 (1 µmol/L) and the effects of sildenafil on waves examined (Figure 8C). Under PKG inhibition, sildenafil failed to suppress waves in all 6 cells tested. In addition, sildenafil had no significant effect on the peak or integral of ICa-L. We, therefore, conclude that the protective effect of sildenafil on waves is PKG dependent.

**DISCUSSION**

We have investigated the effects of sildenafil on arrhythmias both in vivo and in isolated myocytes. As summarized in Figure 8D, there were 5 major findings. (1) Sildenafil suppresses TdP by reducing both the frequency of PVCs and the probability of a PVC initiating TdP. (2) The protection against TdP afforded by sildenafil occurs due to later timing of PVCs such that they occur after resolution of the T wave, protecting from an R on...
Hutchings et al PDE5 Inhibition Suppresses Calcium Waves

T effect. (3) In isolated myocytes, PDE5 inhibition suppresses Ca\textsuperscript{2+} waves and triggered APs by reducing SR content. (4) The reduction in SR content results from a decreased SERCA activity with additional contributions from reduced background Ca\textsuperscript{2+} entry. (5) The effects of sildenafil on Ca\textsuperscript{2+} waves are PKG dependent.

Comparison With Previous Work

An antiarrhythmic effect has been demonstrated previously when sildenafil was administered before myocardial ischemia, although the underlying mechanisms were not elucidated. In those studies, administration of sildenafil \textapprox{} 20 hours before canine acute coronary ischemia and reperfusion reduced incidence of PVCs, VT, and VF\textsuperscript{39} while in isolated rat hearts, sildenafil decreased VF\textsuperscript{40}. It is possible that the reduction in arrhythmias was not a direct effect of sildenafil but, rather, secondary to an ischemic preconditioning effect since, in the former, sildenafil serum concentrations are expected to have decayed to low levels at the time of arrhythmia\textsuperscript{41}. In the latter, sildenafil substantially reduced infarct size. A number of studies have now implicated PDE5 inhibition in ischemic pre- and postconditioning, cardioprotection and ischemia-reperfusion injury\textsuperscript{42}, reducing infarct size, apoptosis, and postinfarct remodeling, and via a pathway...
involving opening of BK channels.\textsuperscript{42,44} Such effects are likely contributors in explaining reduced arrhythmias in these contexts. To our knowledge, this article is the first to demonstrate direct antiarrhythmic actions of PDE5 inhibition. While we used a doxetilide-induced model of QT prolongation and ventricular arrhythmia, the cellular mechanisms underlying the antiarrhythmic effects of PDE5 inhibition suggest it may additionally hold antiarrhythmic potential in other conditions causing Ca\textsuperscript{2+}-dependent arrhythmia, such as heart failure (HF), catecholaminergic polymorphic ventricular tachycardia, and digitalis toxicity.

**Cellular Mechanisms of the Antiarrhythmic Effects of Sildenafil**

PDE5 inhibition reduced $I_{\text{Ca-L}}$ as noted previously.\textsuperscript{46} While it is conceivable that the modest decrease in $I_{\text{Ca-L}}$ could contribute to the antiarrhythmic effect of sildenafil, it does not appear to be the predominating mechanism. Some previous work has demonstrated that pharmacological block of L-type Ca channels suppresses EADs, but the dose used also decreased APD.\textsuperscript{46,48} In our experiments and a previous study,\textsuperscript{46} sildenafil did not change APD. Additionally, sildenafil also slowed the inactivation of $I_{\text{Ca-L}}$, presumably because of the smaller Ca\textsuperscript{2+} transients reducing Ca\textsuperscript{2+}-dependent inhibition. Given these opposing actions, the overall effect of sildenafil on EADs is difficult to predict. Furthermore the decrease of $I_{\text{Ca-L}}$ would not be expected to decrease SR Ca content\textsuperscript{46} and, as such, would not be antiarrhythmogenic. It should also be noted that, although $I_{\text{Ca}}$ reduction is a trigger for EADs and sildenafil was found to suppress EADs in vivo, our cellular findings using elevated external Ca\textsuperscript{2+} or reduced external K\textsuperscript{+} suggest that arrhythmias appear to be most dependent on DADs. Specifically, it is the abolition of the DAD that is associated with the antiarrhythogenic effects of sildenafil. We cannot, however, exclude the possibility that abolition of EADs also contributes to the removal of arrhythmias in vivo. Finally, the abolition by sildenafil of EADs in vivo may contribute to decreasing cell and, therefore, SR Ca\textsuperscript{2+} loading, either directly by stopping reactivation of the L-type Ca current or, indirectly, by removing the effect of these depolarizations to decrease the activity of NCX.

It is perhaps surprising that the decrease of both the amplitude and integral of the L-type Ca current (Figure 6) is not associated with an effect on APD. In this context, we can only speculate that the slowed inactivation and consequent increase of inward current at late times during the AP plateau may result in sildenafil increasing APD.

Our experiments using thapsigargin (Figure VI in the Data Supplement) show that the degree of inhibition of SERCA by sildenafil is sufficient to suppress Ca\textsuperscript{2+} waves in the majority of cells indicating a major role for reduced SR Ca\textsuperscript{2+} content. In those cells continuing to display waves, the decrease in SERCA prolongs the time taken for the SR to reach threshold, thus delaying onset of the wave. Plausibly, this may explain the delayed PVCs and reduction in R on T events observed in sildenafil. Our experiments also point to another SERCA-independent mechanism; sildenafil reduces background Ca\textsuperscript{2+} entry, which is expected to decrease SR Ca\textsuperscript{2+} content and Ca\textsuperscript{2+} waves.\textsuperscript{15} However, we are unable to quantify background Ca\textsuperscript{2+} entry without raising external Ca\textsuperscript{2+}, and, therefore, the contribution to the effects of sildenafil under more physiological conditions remains to be determined. Our finding that inhibiting SERCA suppresses Ca\textsuperscript{2+} waves and thence arrhythmias is in agreement with previous work where SR Ca\textsuperscript{2+} content is reduced below a threshold level.\textsuperscript{49} However, it has also been reported that decreasing SERCA can allow Ca\textsuperscript{2+} waves to propagate more easily\textsuperscript{50} and, correspondingly, increasing SERCA function can prevent Ca\textsuperscript{2+}-dependent arrhythmias in the setting of severe SR leak, by restricting SR Ca\textsuperscript{2+} release to miniwaves and Ca\textsuperscript{2+} sparks, thus preventing propagation of cell-wide waves.\textsuperscript{51}

In scenarios where SERCA inhibition is insufficient to reduce SR content below threshold, it is conceivable that it could aggravate arrhythmias by organizing cell-wide Ca\textsuperscript{2+} waves, and it will be important, therefore, to test the role of sildenafil under these conditions as well.

Our findings raise several important questions. First, while we have characterized the mechanistic components underlying antiarrhythmic effects of sildenafil, the signaling pathways controlling these events require further exploration. The process appears to be PKG-dependent given that the sildenafil suppression of waves was abolished by KT5823. Exactly how manipulation of the cGMP-PKG axis reduces SR Ca\textsuperscript{2+} content is an important unanswered question. Indeed this is at odds with previous reports of PKG increasing SERCA function via phosphorylation of PLB (phospholamban).\textsuperscript{52} One possibility is that PKG can activate protein phosphatase 1,\textsuperscript{53,54} which could lead to a dephosphorylation of phospholamban and reduce SERCA activity. In our experiments, sildenafil also decreased background Ca\textsuperscript{2+} influx. A background Ca\textsuperscript{2+} influx has been reported before in cardiac myocytes and is sensitive to gadolinium.\textsuperscript{37,55} Transient Receptor Potential (TRP) channels are expressed in cardiac myocytes, are inhibited by gadolinium, and PKG, which is activated by PDE5 inhibition, has been shown to acutely decrease Ca\textsuperscript{2+} entry via TRPC6 inhibition in rat neonatal cardiac myocytes.\textsuperscript{56} Thus, this appears to be a plausible molecular mechanism for the sildenafil decrease in background influx and by changing cytosolic Ca\textsuperscript{2+} and activation of CaMKII, could conceivably modify PLB phosphorylation.

Given the potential roles for cross talk effects between cGMP and the cAMP-PKA (protein kinase A) axis, whether the antiarrhythmic effects of PDE5 inhibition extend to catecholamine-induced arrhythmia is an important consideration in future studies. While Lee et al\textsuperscript{53} demonstrated that sildenafil suppressed
β-adrenergic stimulation of contractility in mouse hearts via PKG phosphorylation of cTnI (cardiac troponin I), the effect PDE5 inhibition has on Ca\textsuperscript{2+} cycling under these conditions is difficult to predict given that cGMP has the capacity to inhibit PDE3 and thus has the potential to increase both $I_{Ca,L}$ and $k_{SERCA}$.

While sildenafil has selectivity for PDE5, the concentrations used in this study (1 µmol/L and 20 nmol/L) may to a lesser extent also inhibit other PDEs including PDE1, PDE6, and PDE11. Effects on PDE6 and PDE11 are unlikely to be relevant as these are not expressed in the heart.\textsuperscript{58,59} Expression of PDE1 has been confirmed in the heart and has been implicated in pathological hypertrophy.\textsuperscript{60} Given that sildenafil has an IC\textsubscript{50} (the half maximal inhibitory concentration) for PDE1 of 350 nmol/L,\textsuperscript{57} sildenafil at 1 µmol/L is expected to cause substantial inhibition. Nevertheless, the effects of sildenafil were also reproduced at 20 nmol/L (Figure 4D through 4F; Figure IX in the Data Supplement) where inhibition of PDE1 is expected to be minimized. During in vivo experiments, serum and myocardial concentrations of sildenafil were not determined following administration of intravenous sildenafil, and as such, we cannot be certain about the degree of selectivity in vivo. The dose used (10 mg) was, however, similar to that used clinically (see below).

**Clinical Relevance**

Accompanying sildenafil suppression of waves was a reduction in the amplitude of the Ca\textsuperscript{2+} transient by reduced SR Ca\textsuperscript{2+} content and decreased trigger for SR release by $I_{Ca,L}$, which would be expected to decrease contractility. In addition, PDE5 inhibitors reduce arterial BP. These are important clinical considerations given that frequently encountered proarrhythmic states are accompanied by impaired ventricular systolic function (eg, heart failure, myocardial infarction) and hemodynamic disturbance. Paradoxically, however, PDE5 inhibitors appear to improve contractile function in clinical and preclinical models of systolic HF and in animal models of myocardial ischemia (MI). In HF, PDE5 inhibitors have neutral effects when administered acutely and overall positive effects on ventricular function following chronic treatment.\textsuperscript{61–65} After MI, PDE5 inhibitors appear to improve contractile function, an effect that may relate to infarct size reduction and preventing adverse remodeling.\textsuperscript{66} While there is reason to be cautious when acutely administering sildenafil in patients with poor contractile function and low arterial BP, the doses used in our in vivo experiments are comparable (even allowing for the sheep weighing half of a human) to those safely encountered in our in vivo experiments are comparable (even allowing for the sheep weighing half of a human) to those safely used in clinical trials. The heart failure.\textsuperscript{65} Furthermore, while its use as an acute antiarrhythmic has never been tested in humans, it is conceivable that its acute negative inotropic and hypotensive effects may be outweighed by acute antiarrhythmic and longer term contractile remodeling effects.\textsuperscript{62,67} One noteworthy finding in our observations of cells showing Ca\textsuperscript{2+} waves is that although Ca\textsuperscript{2+} transient amplitude is reduced, this effect is relatively modest (−26%), yet abolishing waves is expected to improve overall contractile performance by enhancing diastolic performance.\textsuperscript{71,72} Nevertheless, how PDE5 inhibition induces remodeling and improves contractile function in chronic treatment to overcome acute negative inotropic effects, is an important area for further investigation.

In conclusion, we have demonstrated that PDE5 inhibition with sildenafil acutely suppresses Ca\textsuperscript{2+}-dependent triggered arrhythmias in vivo via the suppression of Ca\textsuperscript{2+} waves in cardiac myocytes. Mechanistically, this is achieved via an acute reduction in SERCA function, as well as decreased background Ca\textsuperscript{2+} entry, and depends on a signaling pathway involving PKG. We propose the described effects are both directly clinically relevant and highlight a novel mechanism of arrhythmia suppression not reported previously.

**ARTICLE INFORMATION**

Received February 15, 2021; revision received July 1, 2021; accepted July 8, 2021.

**Affiliation**

Unit of Cardiac Physiology, Division of Cardiovascular Sciences, Faculty of Biology Medicine and Health, University of Manchester, Manchester Academic Health Sciences Centre, United Kingdom.

**Sources of Funding**

This work was supported by grants from the British Heart Foundation (FS/15/28/31476, FS/12/57/29717, CH/2000004/12801, FS/12/34/29565, and PG/10/89/28630) and the Medical Research Council (MR/K501211/1). D.C. Hutchings and C.M. Pearman were supported by clinical lectureships from the National Institute for Health Research (NIHR).

**Disclosures**

None.

**Supplemental Materials**

Expanded Materials and Methods
Data Supplement Tables I–V
Data Supplement Figures I–X
References 73–79
Major Resources Table

**REFERENCES**

1. Jervell A, Lange-nielsen F. Congenital deaf-mutism, functional heart disease with prolongation of the Q-T interval and sudden death. Am Heart J. 1957;54:59–68. doi: 10.1016/0002-8703(57)90079-0
2. Moss AJ, Schwartz RJ, Crampton RS, Tzivoni D, Locati EH, MacCluer J, Hall WJ, Weltkamp L, Vincent GM, Garson A Jr. The long QT syndrome. Prospective longitudinal study of 328 families. Circulation 1991;84:1136–1144. doi: 10.1161/01.cir.84.11.1136
3. January CT, Riddle JM. Early afterdepolarizations: mechanism of induction and block. A role for L-type Ca\textsuperscript{2+} current. Circ Res. 1989;64:977–990. doi: 10.1161/01.res.64.5.977
4. Ntimec J, Kim JJ, Gabris B, Salama G. Calcium oscillations and T-wave lability precede ventricular arrhythmias in acquired long QT type 2. Heart Rhythm. 2010;7:1686–1694. doi: 10.1016/j.hrthm.2010.06.022
5. Volders PG, Vox MA, Stablo B, Spildo KR, de Groot SH, Gorgels AP, Wellens HJ, Lazzara R. Progress in the understanding of cardiac early afterdepolarizations and Torsades de Pointes: time to revise current concepts. Cardiovasc Res. 2000;46:376–392. doi: 10.1016/s0008-6363(00)00022-5

Circulation Research. 2021;129:650–665. DOI: 10.1161/CIRCRESAHA.121.318473 September 3, 2021 663
6. Kim JJ, Némec J, Li Q, Salama G. Synchronous systolic subcellular Ca²⁺-elevations underlie ventricular arrhythmia in drug-induced QT type 2. Circ Arrhythm Electrophysiol. 2015;8:703–712. doi: 10.1161/CIRCEP.114.002214

7. Fabiato A. Simulated calcium current can both cause calcium loading in and trigger calcium release from the sarcoplasmic reticulum of a skinned canine cardiac Purkinje cell. J Gen Physiol. 1985;85:291–320. doi: 10.1085/jgp.85.2.247

8. Fabiato A. Time and calcium dependence of activation and inactivation of calcium-induced release of calcium from the sarcoplasmic reticulum of a skinned canine cardiac Purkinje cell. J Gen Physiol. 1985;85:247–289. doi: 10.1085/jgp.85.2.247

9. Wier WG, Cannell MB, Berlin JR, Marban E, Lederer WJ. Cellular and subcellular heterogeneity of Ca²⁺ in single heart cells revealed by fura-2. Science. 1985;229:326–328. doi: 10.1126/science.3798114

10. Trafford AW, O’Neill SC, Eisner DA. Factors affecting the propagation of locally activated systolic Ca transients in rat ventricular myocytes. Pflugers Arch. 1993;425:181–183. doi: 10.1007/BF00374521

11. Ferrier GR, Saunders JH, Mendez C. A cellular mechanism for the generation of ventricular arrhythmias by catecholamines. Circ. Res. 1973;32:600–609. doi: 10.1161/01.res.32.5.600

12. Mechmann S, Pott L. Identification of Na-Ca exchange current in single cardiac myocytes. Pflugers Arch. 1983;419:597–599. doi: 10.1007/BF019597a0

13. Lederer WJ, Tsien RW. Transient inward current underlying arrhythmogenic effects of cardiotonic steroids in Purkinje fibers. J Physiol. 1976;263:73–100. doi: 10.1113/jphysiol.1976.sp011622

14. Venetucci LA, Trafford AW, Eisner DA. Increasing ryanodine receptor open probability does not produce arrhythmogenic calcium waves: threshold sarcoplasmic reticulum calcium content is required. Circ. Res. 2007;100:105–111. doi: 10.1161/01.res.0000292828.17939.00

15. Díaz ME, Trafford AW, O’Neill SC, Eisner DA. Measurement of sarcoplasmic reticulum Ca²⁺ content and sarcocellular Ca²⁺ fluxes in isolated rat ventricular myocytes during spontaneous Ca release. J Physiol. 1997;501(pt 1):193–216. doi: 10.1111/j.1469-7793.1997.tb00300.x

16. Jiang D, Xiao B, Yang D, Wang R, Chi P, Zhang L, Cheng H, Chen SR. RYR2 mutations linked to ventricular tachycardia and sudden death reduce the threshold for store-overload-induced Ca²⁺ release (SOICR). Proc Natl Acad Sci USA. 2004;101:13062–13067. doi: 10.1073/pnas.0402388101

17. Orchard CH, Eisner DA, Allen DG. Oscillations of intracellular Ca²⁺ in mammalian cardiac muscle. Nature. 1983;304:735–738. doi: 10.1038/304735a0

18. Fernández-Velasco M, Rueda A, Rizzi N, Benthap JT, Colombi B, Napoliolito C, Priori SG, Richard G, Gomez AM. Increased Ca²⁺ sensitivity of the ryanodine receptor mutant RYR2A4496C underlies catecholaminergic polymorphic ventricular tachycardia. Circ. Res. 2009;105:201–209, 12p following 209. doi: 10.1161/CIRCRESAHA.108.177493

19. Kashimura T, Briston SJ, Trafford AW, Napoliolito C, Priori SG, Eisner DA, Venetucci LA. In the RYR2(R4496C) mouse model of CPVT, β-adrenergic stimulation induces Ca waves by increasing SR Ca content and not by decreasing the threshold for Ca waves. Circ. Res. 2010;107:1483–1489. doi: 10.1161/CIRCRESAHA.110.227744

20. Kubalova Z, Terentyev D, Vatchenko-Karpinski S, Nishijima Y, Györke I, Terentyeva R, da Cuñha DN, Sridhar A, Feldman DS, Hamlin RL, et al. Abnormal intrastore calcium signaling in chronic heart failure. Circ Res. 2012;59:1921–1927. doi: 10.1161/CircRes.111.963025

21. Volders PG, Kucilar A, Vos MA, Spidro R, Wellens HJ, Lazzara R, Szabo B. Similarities between early and delayed afterdepolarizations induced by isoproterenol in canine ventricular myocytes. Cardiovasc Res. 1997;34:348–358. doi: 10.1016/s0008-6363(96)00270-2

22. Takimoto E, Champion HC, Belardi D, Moslehi J, Mongillo M, Mergia E, Mosleh DC, Zhu G, Ayers K, Czocco M, et al. cGMP-catalysed by phosphodiesterase 5A regulates cardiac adrenergic stimulation by NO- dependent mechanism. Circ. Res. 2005;96:100–109. doi: 10.1161/01.res.0000152262.22968.72

23. Lee DI, Vahebi S, Tocchetti GC, Barouh LA, Solaro RJ, Takimoto E, Kass DA. PDE5A suppression of acute beta-adrenergic activation requires modulation of myocyte beta-s signaling coupled to PKG-mediated troponin I phosphorylation. Basic Res. Cardiol. 2010;105:337–347. doi: 10.1007/s00395-010-0048-5

24. Borlaga BA, Melenovsky V, Marhin T, Fitzgerald P, Kass DA. Sildenafil inhibits β-adrenergic-stimulated cardiac contractility in humans. Circulation. 2005;112:2642–2649. doi: 10.1161/CIRCULATIONAHA.105.540500

25. Klkenny C, Browne WJ, Cuthil IC, Emerson M, Altham DG. Improving bio- science research reporting: the ARRIVE guidelines for reporting animal research. PLoS Biol. 2010;8:e1000412. doi: 10.1371/journal.pbio.1000412

26. Dibb KM, Ruecksehosslo U, Eisner DA, Isenberg G, Trafford AW. Mechanisms underlying enhanced cardiac excitation contraction coupling observed in the senescent sheep myocardium. J Mol Cell Cardiol. 2004;37:1171–1181. doi: 10.1016/j.yjmcc.2004.09.005
62. Takimoto E, Champion HC, Li M, Belardi D, Ren S, Rodriguez ER, Bedja D, Gabrielson KL, Wang Y, Kass DA. Chronic inhibition of cyclic GMP kinase phosphorylates phospholamban in isolated sarcoplasmic reticulum vesicles. *Cardiovasc Res*. 2001;15:1718–1726. doi: 10.1096/fj.00-0538com

63. Hutchings DC, Anderson SG, Caldwell JL, Trafford AW. Phosphodiesterase-5 inhibitors and the heart: compound cardioprotection? *Heart*. 2018;104:1244–1250. doi: 10.1136/heartjnl-2017-312865

64. Lawless M, Caldwell JL, Radcliffe EJ, Smith CER, Mellers MP, Hutchings DC, Woods LS, Church SJ, Unwin RD, Kirkwood GJ, et al. Phosphodiesterase-5 inhibition improves contractile function and restores transverse tubule loss and catecholamine responsiveness in heart failure. *Sci Rep*. 2019;6:6801. doi: 10.1038/s41598-019-42592-1

65. Guazzi M, Vicenzi M, Arena R, Guazzi MD. PDE5 inhibition with sildenafil improves left ventricular diastolic function, cardiac geometry, and clinical status in patients with stable systolic heart failure: results of a 1-year, prospective, randomized, placebo-controlled study. *Circ Heart Fail*. 2011;4:88–97. doi: 10.1161/CIRCHEARTFAILURE.110.0044694

66. Saloum FN, Abbate A, Das A, Houser JE, Mudrick CA, Qureshi IZ, Hoke NN, Roy SK, Brown WR, Prabhakar S, et al. Sildenafil (Viagra) attenuates ischemic cardiomyopathy and improves left ventricular function in mice. *Am J Physiol Heart Circ Physiol*. 2008;294:H1398–H1406. doi: 10.1152/ajpheart.91438.2007

67. Vachieri JL, Huez S, Gillies H, Layton G, Hayashi N, Gas X, Naeije R. Safety, tolerability and pharmacokinetics of an intravenous bolus of sildenafil in patients with pulmonary arterial hypertension. *Br J Clin Pharmacol*. 2011;71:289–292. doi: 10.1111/j.1365-2125.2010.03831.x

68. Goldstein I, Lue TF, Padma-Nathan H, Rosen RC, Wicker PA, Oral sildenafil in the treatment of erectile dysfunction. Sildenafil Study Group. *N Engl J Med*. 1996;338:1397–1404. doi: 10.1056/NEJM199605163381215

69. Anderson SG, Hutchings DC, Woodward M, Rahimi K, Rutter MK, Kirby M, Hackett G, Trafford AW, Heald AH. Phosphodiesterase type-5 inhibitor use in type 2 diabetes is associated with a reduction in all-cause mortality. *Heart*. 2016;102:1750–1756. doi: 10.1136/heartjnl-2015-309229

70. Guazzi M, Tumminello G, Di Marco F, Fiorentini C, Guazzi MD. The effects of phosphodiesterase-5 inhibition with sildenafil on pulmonary hemodynamics and diffusion capacity, exercise ventilatory efficiency, and oxygen uptake kinetics in chronic heart failure. *J Am Coll Cardiol*. 2004;44:2339–2348. doi: 10.1016/j.jacc.2004.09.041

71. Allen DG, Eisner DA, Pifolos JS, Smith GL. The relationship between intracellular calcium and contraction in calcium-overloaded papillary muscles. *J Physiol*. 1985;364:169–182. doi: 10.1113/jphysiol.1985.sp015737

72. Capogrossi MC, Suarez-Isla BA, Lakatta EG. The interaction of electrically excitable and contractile function in cardiac myocytes studied with confocal microscopy and membrane capacitance measurements: species-dependence and developmental effects. *Biophys J*. 1996;70:1494–1504. doi: 10.1016/S0006-3495(96)79711-4

73. Negretti N, Varro A, Eisner DA, Estimate of net calcium fluxes and sarcoplasmic reticulum calcium content during systole in rat ventricular myocytes. *J Physiol*. 1995;486:581–591. doi: 10.1113/jphysiol.1995.sp020836

74. Díaz ME, Graham HK, Trafford AW. Enhanced sarcocellular Ca2+ efflux reduces sarcoplasmic reticulum Ca2+ content and systolic Ca2+ in cardiac hypertrophy. *Cardiovasc Res*. 2004;62:539–547. doi: 10.1016/j.cardiores.2004.01.038

75. Pearman CM, An Excel-based implementation of the spectral method of action potential alternans analysis. *Physiol Rep*. 2014;2:e12194. doi: 10.1484/phy2.12194

76. Postema PG, De Jong JS, Van der Blatt IA, Wilde AA. Accurate electrocardiographic assessment of the QT interval: teach the tangent. *Heart Rhythm*. 2008;5:1015–1018. doi: 10.1016/j.hrthm.2008.03.037

77. Lepeschkin E, Surawicz B. The measurement of the QT-interval of the electrocardiogram. *Circulation*. 1956;2:378–388. doi: 10.1161/01.che.63.3.378

78. Hintseer M, Thomsen MB, Beckmann BM, Puefer A, Schimpf R, Wichmann HE, Steinbeck G, Vos MA, Kaab S. Beat-to-beat variability of QT intervals is increased in patients with drug-induced long-GT syndrome: a case control pilot study. *Eur Heart J*. 2008;29:185–190. doi: 10.1093/eurheartj/ehn586