

Subcellular Mechanisms Underlying Digitalis-Induced Arrhythmias: Role of Calcium/Calmodulin-Dependent Kinase II (CaMKII) in the Transition from an Inotropic to an Arrhythmogenic Effect



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Cardiotonic glycosides or digitalis are positive inotropes used in clinical practice for the treatment of heart failure, which also exist as endogenous ligands of the Na^+/K^+ ATPase. An increase in the intracellular Ca^{2+} content mediates their positive inotropic effect, but has also been proposed as a trigger of life-threatening arrhythmias. Although the mechanisms involved in the positive inotropic effect of these compounds have been extensively studied, those underlying their arrhythmogenic action remain ill defined. Recent evidence has placed posttranslational modifications of the ryanodine receptor (RyR2), leading to arrhythmogenic Ca^{2+} release, in the centre of the storm. In this review we will examine, in depth, the mechanisms that generate the arrhythmogenic substrate, focussing on the role played by the RyR2 and how its CaMKII-dependent regulation may shift the balance from an inotropic to an arrhythmogenic Ca^{2+} release. Finally, we will provide evidence suggesting that stabilising RyR2 function could result in a potential new strategy to prevent cardiotonic glycoside-induced arrhythmias that could lead to a safer and more extensive use of these compounds.

Keywords

Cardiotonic glycosides • CaMKII • RyR2 • Arrhythmia • Calcium handling

Basic and Clinical Relevance of Cardiotonic Glycosides

Cardiotonic glycosides (CGs) are a group of secondary metabolites of plants belonging to the genus *Digitalis*. These compounds selectively bind to and inhibit the sarcolemmal Na^+/K^+ -ATPase (NKA) leading to changes in Ca^{2+} homeostasis which result in increased myocardial contractility. For this reason, CGs have been used as inotropes for the treatment of heart failure (HF) over the last 200 years [1–3]. The relevance of these compounds exceeds their pharmacological

interest given that circulating endogenous CGs, which are structurally, biologically, and immunologically indistinguishable from the plant-derived digitalis have been described [4]. More importantly, endogenous CGs, such as the ouabain-like factor, have been shown to be elevated in the transition from cardiac hypertrophy to heart failure [5,6] and to have a central role in the development of essential hypertension [7,8].

The use of CGs for the treatment of HF has been debated for a long time given their narrow therapeutic range, determined by their toxic effects, which include an enhanced

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propensity for arrhythmias [1,2]. A remarkable step in the knowledge of the clinical role of CGs came from the publication of the Digitalis Investigation Group trial, which involved patients with chronic heart failure and in sinus rhythm [9]. This study demonstrated that, although the clinically used CG, digoxin, did not show significant effects on mortality, it was effective at reducing the hospitalisations derived from HF worsening. While this study did not find differences in the incidence of hospitalisation caused by ventricular arrhythmias or cardiac arrest, differences in “mortality for other cardiac causes”, which could include deaths associated with arrhythmias, were observed. Consistently, the AFFIRM trial showed that digoxin therapy was associated with higher cardiovascular and arrhythmic mortality [10]. Indeed, the incidence of CG-induced arrhythmias is not well defined. However, an incidence of 10% at a level of 1.7 ng/mL of digoxin has been suggested [11]. In addition, a remarkable observation is that CGs have been shown to predispose for the development of ventricular arrhythmias during ischaemic episodes [12,13].

At present, the use of digoxin has decreased considerably but it remains in the current guidelines for the treatment of patients with atrial fibrillation or to improve symptoms and haemodynamics of patients with systolic dysfunction [3,14].

The interest in outlining the beneficial and deleterious effects of CGs on cardiac function has grown over the years and multiple reports from our, and other, laboratories have emerged delineating the mechanisms involved in the inotropic and arrhythmic effects of these compounds. In the following sections of this review we will summarise the basic mechanisms that explain the CG-induced positive inotropic effect and examine, in depth, the events that occur at the cardiac myocyte level that generate the substrate for the development of arrhythmias.

Mechanisms Underlying the CG-induced Positive Inotropic Effect

In cardiac muscle, a rise in intracellular Ca^{2+} couples membrane depolarisation with contraction in a process termed excitation-contraction coupling (ECC). The rise in Ca^{2+} involves Ca^{2+} influx through voltage-dependent L-type Ca^{2+} channels which triggers Ca^{2+} release from the intracellular Ca^{2+} store, the sarcoplasmic reticulum (SR), via opening of the ryanodine receptors (RyR2).

During diastole, RyR2 are closed and low cytosolic Ca^{2+} concentration is restored mainly by its active pumping into the SR by the SR Ca^{2+} ATPase (SERCA), and by its extrusion to the extracellular space through the sarcolemmal $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX).

The electrogenic NCX extrudes one Ca^{2+} in exchange for three Na^+ ions using the electrochemical Na^+ gradient which is maintained by the NKA, an oligomeric membrane protein that catalyzes the coupled active transport of Na^+ and K^+ across the plasmalemma of most mammalian cells.

CGs bind to the NKA inhibiting the transport of Na^+ and K^+ across the plasmalemma, resulting in an elevation of the intracellular Na^+ concentration which affects NCX function by reducing the driving force for Ca^{2+} extrusion. Given that the NCX represents the main route for Ca^{2+} extrusion from the cardiac myocyte, NKA inhibition promotes the accumulation of cytosolic Ca^{2+} leading to an increase in SR Ca^{2+} load. Thereby, more Ca^{2+} stored in the SR allows for greater Ca^{2+} release upon stimulation, resulting in a positive inotropic effect [15].

Interestingly, additional mechanisms have been suggested to contribute to the positive inotropic effect of CGs, among these, direct actions of these compounds on RyR2 function, which result in increased SR Ca^{2+} release have been reported [16]. Nevertheless, whether cardiac glycosides can directly affect RyR2 activity in intact myocytes is controversial. Nishio et al. reported that the CG, ouabain, exerts positive inotropic effects even in Na^+ free conditions (independently of NKA inhibition), thus suggesting direct RyR2 Ca^{2+} sensitisation [17]. However, Altamirano et al. showed that the positive inotropic effect of several CGs, including digoxin, was completely prevented by removal of extracellular Na^+ [15]. Nevertheless, evidence indicating that altered RyR2 sensitivity can only cause a transitory positive inotropic effect [18] suggests that direct RyR2 actions of CGs would not be the primary mechanism responsible for the CG-induced positive inotropic effect.

Delayed-after Depolarisations Initiate Life-threatening Arrhythmias During CG Toxicity

Besides bradycardia and parasympathetic-like alterations in heart rhythm promoted by CGs [2], there are arrhythmias such as ventricular tachycardia/fibrillation that, although infrequent, can compromise heart function and promote sudden death during CGs toxicity [2,4]. The development of these arrhythmias depends on the generation of ectopic foci of discharge from groups of cardiac myocytes or purkinje fibres which arise from transient depolarisations in their membrane potential, termed delayed-after depolarisations or DADs [19].

At the cellular level, the development of DADs occurs when Ca^{2+} from the SR is spontaneously released during diastole in the form of a Ca^{2+} wave and subsequently extruded by the forward mode of electrogenic NCX which generates a depolarising current (Iti or transient inward current) [20]. These depolarizing currents, when sufficiently large, lead to DADs that may reach threshold and trigger spontaneous action potentials, which can originate extrasystoles and ventricular arrhythmias [21]. Taking into account the proposed sequence of events which culminate in arrhythmias at the tissular level, we will further discuss the mechanism of diastolic spontaneous SR Ca^{2+} release (SR Ca^{2+} leak), as a trigger of DADs in cardiac ventricular myocytes.

Sarcoplasmic Reticulum Ca^{2+} Leak as the Arrhythmogenic Event in Digitalis Toxicity

SR Ca^{2+} leak through RyR2 is known to reduce cardiac contractility, impair relaxation and promote arrhythmias [20]. Indeed, enhanced spontaneous SR Ca^{2+} release has been observed in different pathological situations such as heart failure [22,23], atrial fibrillation [24] and congenital arrhythmias [25].

The magnitude of SR Ca^{2+} leak depends on two main factors: 1) SR Ca^{2+} load and 2) RyR2 open probability. In this regard, the arrhythmic effect of NKA inhibition has been proposed to occur when Ca^{2+} entry via the NCX increases SR Ca^{2+} load until Ca^{2+} storage capacity is exceeded, so that oscillations of release-uptake cycles arise to re-establish the Ca^{2+} equilibrium between the cytosol and the SR [26]. These oscillations would result in diastolic Ca^{2+} release to cytosol, which as mentioned previously, would be extruded by the NXC generating the depolarising current that leads to the development of arrhythmogenic DADs. However, several lines of evidence suggest that increased SR Ca^{2+} load, in itself, is not sufficient to promote arrhythmogenic SR Ca^{2+} release. For example, phospholamban (PLN) knock-out mice, which have a fully loaded SR, have not proven to be prone to arrhythmias under basal conditions [27,28]. Thus, in addition to an increase in SR Ca^{2+} load, an increase in RyR2 open probability would also be required to enhance SR Ca^{2+} leak, generating the substrate for triggering Ca^{2+} waves, DADs and eventually arrhythmias in the context of digitalis toxicity.

In the following section of this review we will present the available evidence showing that altered RyR2 function may be at the basis of digitalis-induced arrhythmias.

Effect of Digitalis on RyR2 Function

Posttranslational modifications such as phosphorylation and oxidation have been shown to affect RyR2 function and its open probability.

There are at least two phosphorylatable sites on the RyR2 which have been related to a proarrhythmogenic increase in the open probability of the channel, Serine (Ser) 2808 and Ser2814 [23,29]. Ser2808 is targeted by Protein Kinase A (PKA) which is the intracellular mediator of the β -adrenergic stimulus. However, several studies have demonstrated that CGs do not induce phosphorylation of Ser2808 [30,31]. Ser2814 is phosphorylated by CaMKII, which is a central signalling molecule that senses cytoplasmic Ca^{2+} levels and regulates Ca^{2+} homeostasis.

This kinase contains a regulatory domain that controls activation of the enzyme, and a catalytic domain that associates with substrates and performs the kinase function. Under resting conditions, the regulatory and catalytic domains are closely associated, blocking substrate binding and resulting in auto-inhibition of the kinase. If intracellular

Ca^{2+} concentration rises, calcified Calmodulin ($\text{Ca}^{2+}/\text{CaM}$) binds to CaMKII at the regulatory domain. $\text{Ca}^{2+}/\text{CaM}$ binding disrupts the association of the regulatory and catalytic domains, causing a conformational shift that relieves auto-inhibition and activates the kinase [32].

A role of CaMKII in DAD formation has been previously described by Wu et al., reporting that CaMKII triggers an NCX-dependent arrhythmogenic transient inward current through its effect on SR Ca^{2+} release [33]. Given the increase in $[\text{Ca}^{2+}]_i$ promoted by NKA inhibition it is conceivable that CaMKII could be involved in at least part of the actions of CG on Ca^{2+} handling and arrhythmogenesis. Indeed, we demonstrated that CGs activate CaMKII in rodent hearts treated with low non-toxic doses of ouabain administrated chronically or high-toxic doses administrated acutely [34,35]. Moreover, we showed that CaMKII inhibition is able to prevent ouabain- and digoxin-induced apoptosis [34] and spontaneous contractile activity in isolated ventricular myocytes and arrhythmias in mice [35]. In this context, CaMKII activation is associated with the phosphorylation of the RyR2 at site Ser2814 and phospholamban (PLN), the regulatory protein associated with the SR Ca^{2+} pump, SERCA, at site Thr17.

Targeting these proteins CaMKII would promote an increase in both SR Ca^{2+} load and the sensitivity of the RyR2 for Ca^{2+} release [36]. Nevertheless, our results demonstrate that although ouabain enhances PLN phosphorylation, which would increase SERCA activity and favour SR Ca^{2+} load, this phosphorylation is not required for the digitalis-induced increase in SR Ca^{2+} load [35] which would occur simply by the Na^+ overload-dependent activation of the reverse mode NCX. Interestingly, Fig. 1 shows that although CaMKII inhibition can prevent CG-induced arrhythmias it does not affect their positive inotropic effect. These results are consistent with those using the more specific CaMKII inhibitor, AIP, and with those using myocytes from mice over-expressing the inhibitory protein AIP targeted to the SR (SR-AIP mice). In contrast, CG-induced arrhythmias could not be prevented by KN92, the inactive analog of KN93 [35].

The fact that CG-induced SR Ca^{2+} loading occurs independently of CaMKII activation may explain why we, and others, have observed that CaMKII inhibition does not affect their positive inotropic effect [31,35]. We propose that similar to other models of Ca^{2+} -leak/DAD-triggered arrhythmias [37,38], the increase in SR Ca^{2+} load (which occurs independently of CaMKII activity) is necessary but not sufficient for the initiation of arrhythmogenic Ca^{2+} waves. More likely, the increase in SR Ca^{2+} load, together with an increase in RyR2 opening probability resulting from RyR2 post-translational modifications would be the required scenario for the generation of the arrhythmogenic substrate. These results support the role of the RyR2 as a key target in digitalis-induced arrhythmias.

The other mechanism that is known to modulate RyR2 opening is its oxidation at the level of specific methionine residues [39,40]. RyR2 contains multiple thiols [41] that can be affected by redox modification. RyR2 thiol oxidation increases the sensitivity of the channel to luminal Ca^{2+} , thus

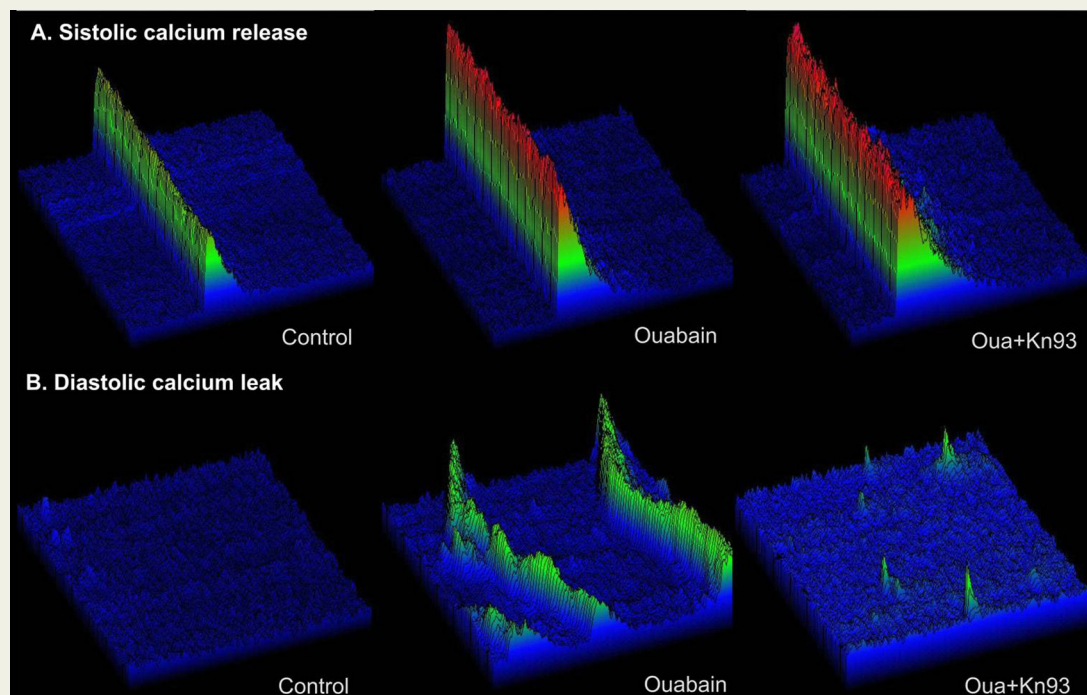


Figure 1 3D reconstruction of representative $[Ca^{2+}]$ -sensitive Fluo-3 fluorescence, obtained in the absence or presence of either ouabain alone or ouabain plus the CaMKII inhibitor KN93 **A:** typical image of electrically stimulated Fluo-3 transients showing systolic Ca^{2+} release. Note that KN93 fails to prevent the ouabain-induced increase in the Ca^{2+} transient. **B:** typical no-stimulated Fluo-3 fluorescence showing that ouabain enhances diastolic Ca^{2+} release and that this increase is prevented by KN93.

lowering the critical SR Ca^{2+} content at which spontaneous Ca^{2+} release occurs [40]. Consistent with this mode of regulation, Ho et al. demonstrated, in cardiac myocytes, that the pro-arrhythmic effects of CGs include alterations in RyR2 function caused by oxidative changes [42]. Clearly, this mechanism would require CGs to promote an increase in ROS production. Indeed, the mechanism for digitalis-induced ROS production was previously described by several groups which have demonstrated that after binding, CGs are able to promote conformational changes of NKA that activate intracellular signalling cascades that are independent of changes in Na^+ and Ca^{2+} homeostasis [43,44]. These cascades have been shown to involve the activation of Src, epidermal growth factor receptor (EGFR) and phosphoinositol kinase 3 (PI3K), culminating in ROS production via NADPH Oxidase 2 (NOX2) [36–38] which could serve as a trigger for further release of ROS from the mitochondria, by a mechanism termed “ROS-induced ROS release” [45].

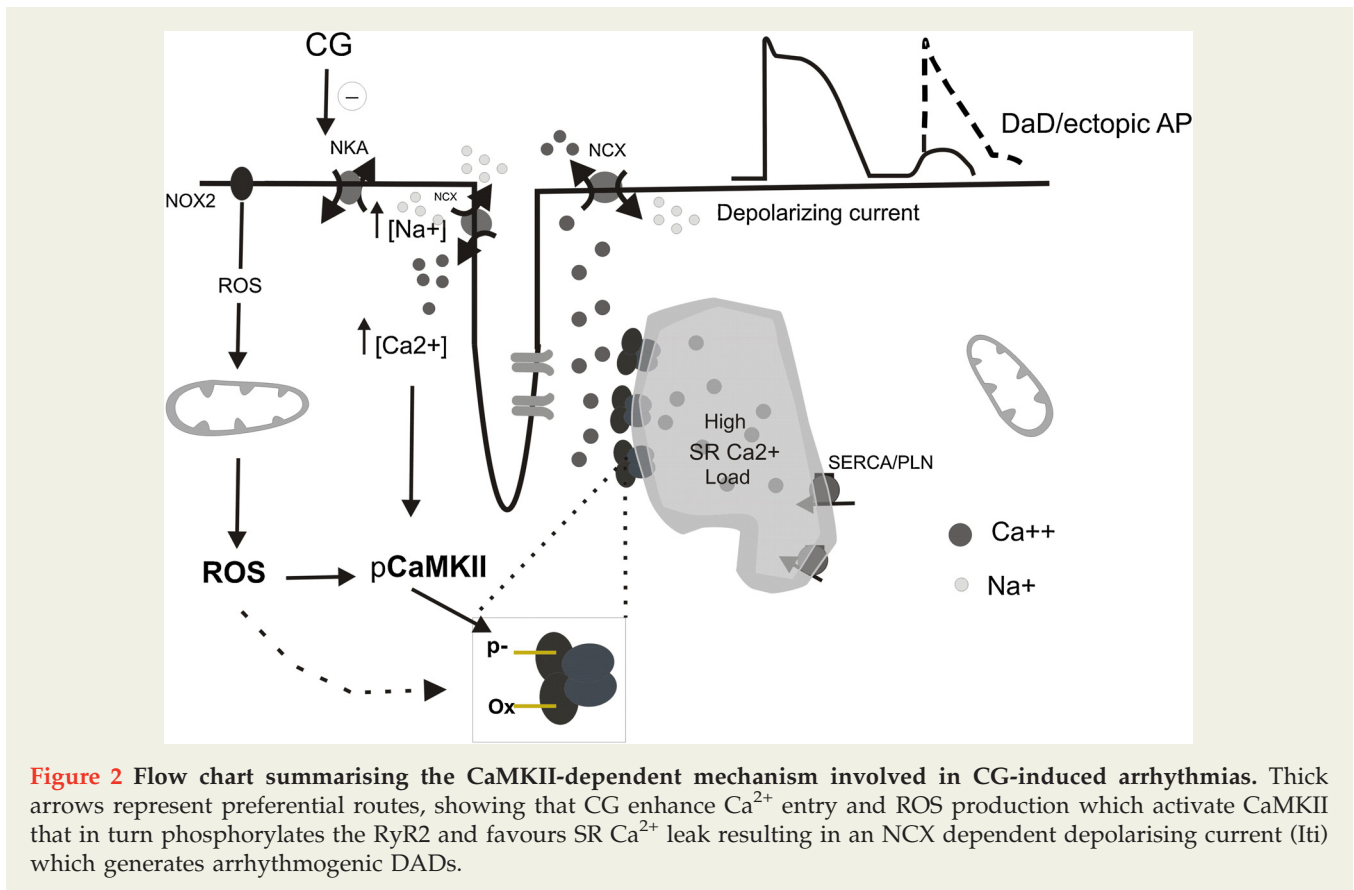
However, the relevance of the direct oxidation of the RyR2 remains unclear in the setting of digitalis toxicity. Considering that CaMKII can be activated through oxidative modification [46] ROS could also be affecting RyR2 function through ROS-dependent CaMKII sensitisation [47]. Indeed, taking into account this mechanism of CaMKII activation and in agreement with our original findings showing that CaMKII dependent RyR2 phosphorylation is a crucial determinant of CG-induced arrhythmias, Ho et al. proposed an

integrative model in which digitalis-dependent arrhythmogenesis would be due to oxidative activation of CaMKII which would lead to phosphorylation of the RyR2 enhancing SR Ca^{2+} leak. Supporting this conclusion, experiments in transgenic mice in which site Ser2814 of the RyR2 could not be phosphorylated (2814A mice), provided unequivocal evidence of the importance of phosphorylation of this site in CG-induced arrhythmias [31]. Myocytes isolated from 2814A mice did not show enhanced SR Ca^{2+} leak when exposed to digitoxin compared to myocytes from wild-type mice. These experiments additionally demonstrate that it is the phosphorylation rather than oxidation of RyR2 that is required for the increase in channel spontaneous activity and arrhythmogenesis in the context of digitalis toxicity.

As can be observed in the scheme shown in Fig. 2, CG-induced Ca^{2+} and/or ROS-dependent CaMKII activation results in RyR2 Ser2814 phosphorylation, which increases the sensitivity of this channel for spontaneous Ca^{2+} release, which in addition to the increase in SR Ca^{2+} load, promotes an arrhythmic Ca^{2+} release in cardiac myocytes.

Role of Mitochondria in CG-induced Arrhythmias

Herein we described SR function as having a fundamental role in CG-induced arrhythmias. However, mitochondria



have also been involved in the pathogenesis of these arrhythmias. As mentioned above, mitochondria are the source of ROS release which is triggered by Ca^{2+} -independent signalling after CG binding to NKA that could oxidise and activate CaMKII resulting in enhanced SR Ca^{2+} leak, DADs and triggered arrhythmias [31].

Mitochondria also participate in cellular Ca^{2+} handling by uptaking cytosolic Ca^{2+} through the mitochondrial Ca^{2+} uniporter [48]. Thus, when cardiac Ca^{2+} cycling increases as a result of an enhancement in workload, an increase in mitochondrial Ca^{2+} accumulation occurs, which is critical for maintaining NADH/NAD⁺ redox potential due its role in the activation of several enzymes of the tri-carboxylic acid cycle. This mechanism allows energy supply to be matched to energy demand when Ca^{2+} cycling is enhanced.

The main pathway for mitochondrial Ca^{2+} extrusion is the mitochondrial $\text{Na}^+/\text{Ca}^{2+}$ exchanger (mNCX). Interestingly, the inhibition of mNCX has also been shown to prevent CG-induced arrhythmias in a guinea pig model [43]. The mechanism proposed by Liu et al. to explain the antiarrhythmic effect of mNCX inhibition is that CGs, by elevating cytosolic $[\text{Na}^+]_i$, blunt mitochondrial Ca^{2+} accumulation via the activation of Ca^{2+} extrusion through the mNCX, resulting in net oxidation of NADH and the uncoupling of energy production from increased demand, which ends up increasing ROS production and promoting arrhythmogenesis [49].

Therefore, mNCX inhibition, by preventing mitochondrial Ca^{2+} leakage, would restore mitochondrial energy production,

reducing mitochondrial ROS release and arrhythmias. However, an alternative explanation for the ability of mNCX inhibition to prevent cardiotoxic glycoside-induced arrhythmias would be related to the capacity of mitochondria to act as an intracellular Ca^{2+} buffering system. Thus, during Na^+ overload, mNCX activation could increase $[\text{Ca}^{2+}]_i$ in the cytosol, providing an additional source of Ca^{2+} for loading the SR and for CaMKII activation. Indeed, in the presence of mNCX inhibition, less CaMKII activation could be expected due to either less ROS production and/or less cytosolic Ca^{2+} accumulation.

Finally, during CG treatment, a crosstalk between mitochondrial function and CaMKII activity is also possible given that recent evidence indicates that CaMKII is able to phosphorylate the mitochondrial Ca^{2+} uniporter and increase the rate of mitochondrial Ca^{2+} uptake [50]. Consistently, results from our laboratory indicate that chronic treatment of cardiac myocytes with sub-arrhythmogenic doses of CGs promotes mitochondrial pathway-dependent apoptosis, in a CaMKII-dependent manner [34].

RyR2 Stabilisation as an Antiarrhythmic Option during Inotropic Support

The evidence provided herein highlights the potential benefit of CaMKII inhibition as a therapeutic strategy to prevent

CG-induced arrhythmias, without altering their positive inotropic effect. However, the ubiquitous nature of CaMKII and its effects on different protein targets precludes its inhibition as a therapeutic tool. Albeit not impossible, pharmacological CaMKII inhibition would require cardiac-specific CaMKII inhibitors which are at present unavailable. In addition, a target-specific therapy would be desirable, taking into account the existence of multiple targets for CaMKII activity. For example, the phosphorylation of site Thr17 of phospholamban plays a key role in the β -adrenergic response and mediates the recovery of contractility after cardiac acidosis [51,52].

The demonstration of RyR2 being a crucial player in the development of arrhythmias during CG toxicity allows us to postulate an alternative therapeutic approach, which involves the concept of “RyR2 stabilisation”. The term “stabilisation” refers to the possibility to reduce RyR2 spontaneous diastolic opening without affecting systolic release. Thus, the use of compounds that are able to stabilise the RyR2 could prevent the adverse arrhythmogenic effects of GCs in the absence of detrimental effects on inotropy. We are aware of only one report that tested this possibility in the setting of CG treatment [30]. Using the multi-channel blocker JTV-519 (K201) which has been shown to stabilise the RyR2, Sacherer et al. showed, in mice myocytes and in non-failing human myocardium treated with ouabain, that JTV-519 was able to reduce SR Ca^{2+} leak. However, JTV-519 also reduced the positive inotropic response to digitalis, apparently due to the simultaneous L-type Ca^{2+} current-inhibitory effect of this compound. Despite this effect, JTV-519 improved the inotropic response of human cardiac strips by reducing diastolic contracture (a process dependent on an increase of diastolic cytosolic Ca^{2+} levels which would be reduced by JTV-519). These results, using JTV-519, together with additional reports showing that alternative RyR2 stabilisers such as VKII86 or tetracaine (in non Na^+ channel blocking doses) can reduce DAD-triggered arrhythmias [37,38], provide proof of concept that RyR2 stabilisers could be a promising approach to enhance the benefit and safety of CG treatment. Indeed, further work is warranted to find the “ideal” RyR2 stabiliser which should be a RyR2-specific modulator without effects on sarcolemmal currents, able to reduce spontaneous openings during diastole but without decreasing the efficacy of Ca^{2+} -induced Ca^{2+} release that triggers contraction.

Concluding Remarks

In summary, this review highlights the critical and previously unrecognised role of CaMKII in CG toxicity. The available evidence indicates that CGs increase Ca^{2+} and ROS which activate CaMKII resulting in an increase in the open probability of the RyR2, lowering the threshold for spontaneous release and predisposing the heart for DAD triggered arrhythmias. These results highlight the need for a redefinition of the mechanisms underlying digitalis-induced arrhythmias in general, attributed almost exclusively to an increase in SR Ca^{2+} load. These findings could help to explain the enhanced propensity

for fatal arrhythmias observed in heart failure patients, where high levels of endogenous ouabain-like compounds and CaMKII expression have been reported [53].

Importantly, the evidence provided herein suggests that CaMKII inhibitors could potentially be used as an adjunct to digitalis treatment for cardiovascular disease. However, the ubiquitous nature of CaMKII and the absence of organ specific CaMKII inhibitors preclude this possibility. The fundamental observation that CaMKII-dependent phosphorylation of the RyR2 is crucial in the development of arrhythmias during CG toxicity indicates that reducing RyR2 open probability using specific channel stabilisers could arise as a novel approach to ameliorate the usefulness of CG therapy.

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Disclosures

None

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