Phenytoin Reduces Activity of Cardiac Ryanodine Receptor 2; A Potential Mechanism for Its Cardioprotective Action [S]

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ABSTRACT

Phenytoin is a hydantoin derivative that is used clinically for the treatment of epilepsy and has been reported to have antiarrhythmic actions on the heart. In a failing heart, the elevated diastolic Ca^{2+} leak from the sarcoplasmic reticulum can be normalized by the cardiac ryanodine receptor 2 (RyR2) inhibitor, dantrolene, without inhibiting Ca^{2+} release during systole or affecting Ca^{2+} release in normal healthy hearts. Unfortunately, dantrolene is hepatotoxic and unsuitable for chronic long-term administration. Because phenytoin and dantrolene belong to the hydantoin class of compounds, we test the hypothesis that dantrolene and phenytoin have similar inhibitory effects on RyR2 using a single-channel recording of RyR2 activity in artificial lipid bilayers. Phenytoin produced a reversible inhibition of RyR2 channels from sheep and human failing hearts. It followed a hyperbolic dose response with maximal inhibition of ~50%, Hill coefficient ~1, and IC_{50} ranging from 10 to 20 μM. It caused inhibition at diastolic cytoplasmic $[Ca^{2+}]$ but not at Ca^{2+} levels in the dyadic cleft during systole. Notably, phenytoin inhibits RyR2 from failing human heart but not from healthy heart, indicating that phenytoin may selectively target defective RyR2 channels in humans. We conclude that phenytoin could effectively inhibit RyR2-mediated release of Ca^{2+} in a manner paralleling that of dantrolene. Moreover, the IC_{50} of phenytoin in RyR2 is at least threefold lower than for other ion channels and clinically used serum levels, pointing to phenytoin as a more human-safe alternative to dantrolene for therapies against heart failure and cardiac arrythmias.

SIGNIFICANCE STATEMENT

We show that phenytoin, a Na channel blocker used clinically for treatment of epilepsy, is a diastolic inhibitor of cardiac calcium release channels [cardiac ryanodine receptor 2 (RyR2)] at doses threefold lower than its current therapeutic levels. Phenytoin inhibits RyR2 from failing human heart and not from healthy heart, indicating that phenytoin may selectively target defective RyR2 channels in humans and pointing to phenytoin as a more human-safe alternative to dantrolene for therapies against heart failure and cardiac arrhythmias.

Introduction

Proper cardiac contraction and rhythm relies on the balance of Ca^{2+} fluxes across the sarcoplasmic reticulum (SR) and sarcolemma of myocytes (Bers, 2006; Eisner et al., 2017). During an action potential, Ca^{2+} influx into the cell via L-type Ca^{2+} channels activates RyR2 and the release of Ca^{2+} from the SR by a process called Ca^{2+}-induced Ca^{2+} release (Fabiato, 1983). The combination of Ca^{2+} influx and SR release increases the bulk cytoplasmic Ca^{2+} concentration $[Ca^{2+}]_c$ to ~1 μM (Shannon et al., 2003). During Ca^{2+} release, the concentration of free $[Ca^{2+}]$ in the SR decreases from ~1 mM to between 0.4 and 0.7 mM (Zima et al., 2008). Modeling studies predict that during Ca^{2+} release, $[Ca^{2+}]$ in the 14 nm cleft between the SR

and sarcolemma reaches ~100 μM (Laver et al., 2013). With membrane repolarization, Ca^{2+}-induced Ca^{2+} release ceases, RyR2 channels close, and SR Ca^{2+} release terminates. In addition, removal of excess cytoplasmic Ca^{2+} by the Na^+-Ca^{2+} exchanger in the sarcolemma tends to depolarize the membrane (Bers, 2002, 2006; Dibb et al., 2007). Thus, excess SR Ca^{2+} release through "leaky" RyR2 during diastole can lead to both a reduced diastolic SR Ca^{2+} load, systolic Ca^{2+} release, and muscle contraction as seen in heart failure (Yano et al., 2005; Oo et al., 2015) as well as increased sarcoplasmic depolarization, excitability, and cardiac arrhythmias (George et al., 2006, 2007).

Some agents that partially inhibit RyR2 activity reduce diastolic Ca^{2+} leak and protect against inherited adrenergic-induced arrhythmias (flecainide, carvedilol) (Hilliard et al., 2010; Hwang et al., 2011; Pölönen et al., 2018) and arrhythmias in acquired disorders such as heart failure (e.g., dantrolene) (Yano et al., 2005). Dantrolene is a hydantoin derivative that was first used as a skeletal muscle relaxant and is now prescribed for acute treatment of malignant hyperthermia

ABBREVIATIONS: CaM, calmodulin; ICM, ischemic cardiomyopathy; P_o, open probability; RyR2, cardiac ryanodine receptor; SR, sarcoplasmic reticulum.

(Muehlschlegel and Sims, 2009). In failing heart, dantrolene selectively reduces diastolic Ca^{2+} leak without inhibiting Ca^{2+} release during systole and without otherwise affecting the function of normal healthy hearts (Maxwell et al., 2012; Chou et al., 2014). These properties favor a therapeutic role for dantrolene, but unfortunately, dantrolene is hepatotoxic with chronic long-term administration (Paul-Pletzer et al., 2002) and is therefore not suitable as a chronic heart failure therapy. Here, we explore the possibility that other less-toxic hydantoin derivatives produce similar inhibitory actions on RyR2.

Phenytoin (Dilantin) is a hydantoin derivative that is used clinically for the treatment of epilepsy (Twombly et al., 1988) and has been shown to offer protection against cardiac arrythmias (Conn, 1965; Conn et al., 1967; Rosen et al., 1967; Eddy and Singh, 1969). Phenytoin affects several cellular processes, including protein phosphorylation and neurotransmitter release in brain (Pincus and Lee, 1973; Lang et al., 1993). Its antiepileptic action is thought to be through decreasing the hyperexcitability of neurons via Na^+ channel block (IC_{50} = 58 μM in serum) (Lang et al., 1993), which it does without causing sedation or interfering with normal central nervous system function (Pincus and Lee, 1973; Yaari et al., 1986; Lang et al., 1993). In lobster axons, phenytoin was shown to decrease sodium influx (IC_{50} ~200 μM) during stimulation but not at rest (Hasbani et al., 1974). Phenytoin also has an inhibitory action on Ca^{2+} currents in brain slices (IC_{50} ~100 μM) (Pincus and Lee, 1973) and PC12 cells (IC_{50} = 9.6 μM) (Lang et al., 1993). Phenytoin (0.2 mM) reversibly inhibits post-tetanic potentiation, which appears to be through inhibition of presynaptic L-type Ca^{2+} channels and subsequent neurotransmitter release (Dodge and Rahamimoff, 1967; Delorenzo and Glaser, 1976; DeLorenzo, 1977). In neuroblastoma cells, phenytoin causes voltage-dependent inhibition of T-type Ca^{2+} currents (IC_{50} ~100 μM) with no detectable effect on L-type currents (Twombly et al., 1988). The effects of phenytoin on activity of ligand gated intracellular Ca^{2+} channels such as the RyR are unknown. Here, we investigate the direct effects of phenytoin on RyR2 activity using single-channel recording of RyR2 in artificial lipid bilayers to determine its potential as an inhibitor of diastolic Ca^{2+} leak. We also investigate phenytoin dependence on calmodulin (CaM), a component of the RyR2 molecular complex (Balshaw et al., 2001), as our previous investigation found that dantrolene inhibition of RyR2 in lipid bilayers and in permeabilized mouse cardiomyocytes required the presence of physiologic concentrations (100 nM) of CaM (Oo et al., 2015).

Materials and Methods

Heart Tissues and SR Vesicle Isolation. The collection and use of human tissue was approved by the Human Research Ethics Committees of the University of Newcastle (H-2009-0369), The Prince Charles Hospital (Metro North Hospital and Health Service, EC28114), St Vincent's Hospital (H03/118), the Sydney Heart Bank at the University of Sydney (2012-2814), and the University of Canberra (2013/01). Sheep hearts were obtained with approval from the Animal Care and Ethics Committee of the University of Newcastle (approval number A-2009-153). Details of tissue harvesting and SR vesicle isolation are given in Supplemental Table 1 and described previously (Walweel et al., 2017, 2019).

Single-Channel Recording. RyR2 were incorporated into artificial lipid bilayers formed from phosphatidylethanolamine (PE) and phosphatidylcholine (8:2 wt/wt; Avanti Polar Lipids, Alabaster, AL) in n-decane (50 mg/ml; ICN Biomedicals, Irvine, CA). The lipid mixture was applied across a 100-μm–diameter hole in a delrin cup separating cis and trans baths. SR vesicles were added to the cis bath, which was stirred until single channel currents indicated the fusion of an SR vesicle with the bilayer. SR vesicles fuse with the bilayer such that the cytoplasmic domain of RyR2 faces the cis bath (Laver et al., 1995). During stirring, the cis (cytoplasmic) bath contained 250 mM Cs^+ (230 mM $CsCH_3O_3S$, 20 mM CsCl) obtained from Sigma Aldrich (St. Louis, MO) + 1.0 mM $CaCl_2$ obtained from BDH Chemicals (VWR, Radnor, PA). The trans (SR luminal) bath contained 50 mM Cs^+ (30 mM $CsCH_3O_3S$, 20 mM CsCl) + either 0.1 mM $CaCl_2$ or 1 mM $CaCl_2$. When ion channels were detected in the bilayer, the trans $[Cs^+]$ was raised to 250 mM by 50 μl aliquot addition of 4 M $CsCH_3O_3S$. For single-channel recording, the cis solution near the lipid bilayer was replaced by recording solutions flowing from a tube, which was connected via a micromanifold to eight independently controlled syringe pumps. This local perfusion system allowed exposure of RyR2 to multiple serial bathing conditions while simultaneously measuring RyR2 activity (Laver, 2001). All solutions were pH buffered using 10 mM TES (N-tris [hydroxymethyl] methyl-2 aminoethanesulfonic acid) and titrated to pH 7.4 using CsOH (ICN Biomedicals). Cytoplasmic recording solutions were buffered to a redox potential of −232 mV with glutathione disulfide (0.2 mM) and glutathione (4 mM), and luminal solutions were buffered to a redox potential of −180 mV with glutathione disulfide (3 mM) and glutathione (2 mM), which were both obtained from MP Biomedicals (Irvine, CA). A Ca^{2+} electrode (Radiometer, Brea, CA) was used to determine the purity of Ca^{2+} buffers and Ca^{2+} stock solutions as well as free $[Ca^{2+}]$ > 100 nM. Free Ca^{2+} was titrated with $CaCl_2$ and buffered using 4.5 mM BAPTA [1,2-bis (o-aminophenoxy) ethane-N,N,N9,N9-tetraacetic acid obtained from Invitrogen (Carlsbad, CA); free $[Ca^{2+}]$ < 1 μM] or dibromo-BAPTA obtained from Molecular Probes (up to 2 mM; free $[Ca^{2+}]$ between 1 and 10 μM). $MgCl_2$ was obtained from BDH Chemicals (VWR), ATP was obtained from Sigma, and calmodulin was from Sigma Aldrich (prepared from bovine testes) and Enzo Life Sciences (Farmingdale, NY; prepared from pig brain). Dantrolene and phenytoin (powder) were obtained from Sigma Aldrich. They were prepared as stock solutions in dimethylsulfoxide, which was obtained from MP Biomedicals.

Single-channel recordings were made at room temperature (21–23°C). Electric potentials are expressed using standard physiologic convention (i.e., cytoplasm relative to SR lumen at virtual ground). Axopatch 200B amplifier (Axon Instruments/Molecular Devices, Sunnyvale, CA) was used to control and record bilayer potential and currents. The current signal was low-pass filtered at 1 kHz and digitized at 5 kHz. Single-channel parameters, open probability (P_o), and mean open time and mean closed time were measured using a threshold discriminator at 50% of channel amplitude (Channel3 software; N.W. Laver, nic@niclaver.com). P_o of RYRs in multichannel recordings could be measured from the time-averaged current divided by the unitary current and the number of channels. To determine the number of channels in each experiment, the local perfusion system was turned off to produce strong activation of the RyRs by 5 mM Ca^{2+} in the cis bath. The inhibitory action of phenytoin and dantrolene were measured by the ratio of RyR2 P_o during 30–60-second drug exposures to the mean P_o of bracketing 30–60-second periods of vehicle solution. RyR2 gating activity is known to show nonstationary, time-dependent modal behavior (Zahradníková and Zahradník, 1995), and results during such nonstationary periods were excluded from the analysis. Our criterion for nonstationarity was a greater than twofold difference in RyR2 P_o during bracketing periods of vehicle solutions.

Statistics. Significant differences between groups were tested using a Student's t test or ANOVA. Data are presented as means ± S.D. or means ± S.E. as stated in the text. $P < 0.05$ was considered significant, and $P < 0.01$ was considered highly significant. Hill equation Michaelis-Menten fits to dose-response data pooled from

several experiments were optimized using least squares criteria with the MATLAB nonlinear fitting function.

Results

Unless otherwise stated, RyR2 activity was measured in the presence of 100 nM cytoplasmic Ca^{2+} and 2 mM ATP. The inhibitory action of phenytoin and dantrolene were measured by the ratio of RyR2 P_o during 30–60-second drug exposures to the mean P_o of bracketing 30–60-second periods of vehicle solution. Our previous study (Oo et al., 2015) showed that dantrolene inhibition required the presence of CaM. However, phenytoin produced a reversible, concentration-dependent inhibition in sheep RyR2, even in the absence of CaM, with maximal inhibition of ~50%, IC_{50} in the range of 10–20 μM, and Hill coefficients not significantly different to 1 (Fig. 1; Table 1). We found no dependence of fit parameters on either the voltage (+40 and −40 mV, Fig. 1D) or the presence of CaM (Table 1; $P > 0.16$, one-way ANOVA).

The inhibitory effects of dantrolene and phenytoin in the presence of 100 nM CaM are compared in Fig. 1, B, C, E, and F. Both drugs produced reversible partial (~50%) inhibition of sheep RyR2 with Hill coefficients not significantly different to 1, but dantrolene inhibited with an IC_{50} of 0.14 ± 0.01 μM, ~100-fold higher potency than phenytoin. Both phenytoin and dantrolene inhibition did not depend on voltage.
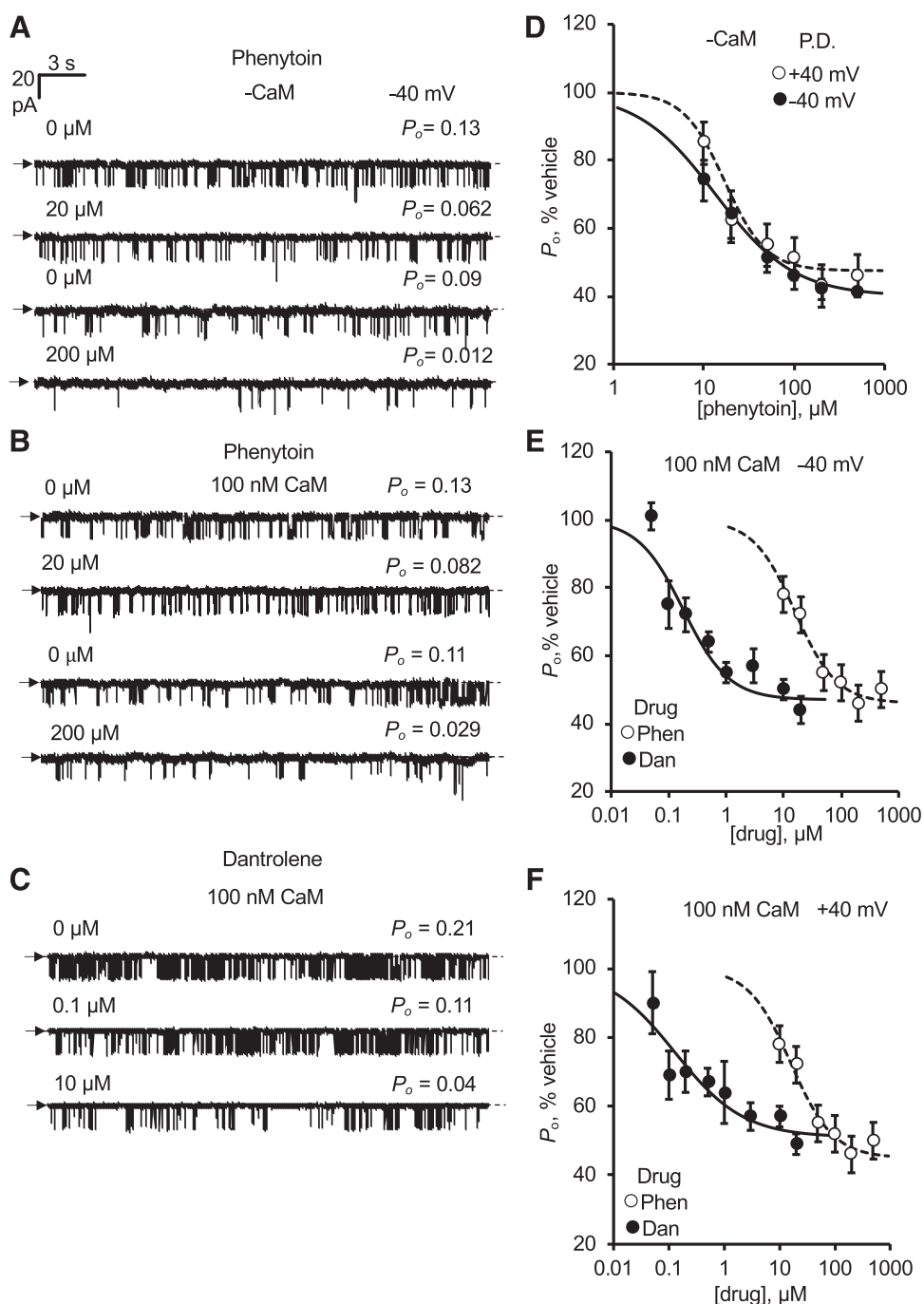

Fig. 1. Phenytoin and dantrolene inhibition of RyR2 from sheep heart. Single-channel recordings of phenytoin inhibition at −40 mV in the absence of CaM (A) and the presence of CaM (B) and dantrolene inhibition at −40 mV in the presence of CaM (C). Traces show 30-second segments of RyR2 activity of RyR2 where channel openings are downward currents steps from the baseline (arrows). Drug concentration is given at the left of each trace, and RyR2 open probabilities (P_o) are given at the right. Scale bars in (A) apply to (A–C). The cytoplasmic bath contains 0.1 μM Ca^{2+} and 2 mM ATP, and the luminal bath contains 0.1 mM Ca^{2+}. (D–F) Averaged data showing dose responses of phenytoin and dantrolene inhibition under conditions listed in (A–C). Error bars are S.E.M. of 13–27 experiments. Solid curves show least-squares fits of the Hill equation to the data compiled from all experiments. An example is shown in Supplemental Fig. 1. The Hill equation and the summary of fit parameters are given in Table 1.

TABLE 1

Hill equation parameters for drug dose responses

Parameters derived from least-squares fits of the Hill equation to the dose responses of P_o in Fig 1. Values are mean $\pm$ S.E.; n = number of samples for each concentration: $P_o = P_{max} + \{(1 - P_{max})/(1 + ([Drug]/IC_{50})^H)\}$.

Drug	CaM nM	Voltage mV	IC$_{50}$ μM	H	P$_{max}$%	n
Phenytoin	100	40	16 ± 4	1.1 ± 0.4	45 ± 5	14–19
Phenytoin	0	40	15 ± 3	1.8 ± 0.6	46 ± 3	13–18
Phenytoin	100	-40	19 ± 6	1.0 ± 0.3	39 ± 6	13–17
Phenytoin	0	-40	13 ± 3	1.0 ± 0.7	40 ± 6	14–27
Dantrolene	100	40	0.13 ± 0.07	0.6 ± 0.3	51 ± 5	13–15
Dantrolene	100	-40	0.14 ± 0.03	1.5 ± 0.5	53 ± 3	14–18

Figure 2 shows a dwell-time analysis of single-channel recordings used for Fig. 1. We investigated phenytoin inhibition under four experimental conditions, including +40 and −40 mV and the presence and absence of CaM (Fig. 2, A and B), as well as dantrolene inhibition in the presence of CaM (Fig. 2, C and D). Near maximal phenytoin inhibition at 100 μM was associated with an approximately twofold increase in the mean closed duration [e.g., mean $\pm$ S.E. (n): 1.2 ± 0.1 ms (13) to 2.6 ± 0.2 ms (22) at −40 mV and 1.1 ± 0.2 ms (15) to 2.3 ± 0.3 ms (15) at +40 mV; $P < 0.03$, 100 μM vs. zero, two-way ANOVA] in the absence of CaM. Mean open duration decreased by ~40% [e.g., 0.9 ± 0.07 ms (13) to 0.6 ± 0.05 ms (22) at −40 mV and 0.95 ± 0.02 ms (15) to 0.68 ± 0.03 ms (15) at +40 mV; $P < 0.01$, 100 μM vs. zero, two way ANOVA] in the absence of CaM. There were no significant differences between the four combinations of experimental conditions ($P > 0.2$, 100 μM vs. zero, presence vs. absence of CaM, and +40 mV vs. −40 mV, three-way ANOVA). Dantrolene inhibition at a concentration of 10 μM, where the effect was near maximal, produced an increase in the mean closed time at +40 mV and −40 mV in the presence of CaM ($P < 0.01$, 10 μM vs. zero, two-way ANOVA) [e.g., 0.8 ± 0.1 ms (7) to 2.2 ± 0.3 ms (8) at −40 mV]. Mean open time decreased by ~40% at +40 and −40 mV in the presence of CaM [e.g., 1.0 ± 0.07 ms (7) to 0.6 ± 0.05 ms (8) at −40 mV; $P < 0.01$, 10 μM vs. zero, two-way ANOVA]. As for dantrolene, there were no significant differences between +40 and −40 mV conditions ($P > 0.3$, +40 mV vs. −40 mV, two-way ANOVA).

Next, we examined how near-maximal inhibition by phenytoin (100 μM) and dantrolene (10 μM) depended on ionic conditions (Fig. 3). Inhibition by phenytoin and dantrolene diminished with increasing cytoplasmic [Ca^{2+}] from ~50% at 0.1 μM (end-diastolic [Ca^{2+}]) to no significant inhibition at 100 μM (systolic [Ca^{2+}]; Fig. 3A). The diminished effects of phenytoin and dantrolene with increasing cytoplasmic [Ca^{2+}] on RyR2 P$_o$ as well as open and closed dwell-times can be seen in Fig. 3B. Because cytoplasmic [Mg^{2+}] is a competitive antagonist that competes with Ca^{2+} binding and activation of RyR2, we checked to see if phenytoin still failed to inhibit RyR2 at 100 μM Ca^{2+} in the presence of physiologic levels of Mg^{2+} (1 mM Mg^{2+} is produced by 3 mM MgCl$_2$ in the presence of 2 mM ATP). In the presence Mg^{2+}, phenytoin still produced no significant inhibition of RyR2 (0.95 ± 0.09, S.D., $n = 12$; Fig. 3, B and C). Thus, it appears that phenytoin and dantrolene are both inhibitors at diastolic cytoplasmic [Ca^{2+}] but not at systolic [Ca^{2+}].

Because RyR2 sensitivity to luminal [Ca^{2+}] is a major determinant of diastolic SR Ca^{2+} leak (e.g., Jiang et al., 2005), we checked to see if RyR2 channels responded to phenytoin with luminal Ca^{2+} close to 1 mM that is achieved during diastole.

Figure 3, B and C shows that in the presence of end-diastolic cytoplasmic [Ca^{2+}], phenytoin had similar inhibitory actions on RyR2 at both 0.1 and 1 mM luminal [Ca^{2+}].

If phenytoin and dantrolene share the same binding site on the RyR2 channel, then phenytoin should not be able to inhibit RyR2 in the presence of saturating concentrations of dantrolene. Hence, we investigated the effect of phenytoin (100 μM) in the presence of dantrolene (10 μM) and did this both in the presence (Fig. 4A) and absence of CaM (Fig. 4B). Grouped data (Fig. 4C) shows that phenytoin inhibition of RyR2 was not alleviated in the presence of saturating concentrations of dantrolene (in the absence of CaM, P$_o$ decreased from 0.28 ± 0.05 to 0.08 ± 0.01; $P < 0.01$, presence vs. absence of phenytoin two-way ANOVA), indicating that phenytoin and dantrolene do not compete for the same binding site.

We investigated the action of phenytoin on RyR2 from human hearts and compared the effects of phenytoin on RyR2 from healthy and failing human [ischemic cardiomyopathy (ICM)] hearts (Fig. 5). Channel recordings show examples in which phenytoin inhibited RyR2 from failing hearts (Fig. 5A) but not from healthy hearts (Fig. 5B). We found phenytoin had a different action on RyR2 from healthy and failing hearts ($P = 0.016$; Fig. 5C). Phenytoin had no significant effect on RyR2 from healthy hearts (H1–H4, $P = 0.6$, paired t test) but caused a ~50% reduction in activity of RyR2 from failing hearts (ICM1–5, $P = 2 \times 10^{-12}$, paired t test; Fig. 5D).

Discussion

We show that the hydantoin, phenytoin, produces a dose-dependent inhibition of RyR2 channels from sheep and human failing hearts that follows a hyperbolic inhibition curve with maximal inhibition of ~50%. However, phenytoin had no effect on RyR2 from healthy human hearts. Our finding reveals a potential new target for phenytoin and new mechanisms by which phenytoin may modulate cell function and cardiac rhythm. Phenytoin inhibition shares several characteristics in common with that of dantrolene. Both produce, at most, partial (~50% with Hill coefficient of ~1) inhibition of RyR2 by means of reduced mean open time and increased mean closed times (Fig. 3B), although phenytoin inhibits with 100-fold lower potency. Both produce inhibition at diastolic cytoplasmic [Ca^{2+}] but not at Ca^{2+} levels that occur in the dyadic cleft during systole. This explains why dantrolene can reduce diastolic SR Ca^{2+} leak and increase SR Ca^{2+} content without interfering with subsequent improvement of the Ca^{2+} transient (Maxwell et al., 2012). If phenytoin inhibition kinetics are fast enough compared with the cardiac cycle, phenytoin could effectively reduce diastolic SR Ca^{2+} leak and improve systolic Ca^{2+} transients like dantrolene. This
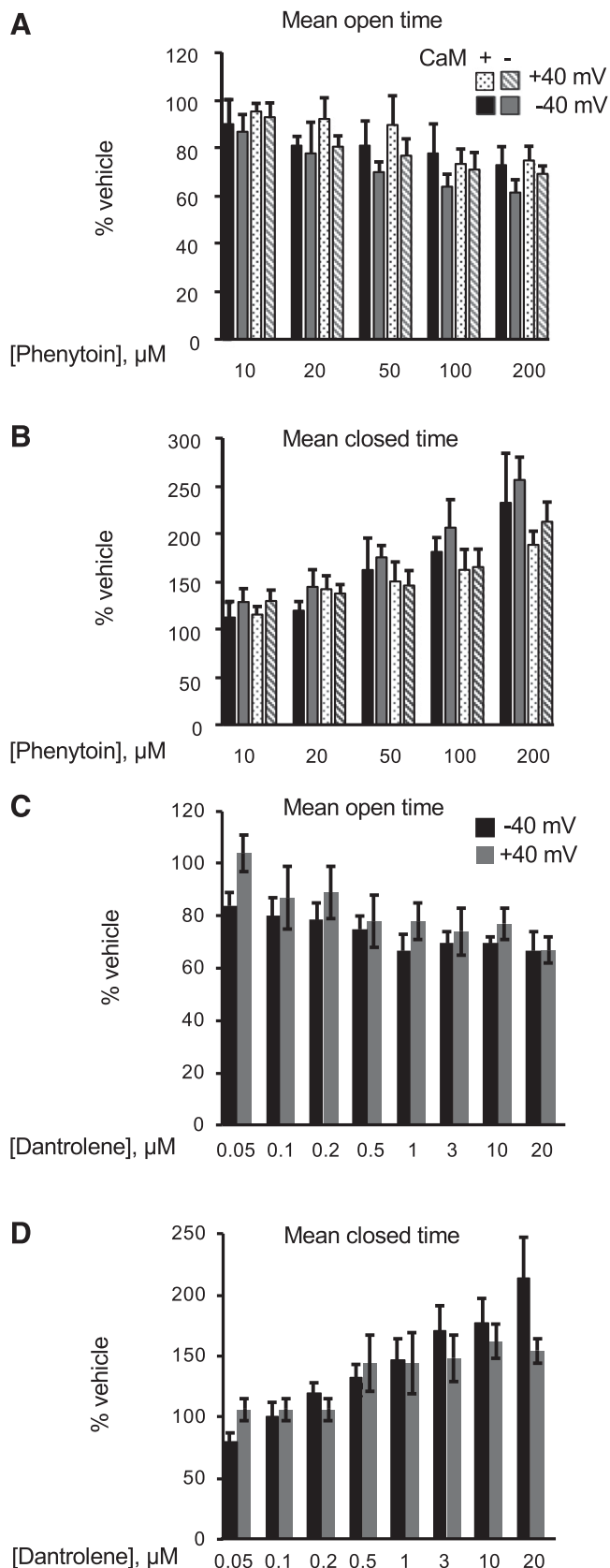
A

B

C

D

Fig. 2. Dwell-time analysis on phenytoin (A and B) and dantrolene inhibition (C and D) of sheep RyR2 channels at $+40$ and -40 mV in the presence and absence of CaM. The cytoplasmic bath contains 0.1 μM Ca^{2+} and 2 mM ATP, and the luminal bath contains 0.1 mM Ca^{2+}. There was no significant difference in open and closed times between all groups for phenytoin ($P > 0.2$, 100 μM vs. zero, presence vs. absence of CaM and $+40$ mV vs. -40 mV, three-way ANOVA) and dantrolene ($P > 0.3$, $+40$ mV vs. -40 mV, two-way ANOVA). Error bars are S.E.M. of n samples; $n = 10$–27 for phenytoin, and $n = 5$–14 for dantrolene.

might explain why phenytoin normalized cardiac rhythms in patients who suffered from ventricular arrhythmias and arrhythmias originating from the atria or atrioventricular node (Conn, 1965; Conn et al., 1967; Rosen et al., 1967; Eddy and Singh, 1969).

A surprising finding of this study was that phenytoin inhibited RyR2 from healthy sheep and failing human hearts but not RyR2 from healthy human hearts. We recently reported the same association for CaM inhibition of RyR2, which resulted from the dependence of CaM inhibition on RyR2 phosphorylation at S2808 and S2814 (Walweel et al., 2019). We previously showed that RyR2 from failing hearts had significant hyper-phosphorylation of S2808 and S2814, a reduced free thiol content, and a reduced interaction with FKBP12.0 and FKBP12.6 compared with healthy hearts (Walweel et al., 2017). Interestingly, sheep RyR2s were also significantly hyper-phosphorylated at S2808 compared with healthy human RyR2, possibly because of higher adrenergic stress in sheep compared with humans prior to heart harvest or because of different adrenergic mechanisms for each species (phosphorylation of S2808 and S2814 are increased by adrenergic stress) (Benkusky et al., 2007; Ferrero et al., 2007). In humans, adrenergic stimulation is predominantly through a stimulatory $G_{s,\alpha}$-protein cAMP-PKA pathway (Kaumann et al., 1999; Molenaar et al., 2000) as opposed to dual $G_{s,\alpha}$-inhibitory G_i-protein pathways in other animals (Zheng et al., 2005). In any case, just how RyR2 phosphorylation, oxidation, or other post-translational modifications affect phenytoin inhibition remains to be determined.

A definitive location of the dantrolene binding site on RyR2 is not known. Labeling of RyR1 peptides has revealed a dantrolene binding site region between aa590 and aa609 on each of the four RyR2 subunits (Paul-Pletzer et al., 2002). Although the corresponding peptide sequence in RyR2 binds dantrolene (aa601–620), dantrolene binding was not detected in the intact RyR2. However, this amino acid sequence is located within 30 Å of the interface of the SPRY splA kinase-ryanodine receptor domain domain of RyR2 and FKBP (Wang et al., 2011; Yuchi et al., 2015). Our finding that both dantrolene and phenytoin inhibition have Hill coefficients of ~1 indicates that their binding to only one of the four putative sites on the RyR2 homotetramer is sufficient for inhibition. The absence of competition between phenytoin and dantrolene inhibition indicates that phenytoin and dantrolene have different binding sites. Separate binding sites are also consistent with a major difference in the actions of phenytoin and dantrolene on RyR2. Namely, phenytoin inhibition is independent of the presence of CaM, whereas dantrolene inhibition is only seen in the presence of CaM.

In spite of different binding sites for phenytoin and dantrolene, our finding that they both inhibit RyR2 from failing hearts and not healthy hearts suggests a common mechanism of action. In RyR2, there is a close proximity (Wang et al., 2007) and interaction between the central domain (residues 2460–2495) and N-terminal domain (residues 590–609) (Yamamoto and Ikemoto, 2002a,b), and disruption (unzipping)
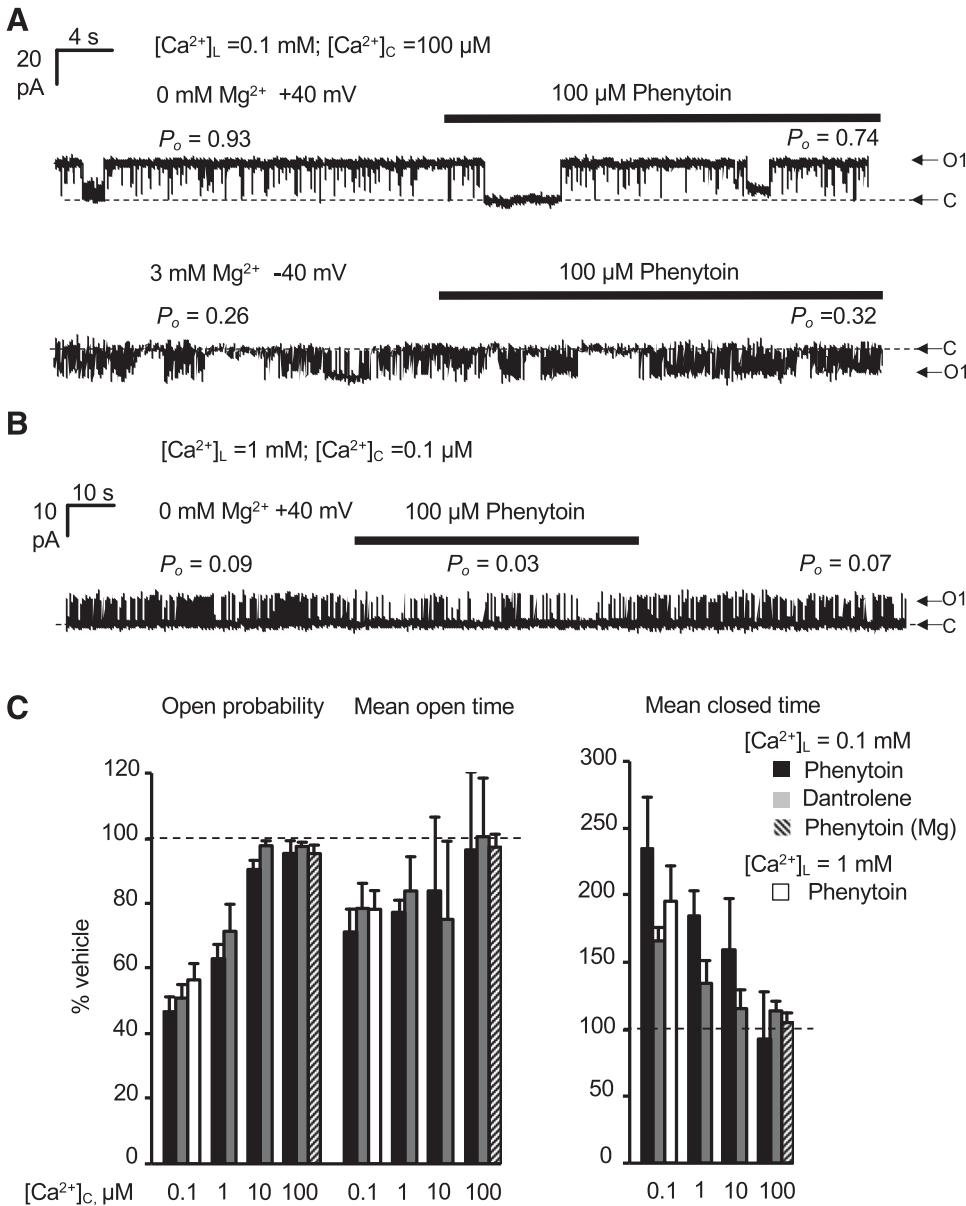
Fig. 3. Phenytoin (100 µM, horizontal bars) and dantrolene (10 µM, + 100 nM CaM) inhibition of sheep RyR2 under different ionic conditions. The cytoplasmic bath contains 0.1 µM Ca^{2+} and 2 mM ATP, and the luminal bath contains 0.1 mM Ca^{2+}. (A) Representative 60-second traces of a channel in 100 µM cytoplasmic Ca^{2+} plus 2 mM ATP (top trace) and with additional 1 mM Mg^{2+} (3 mM $MgCl_2$ + 2 mM ATP, bottom trace). (B) Single-channel recording of phenytoin inhibition (no CaM) in the presence of 1 mM luminal Ca^{2+}. Trace shows 180-second segment of RyR2 activity, showing the lack of action of phenytoin (horizontal bar). Labels indicate current baseline (C) and channel openings (O1) . Open probabilities (P_o) for each bathing condition are given for each trace. (C) Averaged data showing effect of phenytoin and dantrolene on RyR2 open probability and mean open and closed times over a range of cytoplasmic $[Ca^{2+}]$, $[Mg^{2+}]$, and in the presence of 0.1 and 1 mM luminal Ca^{2+}. Error bars are S.E.M. of n samples. For phenytoin, $n = 27, 19, 7, 6$; for dantrolene, $n = 30, 10, 5, 6$ for $[Ca^{2+}]$ (µM) = 0.1, 1, 10, 100, respectively.

of these domain interactions causes malregulation of RyR2 associated with cardiac arrhythmias and heart failure (Ikemoto and Yamamoto, 2002). Dantrolene (1 µM) has been shown to restore the zipped state of RyR2, normalize RyR2 function, and inhibit diastolic SR Ca^{2+} leak in failing heart (Kobayashi et al., 2005, 2009). Within this framework, one can understand why dantrolene and phenytoin can correct the defective interdomain interaction and restore normal RyR2 function (Kobayashi et al., 2009), but they have no effect RyR2 from healthy hearts in which interdomain interactions are already stable. Thus, one could hypothesize that phenytoin, like dantrolene, stabilizes the interdomain interactions and restores the zipped state during diastole in failing hearts.

In order for RyR2 to be a clinically useful target for phenytoin, it would need to be able to readily diffuse across the sarcolemma to reach to the sarcoplasmic reticulum membrane (Milanetti et al., 2016), and it would need to inhibit RyR2 channels with concentrations below those which produce toxic inhibition of other ion channels and transporters. The Caco-2 (human colon

adenocarcinoma) cell monolayer is widely used for the determination of drug intestinal permeability and absorption (Patil et al., 2012). This assay reveals phenytoin to be a highly membrane-permeable drug (Pade and Stavchansky, 1998; Milanetti et al., 2016), which should readily access RyR2 channels in muscle. We show that phenytoin inhibits RyR2 with an IC_{50} ~15 µM, which is substantially lower than its IC_{50} for other muscle ion transporters, such as Na^+ channels [$IC_{50} = 58$ µM (Pincus and Lee, 1973; Yaari et al., 1986; Lang et al., 1993)] and L-type and T-type Ca^{+2} channels [IC_{50} ~100–200 µM (Hasbani et al., 1974; Yatani et al., 1986; Twombly et al., 1988)]. Clinical studies show that therapeutic levels of phenytoin (mean serum concentration of 40–80 µM, 300 mg daily) produces declines in cognitive functions, including performance, concentration, memory, visuomotor functions, and mental speed (Thompson et al., 1981; Andrewes et al., 1986; Pulliainen and Jokelainen, 1994, 1995). These serum concentrations are threefold higher than IC_{50} for phenytoin inhibition of RyR2 channels. Thus, even when account is
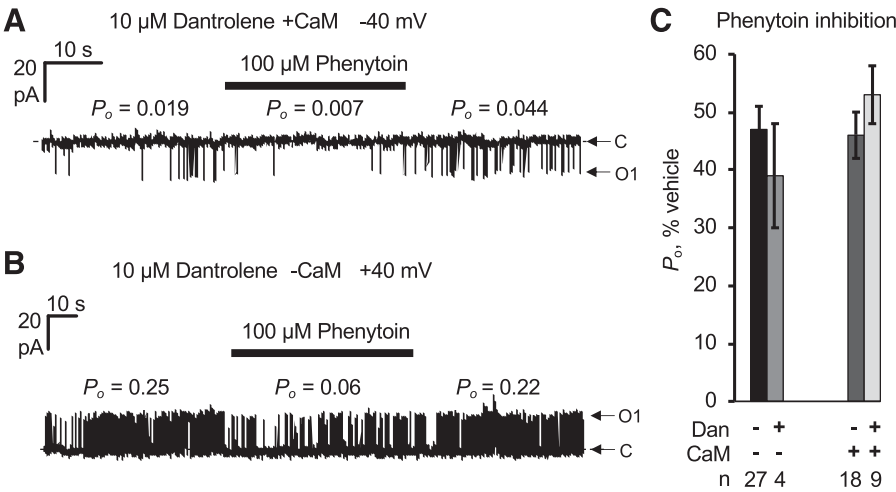
A 10 μM Dantrolene +CaM -40 mV

100 μM Phenytoin

$P_o = 0.019$ $P_o = 0.007$ $P_o = 0.044$

→ C
→ O1

B 10 μM Dantrolene -CaM +40 mV

100 μM Phenytoin

$P_o = 0.25$ $P_o = 0.06$ $P_o = 0.22$

→ O1
→ C

C Phenytoin inhibition

Dan	-	+		-	+
CaM	-	-		+	+
n	27	4		18	9

Fig. 4. Single-channel recordings of phenytoin inhibition in the presence of dantrolene in the presence (A) and absence (B) of CaM. Addition of 100 μM phenytoin is indicated by the horizontal bars. Labels indicate current baseline (C) and single-channel openings (O1). Open probabilities (P_o) for each experimental condition are given. (C) Averaged data showing the relative phenytoin inhibition under four experimental conditions. The cytoplasmic bath contains 0.1 μM Ca^{2+} and 2 mM ATP, and the luminal bath contains 0.1 mM Ca^{2+}. Error bars are S.D. of n experiments.

made for it being a weak acid [pKa 8.06 (Schwartz et al., 1977)], and hence potentially being at a 50% lower concentration intracellularly, it should still be effective as an inhibitor of RyR2 at therapeutic concentrations typically used as an antiepileptic.

A caveat of this study is that, in our measurements, RyR2 are exposed to phenytoin for ~1 minute, whereas phenytoin inhibition of SR Ca^{2+} leak in humans during therapy may occur over days or weeks. Therefore, we cannot rule out further changes in phenytoin effect with maintained exposure.

In summary, we discovered that phenytoin is an inhibitor of RyR2. It acts as a diastolic inhibitor that would reduce Ca^{2+} leak from the SR during diastole without adversely inhibiting SR Ca^{2+} release during systole. Notably, our experiments with human RyR2 indicate that phenytoin inhibits RyR2 from failing human heart and not from healthy heart, suggesting
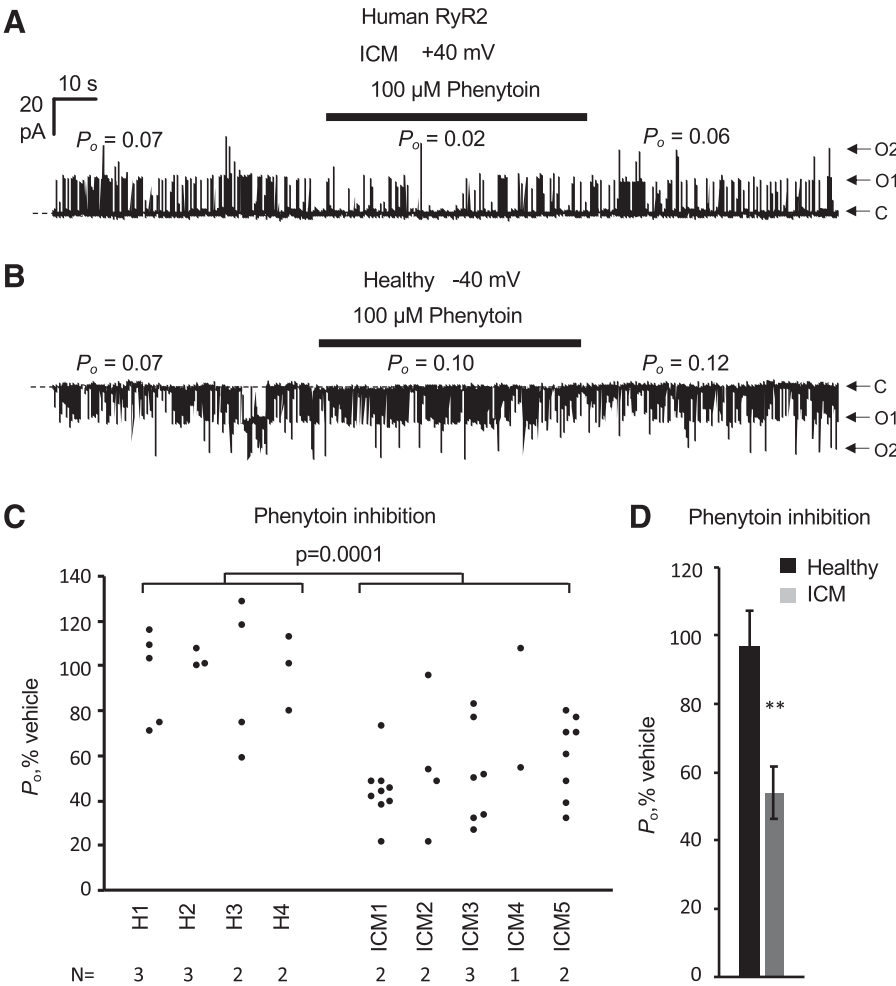
A Human RyR2

ICM   +40 mV

100 μM Phenytoin

$P_o = 0.07$ $P_o = 0.02$ $P_o = 0.06$

→ O2
→ O1
→ C

B Healthy   -40 mV

100 μM Phenytoin

$P_o = 0.07$ $P_o = 0.10$ $P_o = 0.12$

→ C
→ O1
→ O2

C Phenytoin inhibition

p=0.0001

| | H1 | H2 | H3 | H4 | ICM1 | ICM2 | ICM3 | ICM4 | ICM5 |
| N= | 3 | 3 | 2 | 2 | 2 | 2 | 3 | 1 | 2 |

D Phenytoin inhibition

■ Healthy
▨ ICM

**

Fig. 5. Representative 180-second segments of recordings of two channels showing the action of phenytoin on human RyR2 from failing (ICM) hearts (A) and healthy hearts (B). Labels indicate current baseline (C) and openings or one (O1) or two (O2) channels. Scale bars in (A) also apply to (B). Open probabilities (P_o) for each bathing condition are given for each trace. (C) Individual measures of relative inhibition by phenytoin from four healthy (H1–H4) and five failing human hearts (ICM1–ICM5). N = number of independent experiments. $P = 0.0001$ indicates significant difference between four healthy and five ICM hearts using a two-way ANOVA, with each heart nested in heart health status. (D) Average relative inhibition pooled from all human healthy and failing hearts. Error bars are 95% confidence limits and ** indicates a highly significant difference from paired vehicle experiments (i.e., 100%; $P = 2 \times 10^{-12}$, paired t test, $n = 30$). The cytoplasmic bath contains 0.1 μM Ca^{2+} and 2 mM ATP, and the luminal bath contains 0.1 mM Ca^{2+}.

that phenytoin may selectively target defective RyR2 channels in humans. Moreover, phenytoin inhibition of RyR2 is limited to 50% at high doses, which permits a wide therapeutic concentration range. Phenytoin has long been chronically used as an antiepileptic through its action of inhibiting voltage-dependent sodium channels. We demonstrate RyR2 inhibition at doses threefold lower than its IC_{50} for its other known targets in muscle, its therapeutic levels, and levels that produce adverse side effects, pointing to phenytoin as a more human-safe alternative to dantrolene for therapies against heart failure and cardiac arrythmias.

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Authorship Contributions

Participated in research design: Ashna, van Helden, Laver.

Conducted experiments: Ashna.

Contributed new reagents or analytic tools: dos Remedios, Molenaar, Laver.

Performed data analysis: Ashna, Laver.

Wrote or contributed to the writing of the manuscript: Ashna, van Helden, dos Remedios, Molenaar, Laver.

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