

Arrhythmogenic adverse effects of cardiac glycosides are mediated by redox modification of ryanodine receptors

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Non-technical summary Cardiac glycosides (CGs) have been routinely used in the treatment of congestive heart failure (HF). Unfortunately, the therapeutic use of CGs in treating HF is limited by their adverse side effects, including cardiac arrhythmias. The arrhythmic side effects of CGs have been traditionally ascribed to excessive cellular Ca²⁺ retention (Ca²⁺ overload) leading to spontaneous discharges of intracellular Ca²⁺ stores, or Ca²⁺ waves, in turn causing oscillations of the cardiac membrane potential. In the present study, we demonstrate that the proarrhythmic effects of CGs on Ca²⁺ cycling in cardiac myocytes involve alterations in the function of ryanodine receptor calcium channels caused by oxidative changes in the channel structure by reactive oxygen species. Our findings reveal a new mechanism for CG-induced Ca²⁺ waves and suggest a potential target for antiarrhythmic therapy in HF patients treated with CGs.

Abstract The therapeutic use of cardiac glycosides (CGs), agents commonly used in treating heart failure (HF), is limited by arrhythmic toxicity. The adverse effects of CGs have been attributed to excessive accumulation of intracellular Ca²⁺ resulting from inhibition of Na⁺/K⁺-ATPase ion transport activity. However, CGs are also known to increase intracellular reactive oxygen species (ROS), which could contribute to arrhythmogenesis through redox modification of cardiac ryanodine receptors (RyR2s). Here we sought to determine whether modification of RyR2s by ROS contributes to CG-dependent arrhythmogenesis and examine the relevant sources of ROS. In isolated rat ventricular myocytes, the CG digitoxin (DGT) increased the incidence of arrhythmogenic spontaneous Ca²⁺ waves, decreased the sarcoplasmic reticulum (SR) Ca²⁺ load, and increased both ROS and RyR2 thiol oxidation. Additionally, pretreatment with DGT increased spark frequency in permeabilized myocytes. These effects on Ca²⁺ waves and sparks were prevented by the antioxidant *N*-(2-mercapto-propionyl) glycine (MPG). The CG-dependent increases in ROS, RyR2 oxidation and arrhythmogenic propensity were reversed by inhibitors of NADPH oxidase, mitochondrial ATP-dependent K⁺ channels (mito-K_{ATP}) or permeability transition pore (PTP), but not by inhibition of xanthine oxidase. These results suggest that the arrhythmogenic adverse effects of CGs involve alterations in RyR2 function caused by oxidative changes in the channel structure by ROS. These CG-dependent effects probably involve release of ROS from mitochondria possibly mediated by NADPH oxidase.

(Received 1 April 2011; accepted after revision 28 July 2011; first published online 1 August 2011)

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Abbreviations 5-HD, 5-hydroxydecanoate; ALLO, allopurinol; CGs, cardiac glycosides; CsA, cyclosporin A; DADs, delayed afterdepolarizations; DGT, digitoxin; DPI, diphenyliodonium chloride; DTDP, 2,2'-dithiodipyridine; DTT, dithiothreitol; EADs, early afterdepolarizations; EGFR, epidermal growth factor receptor; FCCP, carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone; HF, heart failure; mBB, monobromobimane; mito- K_{ATP} , mitochondrial ATP-dependent K^+ channels; MPG, *N*-(2-mercaptopropionyl) glycine; NCX, Na^+/Ca^{2+} exchanger; NKA, Na^+/K^+ -ATPase; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; PTP, permeability transition pore; ROS, reactive oxygen species; RyR2s, ryanodine receptors; SR, sarcoplasmic reticulum; TMRE, tetramethylrhodamine ethyl ester; XO, xanthine oxidase.

Introduction

Cardiac glycosides (CGs), whose primary target is the Na^+/K^+ -ATPase (NKA), have been commonly used in the treatment of congestive heart failure (HF) (Gheorghide *et al.* 2004). The beneficial impact of CGs has been attributed to positive inotropic effects due to improvements of myocyte Ca^{2+} handling by these reagents. A common view is that inhibition of NKA by CGs results in elevated intracellular $[Na^+]$ which reduces Ca^{2+} extrusion via Na^+/Ca^{2+} exchanger (NCX), thus leading to increased gain of cellular and SR Ca^{2+} and increased myocyte contractility (Bers, 2001; Reuter *et al.* 2002; Altamirano *et al.* 2006). Unfortunately, the therapeutic use of CGs in treating HF is limited by their adverse side effects, including cardiac arrhythmias (Ferrier, 1977). The arrhythmic side effects of CGs have been traditionally ascribed to excessive cellular Ca^{2+} retention (Ca^{2+} overload) leading to spontaneous discharges of intracellular Ca^{2+} stores, or Ca^{2+} waves, in turn causing oscillations of the membrane potential, known as delayed afterdepolarizations (DADs), extra-systolic action potentials and triggered activity (Wier & Hess, 1984; Fujiwara *et al.* 2008; Eisner *et al.* 2009; Weiss *et al.* 2011).

Recently, several groups of investigators have demonstrated that in addition to leading to physiological and pathological changes associated with inhibition of NKA ion transport activity and alterations in ionic balance, binding of CGs to NKA initiates a chain of signalling events that is independent of changes in intracellular $[Na^+]$ and $[Ca^{2+}]$ (Liu *et al.* 2000; Tian & Xie, 2008). In particular, conformational changes on binding of CGs have been reported to initiate a signalling cascade involving the activation of Src kinase and tyrosine phosphorylation of the epidermal growth factor receptor (EGFR) with the concomitant increase in production of reactive oxygen species (ROS) (Tian *et al.* 2003, 2006; Pasdois *et al.* 2007) via mechanisms yet to be fully defined. ROS have been shown to contribute to cardiac arrhythmogenesis and contractile dysfunction through redox modifications of the cardiac Ca^{2+} release channels, or ryanodine receptors (RyR2s), rendering them hyperactive and 'leaky' in various disease settings (Györke & Carnes, 2008; Terentyev *et al.* 2008; Belevych *et al.* 2009; Xie *et al.* 2009). Consequently, the goal of the present study was to test the hypothesis that the arrhythmogenic

adverse effects of CGs involve redox modification of RyR2s resulting from increased production of ROS in cardiac myocytes. In particular, we sought to determine whether exposure of cells to antioxidants reverses the arrhythmogenic effects of CGs on myocyte Ca^{2+} handling and examine the mechanisms of ROS generation by CGs. Our results show for the first time that the arrhythmogenic toxicity of CGs indeed involves alterations in RyR2 function caused by ROS derived from mitochondria.

Methods

Ventricular myocytes from 50 adult LBNF1 male rats (250–300 g) were isolated following standard procedures (Györke *et al.* 1997). Rats were anaesthetized with Nembutal (75 mg kg^{-1} , intraperitoneal injection) and euthanized by exsanguination. All animal procedures were approved by The Ohio State University Institutional Animal Care and Use Committee and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The authors have read, and the experiments comply with, the policies and regulations of *The Journal of Physiology* given by Drummond (2009).

Ca^{2+} imaging and ROS production measurements

Intracellular Ca^{2+} cycling and ROS production in isolated rat ventricular myocytes were monitored by an Olympus Fluoview 1000 confocal microscope using the Ca^{2+} - and ROS-sensitive indicators Fluo-3 and CM- H_2 DCFDA, respectively. The fluorescent probes were excited with the 488 nm line of an argon laser and emission was collected at 500–600 nm for Fluo-3 and 500–560 nm for CM- H_2 DCFDA. Fluo-3 fluorescence was recorded in the line scan mode of the confocal microscope (0.414 μm per pixel, 2 to 5 ms per line). The external, Tyrode solution contained (mM): 140 NaCl, 5.4 KCl, 1 $CaCl_2$, 0.5 $MgCl_2$, 10 Hepes and 5.6 glucose (pH 7.3). Myocytes were paced at 0.3–2 Hz using extracellular platinum electrodes. For Ca^{2+} spark recordings, the cardiac myocytes were permeabilized with saponin (0.01% for 20–30 s). The internal solution contained (mM): 120 potassium aspartate, 20 KCl, 0.81 $MgCl_2$, 1 KH_2PO_4 , 0.5 EGTA

(free $[Ca^{2+}] \sim 50$ nM), 3 MgATP, 10 phosphocreatine, 0.03 Fluo-3 penta potassium salt, 20 Hepes (pH 7.2) and 5 U ml^{-1} creatine phosphokinase. To assess the SR Ca^{2+} load, 20 mM caffeine was applied at the end of the experiments.

Myocyte arrhythmic potential was measured as the number of spontaneous Ca^{2+} waves per second. To test the proarrhythmic effects of CGs, myocytes were exposed to digitoxin (25–100 nM) or ouabain (100 μM). In some experiments, myocytes were pretreated with the reducing reagent and ROS scavenger *N*-(2-mercaptopropionyl) glycine (MPG, 750 μM). To examine the contribution of xanthine oxidase and NADPH oxidase to ROS generation by CGs, we used allopurinol (ALLO, 250 μM) and diphenyliodonium chloride (DPI, 50 μM), respectively. To identify the digitoxin-induced Na^+/K^+ -ATPase cascade, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2, Src family kinase inhibitor), 5-hydroxydecanoate (5-HD, a mito- K_{ATP} channel blocker) and cyclosporin A (CsA, a potent inhibitor of the permeability transition pore in mitochondria) were applied.

RyR2 free thiol content

The content of free thiols in RyR2s was determined using the monobromobimane (mBB) fluorescence method (Terentyev *et al.* 2008). Briefly, samples prepared from isolated myocytes were incubated with 20 mM mBB for 1 h in the dark at room temperature. Subsequently proteins were acetone precipitated and subjected to SDS-PAGE. To examine the effects of DGT on RyR2 redox status, some of the cells were treated with digitoxin (100 nM, 30 min, 37°C). To minimize RyR2 thiol oxidation, some of the samples were incubated with 10 mM dithiothreitol (DTT, reducing agent). As a positive control to maximize RyR2 oxidation, we included 200 μM 2,2'-dithiodipyridine (DTDP, oxidizing agent). mBB fluorescence was normalized to the RyR2 levels quantified using Coomassie Blue staining of gels run in parallel.

Mitochondrial membrane potential measurement

Mitochondrial membrane potential was monitored with a voltage-sensitive fluorescent indicator, tetramethylrhodamine ethyl ester (TMRE). Freshly isolated rat ventricular myocytes were loaded with TMRE (75 nM, 20 min, 37°C) and TMRE fluorescence was measured as a series of X–Y confocal images. TMRE was excited at 543 nm with a helium–neon laser, and the emission signals were collected at 570–650 nm. TMRE fluorescence was normalized to the fluorescence signal obtained in the presence of the mitochondrial uncoupler

carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone (FCCP, 3 μM).

Reagents

All chemicals were obtained from Sigma (St Louis, MO, USA) or Calbiochem (La Jolla, CA, USA) and fluorescent dyes were purchased from Molecular Probes (Eugene, OR, USA).

Statistical analysis

Data are presented as means \pm SEM. Statistical analyses were performed using ANOVA. A level of $P < 0.05$ was accepted as statistically significant.

Results

Disruption of normal Ca^{2+} cycling by DGT

Intracellular Ca^{2+} cycling in paced (at 0.3 Hz) isolated rat ventricular myocytes was monitored in the line scan mode of a confocal microscope using the Ca^{2+} indicator Fluo-3. The effects of DGT at 70 and 100 nM on myocyte Ca^{2+} cycling are illustrated in Fig. 1A and summarized in Fig. 1B–F. Exposure to DGT resulted in significant increases in the amplitude of the Ca^{2+} transients at both 70 and 100 nM (Fig. 1B) without a significant change in the rate of decay of the Ca^{2+} transients (Fig. 1D). At the same time, DGT caused a marked increase in the incidence of arrhythmogenic spontaneous Ca^{2+} waves (SCWs) at 100 nM, although Ca^{2+} wave frequency was unaffected at 70 nM (Fig. 1A and B). The maximal effect of DGT at 100 nM on Ca^{2+} wave frequency was attained within 3–5 min (Supplemental Fig. S1A) and wave frequency was highest at 0.3 Hz pacing rate in the range of 0.3–2 Hz (Supplemental Fig. S1B), conditions used throughout this study. Thus, whereas at 70 nM DGT increased Ca^{2+} transient amplitude without affecting the frequency of Ca^{2+} waves, at 100 nM DGT enhanced both Ca^{2+} transient size and arrhythmogenic propensity.

The positive inotropic and proarrhythmic effects of CGs are generally associated with increased cytosolic and SR Ca^{2+} content. In our experiments, DGT caused no detectable changes in baseline Fluo-3 fluorescence (indexing diastolic $[Ca^{2+}]$) in myocytes lacking Ca^{2+} waves (at 70 nM) or when assessed in areas of line scan images outside of Ca^{2+} waves (at 100 nM) (Fig. 1C). The SR Ca^{2+} content assessed by application of caffeine was significantly reduced by 100 nM DGT, and 70 nM DGT tended to decrease the SR Ca^{2+} content (Fig. 1E). Notably, fractional SR Ca^{2+} release, defined as the ratio of Ca^{2+} transient amplitude to the SR Ca^{2+} content, was increased by DGT at both concentrations (Fig. 1F). Since fractional

release is an indicator of the functional status of the Ca^{2+} release mechanism (Bers, 2001), both the positive inotropic and proarrhythmic effects of DGT in our study appear to arise as a consequence of enhanced functional activity of the SR Ca^{2+} release channels rather than by an increase in the SR Ca^{2+} content.

Clearly, the SR Ca^{2+} content depends on timing of caffeine application with respect to the occurrence of SCWs as Ca^{2+} waves deplete the SR of Ca^{2+} . To examine whether the reduction of the SR Ca^{2+} content by 100 nM

DGT was merely due to SR Ca^{2+} depletion by Ca^{2+} waves, we measured the SR Ca^{2+} content following periods with and without diastolic Ca^{2+} waves. Myocytes were paced at 0.3 Hz. After cessation of pacing, the SR Ca^{2+} content was measured by caffeine application within 10 s during which SCWs occurred in some myocytes but not in others. As expected, the SR Ca^{2+} content was lower following a diastolic Ca^{2+} wave than following an idle diastolic period (Fig. 2A). However, even in the absence of Ca^{2+} waves the SR Ca^{2+} content was lower in DGT-treated myocytes than

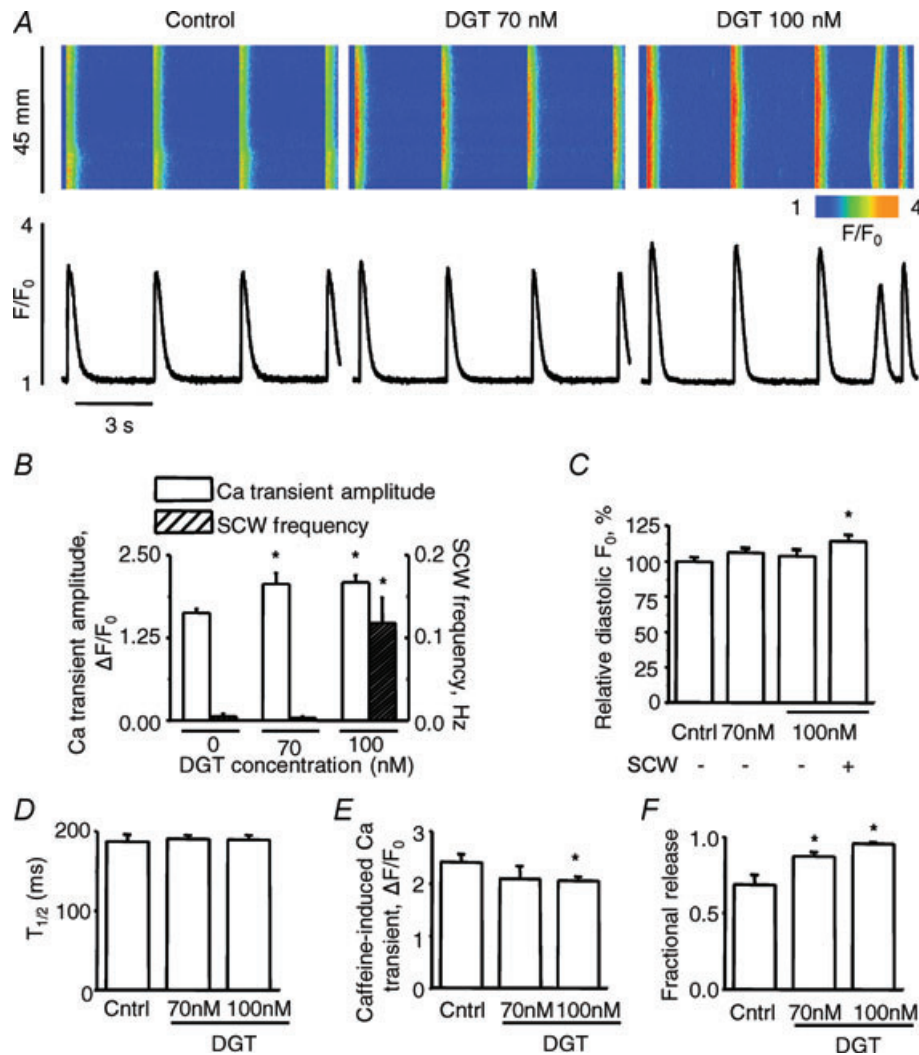


Figure 1. Concentration-, frequency-, and time-dependencies of pro-arrhythmic effects of digitoxin
 A, representative line-scan images (top) and time-dependent profiles (bottom) of spontaneous Ca^{2+} waves (SCWs) under control condition and in the presence of 70 nM digitoxin (DTG) or 100 nM digitoxin, as indicated. B, pooled data for the Ca^{2+} transient amplitude with the frequency of SCWs in control, 70 nM or 100 nM digitoxin. C, bar graph of relative diastolic Fluo-3 fluorescence (F_0) under control condition and in the presence of 70 nM or 100 nM digitoxin. For 100 nM digitoxin, the bars present either total averaged diastolic F_0 or averaged diastolic F_0 excluding image areas containing SCWs as indicated. The values of F_0 represent the mean F_0 obtained during the last 1.5 s of each recording. D, pooled data for the duration of Ca^{2+} transients. E and F, pooled data for the averaged amplitude of caffeine-induced Ca^{2+} transients and fractional SR Ca^{2+} release, calculated as the ratio between the Ca^{2+} transient amplitude and SR Ca^{2+} content under each condition. Data are means \pm SEM from 5 to 43 cells from 2 to 3 heart preparations. * $P < 0.05$ vs. control.

in control myocytes (Fig. 2C). Of note, the amplitude of Ca^{2+} transients was increased in both DGT-treated groups (Fig. 2B) despite the reduced Ca^{2+} loading state of the SR, thus indicating an increase of fractional release (Fig. 2D), similar to the experiments in Fig. 1.

Arrhythmic effects of DGT are prevented by antioxidant pretreatment

To test the possibility that the increased propensity for Ca^{2+} wave generation in DGT-treated myocytes involves ROS, we examined the effects of the antioxidant *N*-(2-mercapto-propionyl) glycine (MPG) on Ca^{2+} cycling. Again, DGT (100 nM) increased the amplitude of Ca^{2+} transients and enhanced the frequency of Ca^{2+} waves (Fig. 3A). Exposure of myocytes to MPG by itself did not result in significant changes in the properties of Ca^{2+} transients, frequency of SCWs or SR Ca^{2+} content (Fig. 3B, C and F). However, as shown in Fig. 3A and B, pretreatment with MPG prevented the increase in SCW frequency by DGT. Notably, MPG did not prevent the increase in Ca^{2+} transient amplitude observed in response to DGT; however, it did avert the decline in the SR Ca^{2+} content caused by DGT, thereby partly normalizing fractional SR Ca^{2+} release (Fig. 3G). These results suggest that the positive inotropic and proarrhythmic effects of DGT in cardiac myocytes involve ROS. Similar results were obtained with another CG, ouabain (Supplemental Fig. S2).

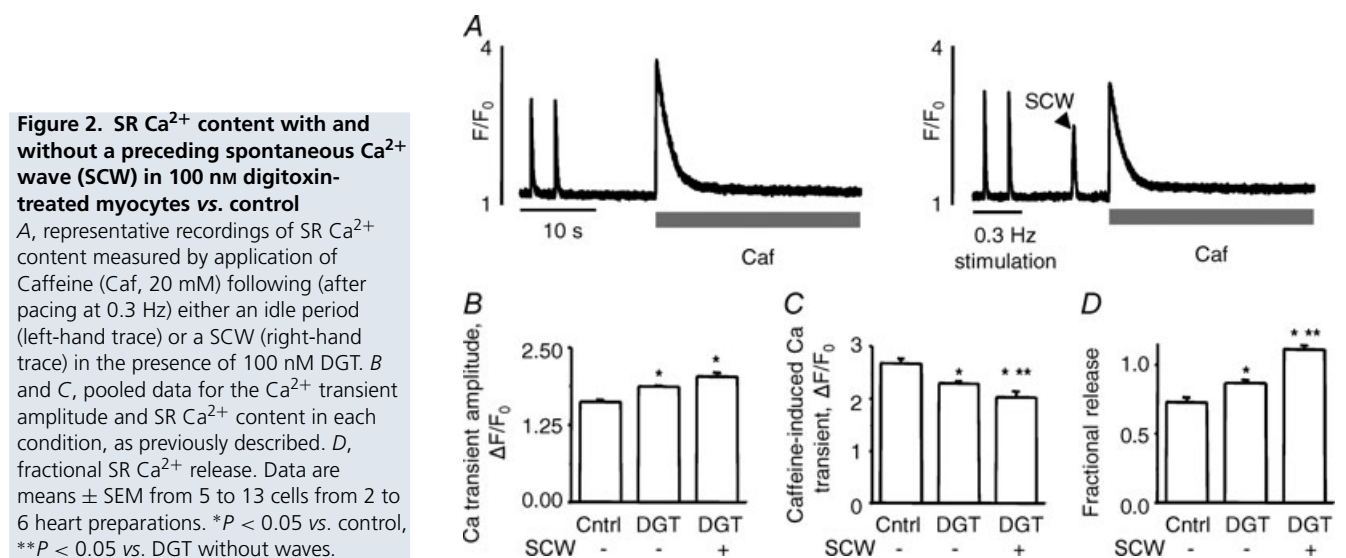
Effects of DGT on Ca^{2+} sparks

To more closely examine the effects of DGT on SR Ca^{2+} release, we performed measurements of Ca^{2+} sparks in saponin-permeabilized myocytes at fixed cytosolic $[\text{Ca}^{2+}]$. In these experiments, myocytes were first exposed to

DGT and then permeabilized with saponin. As shown in Fig. 4A–C, pretreatment with DGT increased the frequency of Ca^{2+} sparks by $\sim 30\%$ and reduced their amplitude by $\sim 15\%$ compared to control; these effects were prevented by MPG. Similar to our observations in intact cells, DGT significantly reduced the SR Ca^{2+} content (by $\sim 15\%$), consistent with the reduced amplitude of Ca^{2+} sparks; this effect was also prevented by MPG (Fig. 4D and E). Thus, as in intact cells, DGT stimulated Ca^{2+} release channel activity in permeabilized cells, in a manner preventable by treatment with the antioxidant MPG. The effects of DGT pretreatment on the properties of Ca^{2+} sparks and SR Ca^{2+} content were mimicked by the oxidizing agent 2,2'-dithiodipyridine (DTDP) (Supplemental Fig. S3). These results further support the notion that DGT affects SR Ca^{2+} release by redox modification of RyR2s.

Increased ROS and RyR2 redox modification caused by DGT

The effects of digitoxin (DGT) on myocyte ROS production were examined using the ROS-sensitive indicator CM- H_2DCFDA . In agreement with previous reports (Xie *et al.* 1999; Liu *et al.* 2000; Tian *et al.* 2003), exposure of myocytes to DGT resulted in a significant increase in the rate of ROS production (Fig. 5). RyR2 contains multiple thiols (Xu *et al.* 1998) that could become modified when myocyte ROS levels are increased. We directly examined the extent of RyR2 modification in myocytes treated with DGT using a mBB fluorescence labelling assay. Based on mBB fluorescence, the fraction of free thiols was indeed decreased significantly in myocytes treated with DGT, indicating increased levels of redox modifications of RyR2 (Fig. 6). The observed DGT-dependent changes in both ROS and RyR2 redox



status were prevented by DPI, an inhibitor of NADPH oxidase, an important ROS generating system in cardiac myocytes (Figs 5 and 6).

Mitochondrial ROS release is involved in DGT-dependent arrhythmogenesis

In addition to NADPH oxidase, the main sources of ROS in myocytes include xanthine oxidase (XO) and mitochondria (Giordano, 2005). To evaluate the potential contribution of these systems to increased ROS production by DGT, we used inhibitors of XO (allopurinol) and mitochondrial ROS release (cyclosporin A, CsA). Whereas

allopurinol failed to produce a significant effect, CsA significantly reduced ROS in DGT-treated myocytes (Fig. 5). In addition, consistent with previous reports (Liu *et al.* 2000; Garlid *et al.* 2003; Tian *et al.* 2003; Pasdois *et al.* 2007), DGT-dependent ROS was significantly inhibited by an inhibitor of mito- K_{ATP} channels, 5-HD, and by an inhibitor of Src kinase, PP2 (not shown). Importantly, these compounds as well as DPI, at the same concentrations at which ROS production was inhibited, also significantly decreased the frequency of DGT-induced SCWs (Fig. 7). These results further support the notion that the increased arrhythmogenic propensity in cardiomyocytes induced by DGT is mediated by ROS. Moreover,

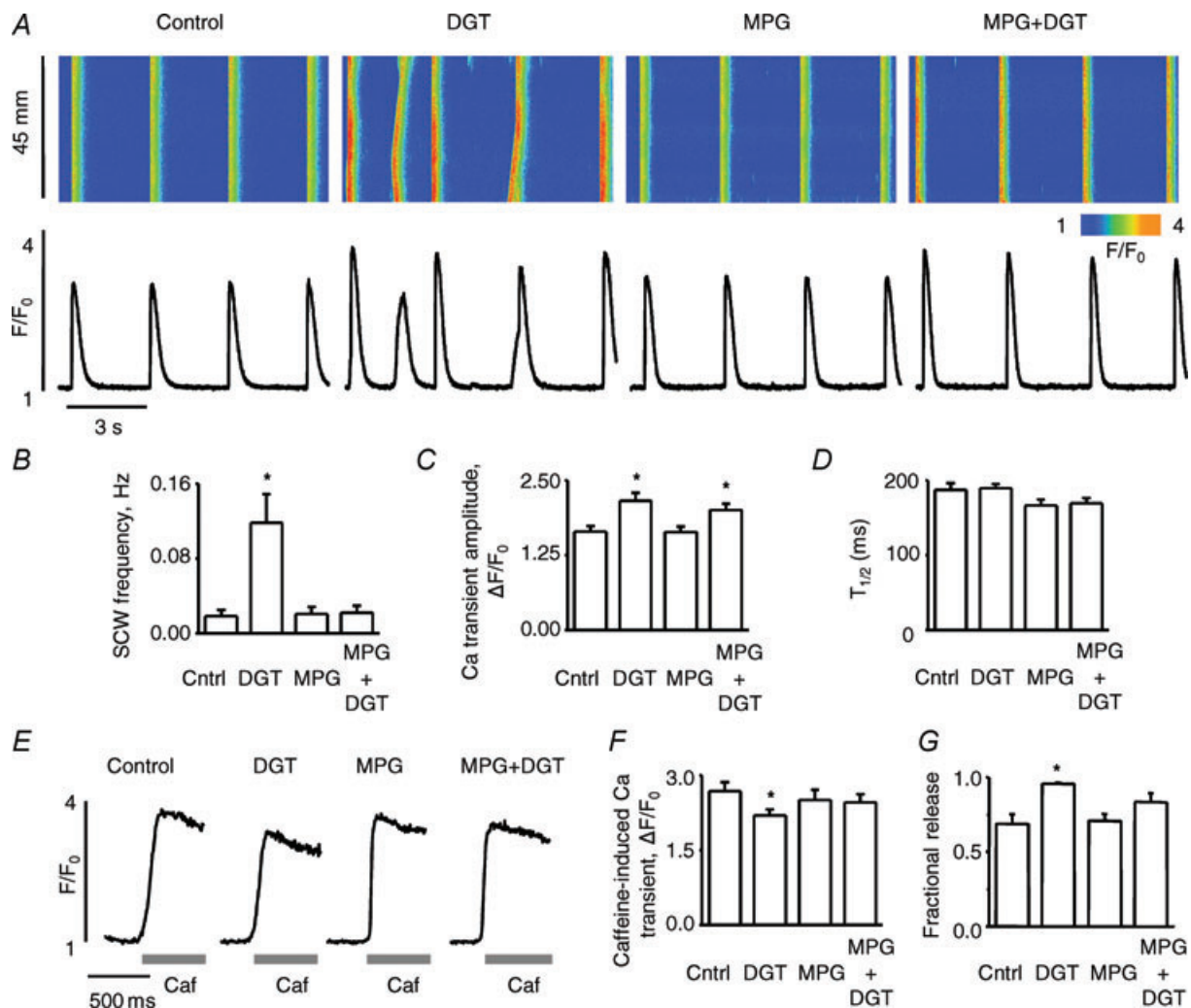


Figure 3. Alterations in Ca^{2+} handling caused by digitoxin and their reversal by the antioxidant, MPG

A, representative line-scan images (top) and time-dependent profiles (bottom) of spontaneous Ca^{2+} waves (SCWs) under control condition and in the presence of 100 nM digitoxin, 750 μ M MPG or 100 nM digitoxin + 750 μ M MPG, as indicated. **B**, pooled data for the frequency of SCWs. **C** and **D**, pooled data for the amplitude and duration, respectively, of Ca^{2+} transients in control, 100 nM digitoxin, 750 μ M MPG or 100 nM digitoxin + 750 μ M MPG, as indicated. **E**, traces of caffeine-induced Ca^{2+} transients under different experimental conditions as indicated at the top. **F**, pooled data for the averaged amplitude of caffeine-induced Ca^{2+} transients under each condition. **G**, bar graph of fractional SR Ca^{2+} release. Data are means \pm SEM from 9 to 31 cells from 6 to 10 heart preparations. * $P < 0.05$ vs. control.

they suggest that the increased generation of ROS by DGT may involve NADPH oxidase and mitochondria but not XO.

To further examine the mechanism of ROS production by CGs, we performed mitochondrial potential measurements using tetramethylrhodamine ethyl ester. As shown in Fig. 8, DGT caused a significant depolarization of the mitochondrial potential, which was prevented by inhibitors of mito- K_{ATP} channels, permeability transition pore and NADPH oxidase, 5-HD, CsA and DPI, respectively. These results provide further evidence for the role of mitochondria in DGT-dependent ROS production and arrhythmogenesis.

Discussion

The main finding of the present study is that the proarrhythmic effects of CGs on Ca^{2+} cycling in cardiac myocytes involve modifications in the RyR2 channel structure by ROS. Furthermore, our results suggest that the CG-induced generation of ROS and proarrhythmic Ca^{2+} waves involve release of ROS from mitochondria which may be mediated by NADPH oxidase. Commonly, spontaneous Ca^{2+} waves in the presence of CGs are attributed to increased cellular accumulation of Ca^{2+}

secondary to the inhibition of the ion transport activity of Na^+/K^+ ATPase. Our findings reveal an alternative mechanism for digitoxin-induced Ca^{2+} waves and suggest a potential target for antiarrhythmic therapy in HF patients treated with CGs.

CG-dependent alterations in myocyte Ca^{2+} signalling involve ROS

Consistent with previous studies of the effects of CGs (Wier & Hess, 1984), DGT increased the amplitude of Ca^{2+} transients without promoting Ca^{2+} waves at relatively low concentrations and increased Ca^{2+} wave frequency (along with enhancing Ca^{2+} transients) at a higher concentration (Fig. 1). Importantly, these stimulatory effects on release were associated with either no change or a decrease in the SR Ca^{2+} content, suggesting that DGT acted on myocyte Ca^{2+} cycling by stimulating RyR2 functional activity rather than through increasing the SR Ca^{2+} load. Increased RyR2 activity was further indicated by increased frequency of Ca^{2+} sparks observed on the backdrop of reduced SR Ca^{2+} content in permeabilized myocytes treated with DGT (Fig. 4). The role of redox modification as a cause of DGT-induced changes in RyR2 function was shown by the following set of results: (1) DGT increased myocyte

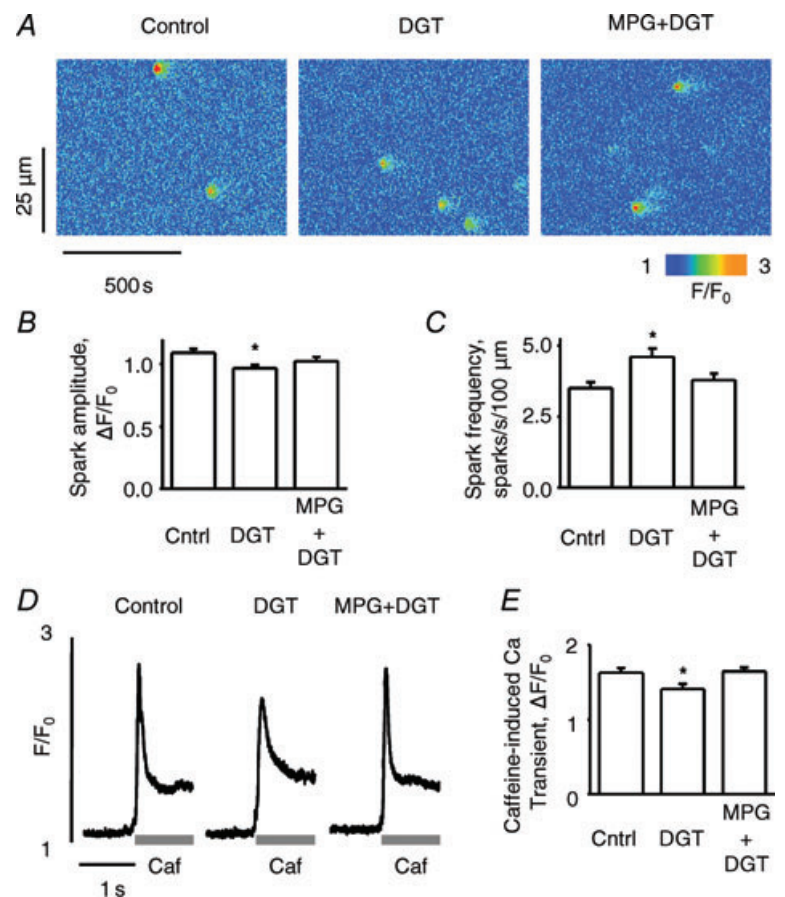


Figure 4. Pretreatment with digitoxin increases the frequency of Ca^{2+} sparks in permeabilized myocytes

A, representative line-scan images of Ca^{2+} sparks in saponin-permeabilized myocytes under control condition and following pretreatment (before permeabilization) with 100 nM digitoxin (DGT) or 100 nM digitoxin + 750 μM MPG, as indicated. B and C, average amplitude and frequency of Ca^{2+} sparks, respectively, for each condition. D, traces of caffeine-induced transients under different experimental conditions as indicated at the top. E, pooled data for the averaged amplitude of caffeine-induced Ca^{2+} transients for each condition. Data are means \pm SEM from 5 to 384 cells from 5 heart preparations. * $P < 0.05$ vs. control.

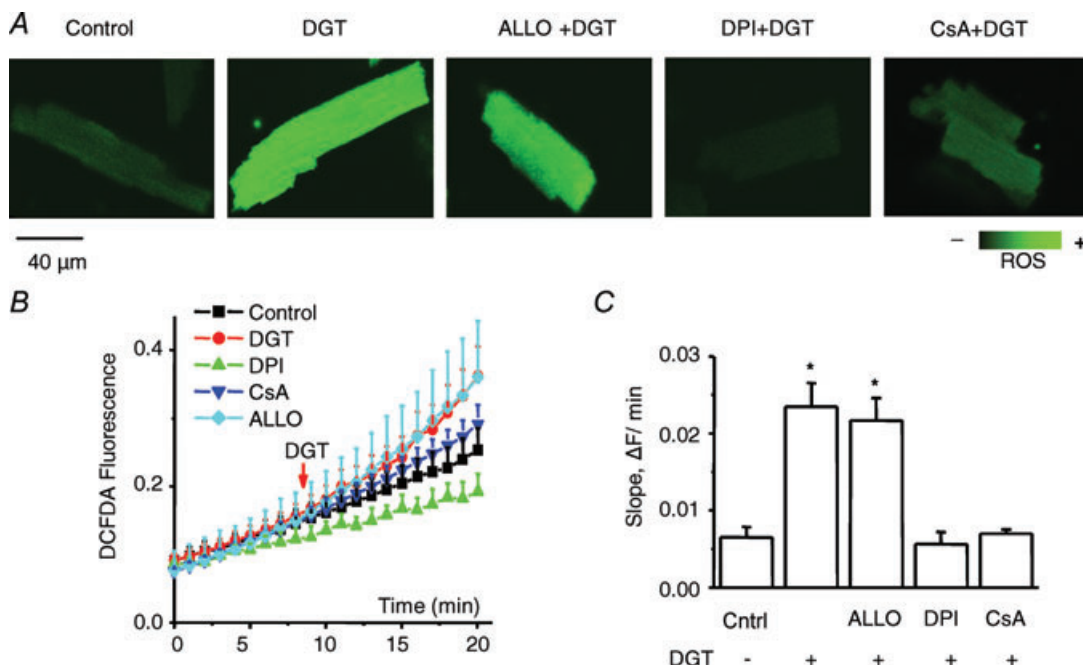


Figure 5. Digitoxin increases ROS in myocytes

A, representative images of ROS generation measured by using the ROS-sensitive dye, CM-H₂DCFDA, for control, 100 nM digitoxin (DGT), 100 nM digitoxin + 250 μM allopurinol (ALLO), 100 nM digitoxin + 50 μM DPI and 100 nM digitoxin + 10 μM CsA, as indicated. B, averaged traces of ROS production in the different groups. C, pooled data for ROS accumulation rates obtained from the slopes in the presence or absence of digitoxin. Data are means ± SEM from 10 to 28 cells from 4 to 8 heart preparations. *P < 0.05 vs. control.

ROS and decreased the level of free, unmodified thiols in RyR2 (Figs 5 and 6); (2) the potentiatory effects of DGT on fractional release and Ca²⁺ waves were reversed by the antioxidant MPG and inhibitors of cellular ROS generating

systems (Fig. 3); and (3) the increased frequency of Ca²⁺ sparks by DGT was observed following the pretreatment and permeabilization of myocytes with the glycoside and was reversed by MPG (Fig. 4); moreover, the effects of DGT on sparks and SR Ca²⁺ content were mimicked by the oxidizing agent DTDP (Supplemental Fig. S3).

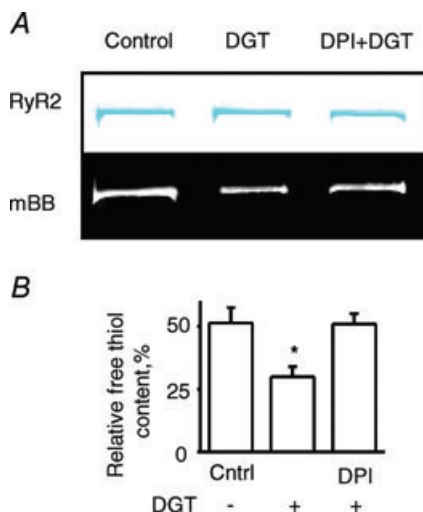


Figure 6. RyR2 thiol oxidation in myocytes by digitoxin

A, representative Coomassie Blue-stained gels (upper panels) and corresponding mBB fluorescence intensity signals (lower panels) of RyR2 from normal, digitoxin-pretreated, and digitoxin-pretreated with DPI myocytes. B, pooled data for free thiol contents normalized by DTT and DTDP. Data are means ± SEM from 6 heart preparations. *P < 0.05 vs. control.

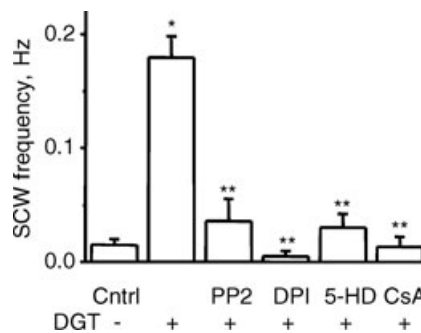
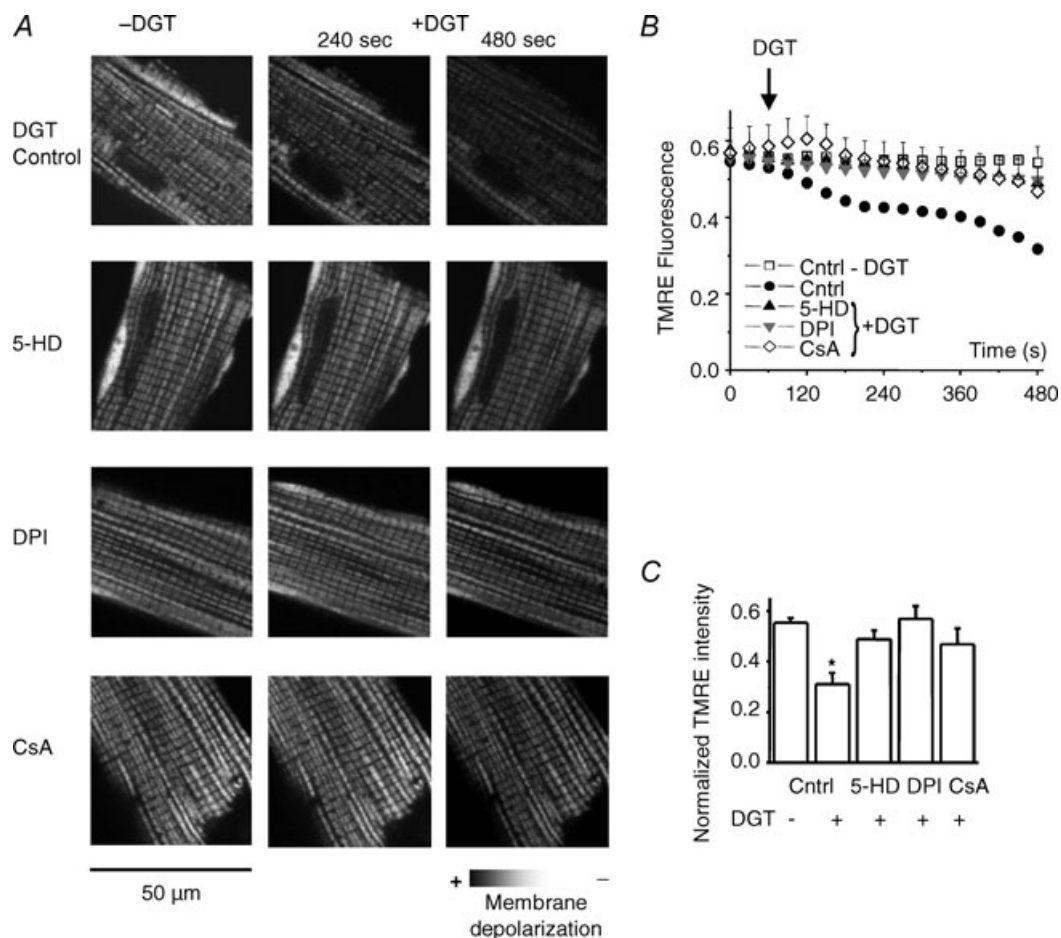


Figure 7. Analysis of the signalling pathways involved in digitoxin-induced increase in myocyte arrhythmic potential

Myocyte arrhythmic potential is assessed as the number of spontaneous Ca²⁺ waves per second in myocytes paced at 0.3 Hz. Pooled data for the frequency of spontaneous Ca²⁺ waves for control, 100 nM digitoxin, and 100 nM digitoxin in the presence of 1 μM PP2, 50 μM DPI, 200 μM 5-HD or 10 μM CsA, as indicated. Data are means ± SEM from 8 to 94 cells from 4 to 10 heart preparations. *P < 0.05 vs. control, **P < 0.05 vs. DGT.

Our present findings are consistent with previous studies that demonstrated that modification of RyR2s by ROS enhances RyR2 functional activity (Boraso & Williams, 1994; Xu *et al.* 1998; Zima & Blatter, 2006; Donoso *et al.*, 2011) and results in increased frequency of spontaneous Ca^{2+} waves and arrhythmogenic electrical events including early or delayed afterdepolarizations (EADs or DADs) in cardiac myocytes (Terentyev *et al.* 2008; Belevych *et al.* 2009; Xie *et al.* 2009). RyR2 thiol oxidation reportedly increases the sensitivity of the channel to luminal Ca^{2+} , thus lowering the critical SR Ca^{2+} content at which spontaneous Ca^{2+} waves occur (Terentyev *et al.* 2008). Spontaneous Ca^{2+} waves and DADs are considered to be the basis of the triggered activity characteristic of CG-induced arrhythmias (Ferrier, 1977; Bers, 2001; Eisner *et al.* 2009).

According to recent evidence, synchronization of Ca^{2+} waves and DADs between individual cardiac cells required for the generation of ectopic action potentials involves temporally aligned SR Ca^{2+} refilling during the Ca^{2+} release–uptake cycle across the myocardium (Wasserstrom *et al.* 2010). Collectively, these results suggest that increased RyR2 function due to redox modification could provide a common mechanism for both the positive inotropic and proarrhythmic effects of CGs in myocytes. Moreover, an excessive SR Ca^{2+} leak could also contribute to the negative inotropic effects of CGs at higher concentrations as hyperoxidation of RyR2 by ROS has been shown to contribute to the increased SR Ca^{2+} leak and depressed cytosolic Ca^{2+} transients in myocytes from failing hearts (Terentyev *et al.* 2008; Belevych *et al.* 2011).



Previously, it has been suggested that CGs enhance SR Ca^{2+} release by acting directly on RyR2 (Rardon & Wasserstrom, 1990; McGarry & Williams, 1993; Sagawa *et al.* 2002; Nishio *et al.* 2004). We believe, however, such direct effects of DGT on RyR2 could not account for most of our results because the DGT-induced increases in the frequency of Ca^{2+} waves and sparks were reversed by an antioxidant treatment (Figs 3 and 4). Nevertheless, our study does not completely rule out the possibility of a direct modulation of RyR2s by CGs which would be expected to complement the stimulatory effects of RyR2 redox modification. Thus, direct action upon RyR2 could explain, at least in part, the residual potentiation of Ca^{2+} transients by DGT in the presence of MPG (Fig. 3). At the same time, it is also possible that RyR2 oxidation contributed to the stimulation of SR Ca^{2+} release by CGs under Na^+ -free conditions, which precludes a mechanism involving alterations of intracellular ionic balances, observed by Nishio *et al.* (2004). Previously, Altamirano *et al.* (2006) reported that ouabain has no effects on frequency and amplitude characteristics of Ca^{2+} sparks when applied directly to permeabilized myocytes. Although this result appears to conflict with direct effects of CGs on RyR2s, the differences between Altamirano *et al.* and our study could be attributed to myocyte permeabilization disrupting the ROS-mediated signalling mechanisms revealed by our experiments. Finally, increased RyR2 phosphorylation by CaMKII could also contribute to the arrhythmogenic effects of CGs (Sapia *et al.* 2010) as a consequence of potential activation of CaMKII either by elevated $[\text{Ca}^{2+}]$ or ROS (Xie *et al.* 2009). However, this possibility seems unlikely for this particular study as RyR2 phosphorylation at the CaMKII phosphorylation site Ser 2814 was not changed following incubation with DGT under our experimental conditions (not shown).

Although glycoside-induced Ca^{2+} waves are commonly attributed to increased Ca^{2+} levels in both extra- and intra-SR compartments, there is a lack of direct experimental evidence for increased SR Ca^{2+} content for either therapeutic or toxic concentrations of CGs, and an elevation in diastolic $[\text{Ca}^{2+}]$ has been shown only at relatively high CG concentrations (Wasserstrom & Aistrup, 2005). In the present study, we found no detectable increases in diastolic Ca^{2+} in areas outside of the propagating Ca^{2+} waves in DGT-treated myocytes (Fig. 1), suggesting that the baseline cytosolic Ca^{2+} was not considerably increased by DGT when assessed independently of SR-derived Ca^{2+} . Moreover, the SR Ca^{2+} content was, in fact, reduced in the presence of the drug (Fig. 1E and F) (or unchanged at lower, purely positive inotropic concentrations of DGT). The reduction in the SR Ca^{2+} content by proarrhythmic concentrations of DGT is attributable to increased diastolic SR Ca^{2+} release in the form of spontaneous Ca^{2+} waves and sparks.

Additionally, oxidation-dependent inhibition of SERCA2 activity (Kennedy *et al.* 2006) could contribute to the decreased SR Ca^{2+} by CGs.

Mechanism of CG-dependent ROS generation

Our findings are also supported by previous reports (Liu *et al.* 2000; Tian *et al.* 2003; Pasdois *et al.* 2007) showing that CGs increase ROS production in cardiac myocytes. While the mechanisms mediating increased ROS remain to be clarified, several laboratories have demonstrated that they involve signalling cascades that are independent of the changes in intracellular Na^+ or Ca^{2+} concentrations (expected as a consequence of inhibition of the activity of NKA) (Tian & Xie, 2008). In particular, it has been established that binding of ouabain to NKA results in the initiation of signalling cascades that involve the activation of Src kinase and opening of the mito- K_{ATP} channels, ultimately leading to increased myocyte ROS (Tian *et al.* 2003, 2006; Pasdois *et al.* 2007). Consistent with these studies, inhibitors of Src and mito- K_{ATP} channels reversed arrhythmogenesis in myocytes in the presence of DGT (Fig. 7). Moreover, DGT caused mitochondrial membrane depolarization, which was prevented by inhibition of mito- K_{ATP} channels and mitochondrial permeability transition pore (Fig. 8), thus further supporting the role of mitochondria in DGT-dependent ROS increase and arrhythmogenesis.

Considering the nearly complete prevention of DGT-dependent ROS, mitochondrial depolarization and arrhythmogenesis by inhibitors of mitochondrial PTP and mito- K_{ATP} , it appears that most of the bioactive ROS under our experimental conditions is derived from the mitochondria. The main potential sources of ROS in cardiac myocytes in addition to mitochondria include XO and NADPH oxidase (Giordano, 2005). While inhibition of XO had no significant effects on ROS, the general NADPH oxidase inhibitor DPI markedly inhibited both ROS and SCWs in DGT-treated cells (Figs 5, 6 and 7). One possible explanation for this result is that mitochondrial ROS release is caused by a relatively small level of ROS supplied by NADPH oxidase. Thus, DPI in addition to inhibiting NADPH oxidase would also prevent the release of ROS from mitochondria. This interpretation is compatible with the concept of ROS-induced ROS generation (Brady *et al.* 2006; Zorov *et al.* 2006) according to which ROS can induce mitochondrial depolarization and subsequent ROS release through mitochondrial PTP in cardiac myocytes. In support of this possibility, NADPH oxidase has been demonstrated to play a role in angiotensin II-mediated cardiac protection by causing mitochondrial ROS release that was sensitive to 5-HD (Kimura *et al.* 2005).

While suggesting a new mechanism for glycoside-dependent arrhythmogenesis, our results

do not rule out a potential role for the classical mechanisms involving changes in cytosolic Na^+ and Ca^{2+} concentrations brought about by inhibition of NKA ion transport activity. Indeed, although not observed in our study, increased diastolic $[\text{Ca}^{2+}]$ resulting from inhibition of the NKA by CGs is likely to contribute to arrhythmogenesis by stimulating RyR2s at high drug concentrations. Additionally, CG-induced elevations in cytosolic $[\text{Ca}^{2+}]$ could facilitate ROS generation by activation of PKC with subsequent stimulation of NADPH oxidase. Thus, PKC could present a site for integration of Ca^{2+} -dependent and Ca^{2+} -independent pathways in activation of NADPH oxidase and ROS production. Additionally, mitochondrial ROS production by CGs could be facilitated by increased mitochondrial Ca^{2+} load and subsequent effects on electron transport chain and mitochondrial NOS (Dedkova & Blatter, 2009). Alternatively, elevated cytosolic $[\text{Na}^+]$ caused by CGs could contribute to increased ROS accumulation by impairing mitochondrial energetic and redox balance via activation of mitochondrial $\text{Na}^+/\text{Ca}^{2+}$ exchanger and blunting mitochondrial Ca^{2+} accumulation (Liu *et al.* 2010).

Limitations

The widely used pharmacological agents DPI, 5-HD and CsA were employed to inhibit NADPH oxidase, mito- K_{ATP} and PTP, respectively. Characteristic of experimentation with pharmacological inhibitors, we cannot rule out the possibility of non-specific and secondary effects in the present study, including inhibition by DPI of mitochondrial complex I (Li & Trush, 1998).

Conclusions

In conclusion, our study shows that the arrhythmogenic effects of CGs on Ca^{2+} cycling in cardiac myocytes involve alterations in RyR2 function caused by oxidative changes in the channel structure by ROS. Furthermore, our data suggest that the CG-dependent ROS is likely to involve release of ROS from the mitochondria possibly mediated by NADPH oxidase. Targeting ROS-dependent alterations in RyR2 function may present a strategy for enhancing the utility of CGs in treating HF.

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Author contributions

H.-T.H, C.A.C, D.T. and S.G. contributed to the conception, design, data interpretation and manuscript preparation. H.-T.H, S.C.W.S and R.T were responsible for experimental work. H.-T.H contributed to data collection and analysis. All authors approved the final version of the manuscript.

Acknowledgements

This work was supported by National Institutes of Health grants (HL074045 and HL063043 to S.G.).