Calcium-Calmodulin Kinase II Mediates Digitalis-Induced Arrhythmias

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Background—Digitalis-induced Na⁺ accumulation results in an increase in Ca²⁺i via the Na⁺/Ca²⁺ exchanger, leading to enhanced sarcoplasmic reticulum (SR) Ca²⁺ load, responsible for the positive inotropic and toxic arrhythmogenic effects of glycosides. A digitalis-induced increase in Ca²⁺i could also activate calcium-calmodulin kinase II (CaMKII), which has been shown to have proarrhythmic effects. Here, we investigate whether CaMKII underlies digitalis-induced arrhythmias and the subcellular mechanisms involved.

Methods and Results—In paced rat ventricular myocytes (0.5 Hz), 50 μmol/L ouabain increased contraction amplitude by 160±5%. In the absence of electric stimulation, ouabain promoted spontaneous contractile activity and Ca²⁺ waves. Ouabain activated CaMKII (p-CaMKII), which phosphorylated its downstream targets, phospholamban (PLN) (Thr17) and ryanodine receptor (RyR) (Ser2814). Ouabain-induced spontaneous activity was prevented by inhibiting CaMKII with 2.5 μmol/L KN93 but not by 2.5 μmol/L of the inactive analog, KN92. Similar results were obtained using the CaMKII inhibitor, autacanotide-2 related inhibitory peptide (AIP) (1 to 2.5 μmol/L), and in myocytes from transgenic mice expressing SR-targeted AIP. Consistently, CaMKII overexpression exacerbated ouabain-induced spontaneous contractile activity. Ouabain was associated with an increase in SR Ca²⁺ content and Ca²⁺ spark frequency, indicative of enhanced SR Ca²⁺ leak. KN93 suppressed the ouabain-induced increase in Ca²⁺ spark frequency without affecting SR Ca²⁺ content. Similar results were obtained with digoxin. In vivo, ouabain-induced arrhythmias were prevented by KN93 and absent in SR-AIP mice.

Conclusions—These results show for the first time that CaMKII mediates ouabain-induced arrhythmogenic/toxic effects. We suggest that CaMKII-dependent phosphorylation of the RyR, resulting in Ca²⁺ leak from the SR, is the underlying mechanism involved. (Circ Arrhythm Electrophysiol. 2011;4:947-957.)

Key Words: cardiotoxic steroids □ arrhythmias □ CaMKII □ heart failure

Cardiotoxic glycosides selectively bind to and inhibit the sarcolemmal Na⁺/K⁺-ATPase and cause an increase in intracellular Na⁺, which in the heart reduces Ca²⁺ extrusion and/or increases Ca²⁺ influx through the Na⁺/Ca²⁺ exchanger (NCX). This increase in Ca²⁺ leads to an increase in sarcoplasmic reticulum (SR) Ca²⁺ load and to a positive inotropic effect, which explains, at least in part, their therapeutic use for heart failure treatment; however, these compounds have associated arrhythmic/toxic effects that conspire against their extensive use in the clinical practice. The arrhythmic effects have been proposed to occur when the SR Ca²⁺ storage capacity is exceeded so that oscillations of release-uptake cycles arise to re-establish the Ca²⁺ equilibrium between the cytosol and the SR. These transient increases in Ca²⁺i (Ca²⁺ waves) activate a transient inward (depolarizing) current (Ii), primarily mediated by the forward-mode NCX current. This Ii is responsible for the generation of delayed after depolarizations (DADs), which, if sufficiently large, may achieve threshold and generate spontaneous action potentials, leading to extrasystoles and ventricular arrhythmias; however, several lines of evidence suggest that increased SR Ca²⁺ load in itself is not sufficient to promote diastolic spontaneous SR Ca²⁺ 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. Moreover, a recent report showed that ouabain-induced DADs could be prevented by using JTV-519, a putative RyR stabilizer, suggesting that the underlying alteration responsible for ouabain-induced arrhythmias was at the level of the RyR rather than on the SR Ca²⁺ load. Indeed, in addition to SR Ca²⁺ overload, an increase in RyR open probability, resulting in enhanced SR...

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Ca\(^{2+}\) leak, is also a well-known substrate for triggering Ca\(^{2+}\) waves, I\(_d\), DADs, and, eventually, arrhythmias.\(^7\) In a previous study, we showed that chronic treatment with low nontoxic doses of the cardiotonic steroid ouabain can induce apoptosis through a mechanism that requires CaMKII activation.\(^8\) In this study, we found that the activation of the NCX during ouabain treatment leads to an increase in intracellular Ca\(^{2+}\), that results in CaMKII activation and culminates in apoptotic cell death. The above-mentioned signaling events could also be involved in cardiotonic steroid-induced arrhythmias, given that CaMKII activation has been shown to increase SR Ca\(^{2+}\) load and leak and induce arrhythmias\(^9\); however, whether CaMKII contributes to glycoside-induced arrhythmias has not been previously assessed.

The aim of this study was to examine whether cardiotonic steroid-induced arrhythmias are CaMKII-dependent and, if so, to determine the underlying mechanisms involved. For this purpose, we assessed the spontaneous contractile activity associated with Ca\(^{2+}\) waves as a proximal direct index of triggered DAD-like arrhythmias in rat myocytes, and we used transgenic mice as an experimental tool to assess the underlying mechanisms of cardiotonic steroid-triggered arrhythmias.

Methods

Myocyte Isolation and Culture

All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No.85-23, revised 1996) and approved by the Institutional Animal Care and Use Committee of La Plata University. Wistar rats (200 to 300 g), BALB/c (wild-type [WT]), or transgenic mice with cardiomyocyte-delimited transgenic expression of SR-targeted CaMKII inhibitor AIP (SR-AIP) or with a double-mutant nonphosphorylated form of phospholamban (PLN-DM), where the mutant PLN has alanine replacing both Ser-16 (PKA site) and Thr-17 (CaMKII site) (Mutant Mouse Regional Resource Center at the University of Missouri, Columbia, MO) were anesthetized by an intra-abdominal injection of sodium pentobarbitone (35 mg [kg body weight])\(^1\). Immediately after plane three of phase III of anesthesia was verified by the loss of the corneal reflex and the appearance of slow deep diaphragmatic breathing, central thoracotomy and heart excision were performed.

Myocytes were isolated by enzymatic digestion, as previously described.\(^1\) Details can be found in the online-only Supplemental Material.

Indo-1 Fluorescence and Cell-Shortening Measurements

Isolated myocytes were loaded with indo-1/AM (17 µmol/L for 9 minutes).\(^1\) See the online-only Supplemental Material for details of indo-1 fluorescence and cell-shortening methods.

The propensity to develop DAD-like arrhythmias was estimated from the number of nonstimulated contractile events (NSE). NSE were defined as spontaneous Ca\(^{2+}\)T increases with subsequent contractions of myocytes.\(^1\) These spontaneous Ca\(^{2+}\)T or Ca\(^{2+}\) waves and the associated contraction can be used as a proximal direct index of triggered DAD-like arrhythmias.\(^1\)

Confocal Imaging of Intact Cardiac Myocytes

Cells loaded with 10 µmol/L Fluo-3 were visualized using a Leica TCS SP5 inverted confocal microscope (Leica, Germany). See the online-only Supplemental Material for details of the confocal imaging.

Western Blot

Homogenates, cytosolic fractions, and SR membranes were prepared from the pulverized ventricular tissue from Langendorff-perfused rat hearts, as previously described.\(^10\) See the online-only Supplemental Materials for details.

Adenoviral Gene Transfer and Transfection Efficiency

Dr Roger J. Hajjar (Mount Sinai School of Medicine, New York, NY) kindly supplied 2 first-generation type 5 recombinant adenoviruses that were used: Ad.βgal, carrying the β-galactosidase and the green fluorescent protein genes, and Ad.CaMKII, carrying both the CaMKIIβ, and the green fluorescent protein genes, each under separate cytomembrane promoters. For details of the infection of myocytes with the adenovirus, see the online-only Supplemental Material.

In Vivo ECG Measurements

Surface ECG were recorded in BALB/c and SR-AIP mice using standard ECG electrodes for the PowerLab 4ST data acquisition system. Find further details in the online-only Supplemental Material.

Ouabain Doses

The concentration of ouabain used in this study (50 µmol/L) has been previously shown to be arrhythmogenic.\(^1\) In the in vivo experiments, 10 mg/kg were injected intraperitoneally. The online-only Supplemental Material details the rationale for using these doses.

Statistical Analysis

The unpaired student t test, Mann-Whitney rank-sum test, Fisher exact test, 1-way ANOVA, or Kruskal-Wallis 1-way ANOVA were used for statistical comparisons when appropriate. Differences were considered significant at P<0.05. Parametric and nonparametric continuous data are expressed as means±SEM and medians±percentiles, respectively, and categorical data are summarized as percents.

Results

Ouabain Induces Spontaneous Contractile Activity, Ca\(^{2+}\) Waves and Activates CaMKII in Rat Myocytes

The effect of 50 µmol/L ouabain on cell contraction and the associated intracellular Ca\(^{2+}\) transient (CaiT) was tested in freshly isolated rat myocytes. The propensity for NSE and spontaneous Ca\(^{2+}\) waves was assessed using the protocol depicted in Figure 1A. Myocytes, field stimulated at 0.5 Hz, were perfused with 50 µmol/L ouabain. After 20 minutes, stimulation was stopped, and myocyte cell length and CaiT were monitored for an additional 10 minutes in the continuous presence of ouabain. The continuous chart recordings show that ouabain administration produced a typical positive inotropic effect, associated with an increase in CaiT and the presence of a large number of NSE and Ca\(^{2+}\) waves during the nonstimulated period compared with control. Overall, ouabain produced a 60±5% increase in contractility (n=6 myocytes from 4 hearts), associated with a 17±3% increase in CaiT amplitude (n=6 myocytes from 4 hearts), and increased the number of NSE from 11±4 to 68±10 events/10 minutes.

We have previously demonstrated that chronic treatment with a low dose of ouabain can activate CaMKII\(^\text{II}\). To evaluate whether CaMKII is also activated by acute ouabain administration, Langendorff-perfused rat hearts were treated for 20 minutes with 50 µmol/L ouabain and then freeze-clamped for Western blotting. As shown in Figure 1B, ouabain effectively increased CaMKII activity (p-CaMKII). Furthermore, the CaMKII inhibitor, KN93, significantly reduced this activation (n=5 hearts).
CaMKII Mediates Ouabain-Induced Spontaneous Activity

Figure 2 shows typical tracings of cell-shortening of myocytes subjected to the protocol depicted in Figure 1A. As shown earlier, in the presence of ouabain, cells develop a large number of spontaneous contractile events during the nonstimulated period. This ouabain-induced spontaneous activity is largely reduced when cells are pretreated with 2.5 μmol/L of the CaMKII inhibitor, KN93. Similar results were obtained in the presence of 1 μmol/L of the more specific CaMKII-inhibitory peptide, AIP (ouabain + AIP). In contrast, the number of NSE was not reduced by the inactive KN93 analog, KN92. Similar results were obtained in experiments performed at 37°C (data not shown). Control experiments showed that the inhibitors used did not significantly affect basal contractility or the number of NSE before the administration of ouabain. The bar graph in Figure 2B shows the overall results of these experiments and additionally indicates that ouabain-induced NSE can be completely prevented by inhibiting SR function with ryanodine and thapsigargin (Ry+TG), indicating a primary role for the SR in ouabain-induced, CaMKII-dependent, spontaneous activity. Interestingly, CaMKII inhibition did not affect the ouabain-induced positive inotropic effect: 168.3±13.3% of control ouabain (n=13 myocytes from 6 hearts) and 159.7±13.3% of...
control ouabain+KN93 (n=14 myocytes from 6 hearts). To further confirm the central role played by CaMKII in ouabain-induced spontaneous activity by nonpharmacologic means, 2 different strategies were followed: (1) we overexpressed CaMKIIe (Ad.CaMKII) in cultured rat myocytes by adenoviral gene transfer, and (2) we employed transgenic mouse myocytes expressing SR-targeted CaMKII inhibitor AIP (SR-AIP). Figure 3A shows that 24 hours after infection, rat myocytes retained their rod shape morphology and functional integrity and presented (nearly 100%) a robust expression of the reporter gene, green fluorescent protein, indicating that our gene of interest was also overexpressed. At this time, CaMKII expression was significantly increased, as confirmed by Western blotting (Figure 3B). Functional experiments were then carried out to examine the susceptibility of these cells to develop ouabain-induced NSE in comparison with cells infected in similar conditions but with the adenovirus carrying the β-galactosidase gene (Ad.βgal). The representative tracings depicted in Figure 3C show that there were no significant differences between infected groups in the basal contraction and the NSE. Of note, spontaneous contractile activity in the absence of ouabain was lower than the one observed in fresh cells. The reason for this is not apparent to us but could be because of the prolonged culture period. As in fresh cells, ouabain increased spontaneous contractile events (NSE) in both β-galactosidase- and CaMKII-expressing cells; however, the incidence of spontaneous contractile activity was significantly higher in Adv.CaMKII cells. In contrast, CaMKII overexpression did not affect the ouabain-induced positive inotropic effect. The bar graphs in Figure 3C show the overall results of these experiments. These data serve to confirm that CaMKII is functionally linked with ouabain-induced NSE and arrhythmogenesis but not with inotropy.
as rat cells; however, ouabain failed to increase the incidence of spontaneous contractile activity in SR-AIP mice myocytes (Figure 4A). Overall results show that ouabain significantly increases the number of NSE in WT controls and that these events evoked by ouabain do not significantly increase in SR-AIP cells (Figure 4B). Figure 4C shows that ouabain produced a similar positive inotropic effect in myocytes isolated from WT or SR-AIP mice.

**Mechanisms Underlying CaMKII-Mediated Ouabain-Induced Arrhythmias**

Ca\(^{2+}\) waves generate spontaneous contractions, but, more importantly, they are also the substrate for DAD-triggered arrhythmias, which are thought to be responsible for digitalis intoxication.\(^{14,15}\) At least 2 factors have been shown to underlie spontaneous SR Ca\(^{2+}\) release or Ca\(^{2+}\) waves: (1) an increase in SR Ca\(^{2+}\) content; \(^{(15)}\) and (2) an increase in the sensitivity of the RyR for Ca\(^{2+}\) release.\(^{(7,15)}\) CaMKII has been shown to enhance both these processes,\(^{(16,17)}\) suggesting its potential involvement in cardiotoxicoid steroid (CTS)-induced arrhythmias; however, whether CaMKII favors ouabain-induced arrhythmogenicity by affecting SR Ca\(^{2+}\) load, the sensitivity of the RyR for Ca\(^{2+}\) release, or both these mechanisms, is unknown.

**Effect of CaMKII Inhibition on Ouabain-Induced Increase in SR Ca\(^{2+}\) Load**

Figure 5A shows the effect of ouabain on the phosphorylation of the CaMKII-dependent PLN residue, Thr17. Ouabain significantly increased Thr17 phosphorylation, and this effect was prevented by 2.5 \(\mu\)mol/L KN93. The traces in Figure 5B show the effect of ouabain in the absence and presence of KN93 on caffeine-induced Ca\(^{2+}\) transients, performed to evaluate SR Ca\(^{2+}\) content. As previously reported by us and others,\(^{(8,15)}\) ouabain significantly increases SR Ca\(^{2+}\) content; however, KN93 failed to affect caffeine-induced SR Ca\(^{2+}\) release. On average, SR Ca\(^{2+}\) load increased by 25±5% in the presence of ouabain (n=6 from 3 hearts) and by 24±6% in the presence of ouabain + 2.5 \(\mu\)mol/L KN93 (n=8 from 4 hearts). These results suggest that although ouabain enhances PLN phosphorylation, which would increase SERCA2a activity and favor SR Ca\(^{2+}\) load, this mechanism is not required for the observed increase in SR Ca\(^{2+}\) content produced by ouabain challenge. To further assess whether ouabain-induced CaMKII-dependent PLN phosphorylation is involved in the generation of spontaneous contractile activity, and therefore in Ca\(^{2+}\) waves and DADs, we used PLN double-mutant (PLN-DM) mouse myocytes. Figure 5C shows overall results, indicating that ouabain significantly and similarly increased the number of NSE in both WT and PLN-DM myocytes, suggesting that targets other than PLN are involved in ouabain-induced, CaMKII-dependent spontaneous activity.

**Effect of CaMKII on RyR Phosphorylation, SR Ca\(^{2+}\) Leak, and Spontaneous Ca\(^{2+}\) Waves**

Figure 6A shows that ouabain significantly increased the phosphorylation of the CaMKII-dependent RyR residue, Ser2814, and that this effect was prevented by 2.5 \(\mu\)mol/L KN93. RyR phosphorylation can increase the sensitivity of the channel for Ca\(^{2+}\) release, promoting diastolic SR Ca\(^{2+}\) leak and Ca\(^{2+}\) waves.\(^{(18)}\) Using confocal imaging, we assessed Ca\(^{2+}\) spark frequency under resting conditions, which reflects Ca\(^{2+}\) leak from the SR.\(^{(18)}\) After 20 minutes of pacing rat myocytes at 0.5 Hz in the presence of ouabain, pacing was stopped, and Ca\(^{2+}\) sparks were measured during 60 s. Figure 6B shows representative fluorescence images and overall results demonstrating that ouabain increases spark frequency. Additionally, we quantified the occurrence of spontaneous Ca\(^{2+}\) waves. Whereas in the absence of ouabain, myocytes hardly showed Ca\(^{2+}\) waves, ouabain treatment significantly enhanced the number of spontaneous Ca\(^{2+}\) waves from 0.03±0.02 to 0.10±0.01 waves* s\(^{-1}\) (n=8 cells from 4 hearts). KN93 prevented both the ouabain-induced increase in Ca\(^{2+}\) spark frequency and in Ca\(^{2+}\) wave occurrence, indicating that CaMKII underlies these events.

**CaMKII Inhibition Prevents Ouabain-Induced Arrhythmias In Vivo**

In the absence of ouabain, WT mice did not exhibit spontaneous arrhythmias as evidenced from continuous in vivo
ECG measurements. As shown in Figure 7A, 10 mg/kg IP administration of ouabain induced a variety of ECG alterations in a background of sinus bradycardia and atrioventricular block because of the vagal effects of ouabain. Among these alterations, ventricular ectopic beats and sustained ventricular tachycardia were the most common. These arrhythmic events were diminished in the presence of KN93 (30 μmol/kg IP; n=9; Figure 7B) and absent in SR-AIP mice (n=5; Figure 7C). Table shows the incidence of ventricular ectopic beats and sustained ventricular tachycardia in the presence of ouabain alone, ouabain+KN93, or SR-AIP mice treated with ouabain. In addition, KN93 significantly reduced ouabain-induced mice mortality. Nine out of 11 (18% survival) mice treated with ouabain died after treatment, whereas 6 out of 9 (67% survival) mice pretreated with KN93 (P=0.04 vs ouabain) and 4 out of 5 SR-AIP (80% survival) mice (P=0.03 vs ouabain) survived ouabain treatment.

Figure 5. Effect of ouabain on sarcoplasmic reticulum (SR) Ca\(^{2+}\) load and phospholamban phosphorylation. A, Representative blots and overall results show that ouabain increases the phosphorylation of PLN at its Thr17 site and that KN93 prevents this increase. Data are expressed as means±SEM from 5 independent experiments from 5 hearts (*P<0.05, 1-way ANOVA, Newman-Keuls). B, Typical tracings and overall results of caffeine pulses performed to estimate SR Ca\(^{2+}\) load in rat myocytes in the absence or presence of either ouabain alone or ouabain+KN93. The bar graph below shows the average values of these experiments (*P<0.05, 1-way ANOVA, Newman-Keuls; n=8 cells from 4 hearts). C, Overall results of the effect of ouabain on the number of nonstimulated events in wild-type (WT) and phospholamban double mutant myocytes (PLN-DM). Data are medians±percentiles from 6 cells from 3 hearts per group (*P<0.05, Mann-Whitney rank-sum test).

CaMKII Inhibition Prevents Digoxin-Induced Spontaneous Activity
To examine whether the observed effect of ouabain on spontaneous activity was common to other related cardiotoxic steroids, we studied the effect of digoxin (DIG) on the number of NSE, SR Ca\(^{2+}\) load, and Ca\(^{2+}\) spark and wave frequency in the absence and presence of KN93. Figure 8 shows that a low, nontoxic but inotropic dose of DIG (10 μmol/L)\(^{19}\) does not increase the number of NSE nor the frequency of spontaneous Ca\(^{2+}\) waves. This dose of DIG showed a tendency to increase SR Ca\(^{2+}\) load and spark frequency; however, these increases did not attain significant levels. In contrast, 75 μmol/L DIG significantly increased the number of NSE and SR Ca\(^{2+}\) load as well as Ca\(^{2+}\) spark and wave frequency. Similar to the results obtained with ouabain, CaMKII inhibition with KN93 did not prevent the DIG-
induced increase in SR Ca$^{2+}$ load but reduced the number of NSE, as well as Ca$^{2+}$ spark and wave frequency.

**Discussion**

Steroidal glycosides extracted from the leaves of plants from the genus Digitalis have been used for the treatment of congestive heart failure for more than 200 years; however, these compounds have a narrow therapeutic window because of the presence of adverse toxic effects, characterized primarily by arrhythmias and, as recently shown by us and others, by apoptosis, which limit their extensive use in the clinical practice. Digitalis-induced arrhythmogenic effects are, as yet, not completely understood. It has been suggested that NCX-mediated Ca$^{2+}$ influx, resulting in SR Ca$^{2+}$ overload, could increase RyR open probability and lead to spontaneous diastolic SR Ca$^{2+}$ release that could activate a transient $I_{di}$, responsible for the generation of DADs, spontaneous action potentials, and ventricular arrhythmias. A digitalis-induced increase in Ca$^{2+}$ could also activate CaMKII, which has been shown not only to favor SR Ca$^{2+}$ load but also to increase the Ca$^{2+}$ sensitivity of the RyR and to induce arrhythmias. Thus, we hypothesized that CaMKII could be involved in glycoside-induced arrhythmogenesis. In the present study, we show that ouabain activates CaMKII, and, for the first time, we demonstrate that ouabain-induced arrhythmias are CaMKII-dependent. Furthermore, we show that ouabain promotes CaMKII-dependent arrhythmogenesis both in vitro and in vivo, and we demonstrate that CaMKII inhibition prevents ouabain-induced arrhythmias without affecting its positive inotropic effect, suggesting a potential therapeutic benefit for CaMKII inhibition during glycoside treatment. Finally, our results indicate that CaMKII-mediated phosphorylation of the RyR, resulting in Ca$^{2+}$ leak from the SR and enhanced Ca$^{2+}$ wave formation, would be the underlying mechanism involved. Highlighting the clinical relevance of our findings, it is noteworthy that we obtained similar results using digoxin, a structurally different cardiotonic steroid routinely used in the clinical practice.
Ouabain Toxicity and CaMKII

It is now generally accepted that ouabain increases intracellular \( \text{Na}^{+}/\text{H}^{+} \) through the inhibition of \( \text{Na}^{+}/\text{H}^{+}/\text{K}^{+} \)-ATPase. The resulting reduction of the transarcolemmal \( \text{Na}^{+}/\text{H}^{+} \) gradient favors the reverse mode of the NCX, which increases intracellular \( \text{Ca}^{2+}/\text{H}^{+} \) load and results in a positive inotropic effect. It has been proposed that when this \( \text{Ca}^{2+}/\text{H}^{+} \) load exceeds the capacity of the SR, abnormal \( \text{Ca}^{2+} \) release occurs, which, in turn, triggers abnormal electric activity and arrhythmic contractions. In the present study, we observed that ouabain increased the activity of CaMKII (p-CaMKII) and that ouabain-induced arrhythmic contractions were significantly reduced by the CaMKII inhibitor KN93 (Figures 1 and 2). These results indicate that CaMKII is involved in ouabain-induced arrhythmogenesis. Further confirming the participation of CaMKII, we showed that (1) KN92, the inactive analog of KN93, failed to affect ouabain-induced NSE; (2) AIP, a structurally different inhibitor of CaMKII, also prevented ouabain-induced NSE; (3) CaMKII overexpression exacerbated ouabain-induced NSE; (4) transgenic mice expressing SR-targeted CaMKII inhibition (SR-AIP) were protected from the toxic effects of ouabain; and (5) in vivo, ouabain failed to induce arrhythmias and death in WT mice pretreated with KN93 and in SR-AIP mice. These findings (combining pharmacological inhibition, genetic manipulation, and in vivo studies) provide substantial evidence indicating that CaMKII is mechanistically involved in ouabain-induced spontaneous activity and arrhythmogenesis.

Mechanisms Underlying Ouabain-Induced CaMKII-Dependent Arrhythmias

Cardiotonic steroid-induced arrhythmias have been shown to be mediated by \( \text{Ca}^{2+} \) waves, resulting in DADs, which generate spontaneous action potentials.\(^{3,6}\) A potential role for CaMKII in DADs formation has been previously reported.\(^{12,20,21}\) Wu and colleagues were the first to describe that CaMKII triggers an NCX-dependent arrhythmogenic transient \( I_{\text{di}} \) through its effect of SR \( \text{Ca}^{2+} \) load/release.\(^{20}\) Said

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**Figure 7.** In vivo ECG of ouabain-induced arrhythmias in the absence and presence of calcium-calmodulin kinase II (CaMKII) inhibition. Representative traces show the progressive effects of ouabain on cardiac rhythm (A), in the presence of KN93 (B), and in SR-AIP mice (C). A shows that ouabain challenge is associated with ventricular escape beats (b), sustained monomorphic (c), and/or polymorphic (d) ventricular tachycardia, followed by death. CaMKII inhibition (KN93 or SR-AIP mice) reduced the occurrence of these events. Note that even though SR-AIP mice presented a pause in sinus rhythm, there were no associated escape ventricular beats (C, b).

**Table. Frequency of Arrhythmias in Ouabain-Treated Mice**

<table>
<thead>
<tr>
<th></th>
<th>WT Mice + Ouabain</th>
<th>WT Mice + Ouabain + KN93</th>
<th>SR-AIP Mice + Ouabain</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of mice</td>
<td>11 (100%)</td>
<td>9 (100%)</td>
<td>5 (100%)</td>
</tr>
<tr>
<td>Sinus bradycardia/AV block</td>
<td>11 (100%)</td>
<td>9 (100%)</td>
<td>5 (100%)</td>
</tr>
<tr>
<td>Ventricular ectopic beats (&gt;1/h)</td>
<td>9 (81.2%)</td>
<td>3 (33.3%)</td>
<td>1 (20%)</td>
</tr>
<tr>
<td>Sustained VT</td>
<td>9 (81.2%)</td>
<td>2 (22.2%)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

Overall data shows the incidence of ventricular ectopic beats (premature or escape beats) and sustained ventricular tachycardia in BALB/c mice in the absence and presence of calcium-calmodulin kinase II inhibition (KN93 and SR-AIP mice). Sustained ventricular tachycardia (VT) was defined as a run of >10 ventricular ectopic beats.

WT indicates wild type; SR, sarcoplasmic reticulum.

ouabain-induced arrhythmic contractions were significantly reduced by the CaMKII inhibitor KN93 (Figures 1 and 2). These results indicate that CaMKII is involved in ouabain-induced arrhythmogenesis. Further confirming the participation of CaMKII, we showed that (1) KN92, the inactive analog of KN93, failed to affect ouabain-induced NSE; (2) AIP, a structurally different inhibitor of CaMKII, also prevented ouabain-induced NSE; (3) CaMKII overexpression exacerbated ouabain-induced NSE; (4) transgenic mice expressing SR-targeted CaMKII inhibition (SR-AIP) were protected from the toxic effects of ouabain; and (5) in vivo, ouabain failed to induce arrhythmias and death in WT mice pretreated with KN93 and in SR-AIP mice. These findings (combining pharmacological inhibition, genetic manipulation, and in vivo studies) provide substantial evidence indicating that CaMKII is mechanistically involved in ouabain-induced spontaneous activity and arrhythmogenesis.

Mechanisms Underlying Ouabain-Induced CaMKII-Dependent Arrhythmias

Cardiotonic steroid-induced arrhythmias have been shown to be mediated by \( \text{Ca}^{2+} \) waves, resulting in DADs, which generate spontaneous action potentials.\(^{3,6}\) A potential role for CaMKII in DADs formation has been previously reported.\(^{12,20,21}\) Wu and colleagues were the first to describe that CaMKII triggers an NCX-dependent arrhythmogenic transient \( I_{\text{di}} \) through its effect of SR \( \text{Ca}^{2+} \) load/release.\(^{20}\) Said
and colleagues showed that post acidosis-induced DAD-triggered arrhythmias could be prevented by CaMKII inhibition and concluded that CaMKII would enhance SERCA2a activity by phosphorylating phospholamban at its Thr17 site, thus increasing SR Ca2+ load and favoring DAD formation.21 Using a protocol similar to the one used in the present study, Curran and colleagues also implicated CaMKII in DAD-triggered arrhythmias of the failing heart.12 In this case, the authors concluded that CaMKII increases the sensitivity of the RyR, lowering the threshold for spontaneous Ca2+ release and thus providing the arrhythmogenic substrate. These studies clearly define the 2 main factors responsible for increasing the propensity for Ca2+ waves and subsequent DADs increased SR Ca2+ load, and/or increased Ca2+ sensitivity of the RyR. We showed that ouabain increased both SR Ca2+ load (Figure 5) and the phosphorylation of the RyR associated with SR Ca2+ leak (Figure 6), indicative of increased Ca2+ sensitivity of the RyR; however, CaMKII inhibition, which prevented ouabain-induced arrhythmias, did not affect SR Ca2+ load, whereas it reduced RyR phosphorylation, Ca2+ leak, and Ca2+ wave propensity (Figure 6). Similar to the conclusion of Curran and colleagues, these results suggest that CaMKII would primarily mediate ouabain-induced arrhythmias through increasing the sensitivity of the RyR to Ca2+. Our results showing that the ouabain-induced increase in PLN Thr17 phosphorylation could be prevented with KN93 seems to be at odds with the failure of KN93 to reduce SR Ca2+ load, given that this phosphorylation is known to enhance SERCA2a activity and SR Ca2+ uptake; however, in the rat, a species with high resting SR Ca2+,22 ouabain can fully load the SR through Ca2+ influx via the NXC, and, therefore, CaMKII-dependent PLN phosphorylation would not further increase SR Ca2+ load. Our results showing that ouabain-induced spontaneous contractile activity was similar between PLN-DM myocytes and WT myocytes (Figure 5) would support the contention that CaMKII-dependent PLN phosphorylation is not involved in ouabain-induced arrhythmias; however, another possible interpretation of our results is that Thr17 phosphorylation of PLN does play a role in the arrhythmic pattern described,
maintaining the increased SR Ca\(^{2+}\) load produced by ouabain, just matching the SR Ca\(^{2+}\) leak produced by RyR phosphorylation. If this were the case, one could further speculate that PLN-DM mice might compensate for the lack of PLN phosphorylation by the increase in i-type Ca\(^{2+}\) channels, typical of these mice.\(^{23}\) In any case, our results showing that KN93 completely prevents ouabain-induced arrhythmias, without affecting SR Ca\(^{2+}\) load, while preventing RyR phosphorylation, SR Ca\(^{2+}\) leak, and Ca\(^{2+}\) wave propensity, would suggest that increased SR Ca\(^{2+}\) load alone is not sufficient to promote ouabain-induced arrhythmias. Interestingly, the inverse conclusion seems to be also true, as suggested by Eisner and colleagues, who elegantly showed that increasing RyR receptor open probability alone is not enough to produce arrhythmogenic diastolic Ca\(^{2+}\) leak and that a parallel increase in SR Ca\(^{2+}\) load is required.\(^{14}\) In the case of ouabain, the NCX would provide Ca\(^{2+}\) to load the SR and activate CaMKII, which, in addition to other targets, would phosphorylate the RyR and increase its Ca\(^{2+}\) sensitivity, lowering the threshold for spontaneous Ca\(^{2+}\) release. Thus, the NCX would couple the arrhythmogenic RyR phosphorylation with SR Ca\(^{2+}\) overload that mediate the spontaneous Ca\(^{2+}\) waves, DADs, and eventually arrhythmias. Indeed, several studies have pointed out the critical role of the NCX in digitalis-induced arrhythmias.\(^{24,25}\)

In a recent report, ouabain-triggered arrhythmias were associated with mitochondrial dysfunction.\(^{26}\) This study convincingly showed that ouabain-induced arrhythmias could be prevented by blocking the mitochondrial NCX. These results, obtained using a different species and by inducing arrhythmias with ouabain plus isoproteenol, make direct comparisons to our results difficult; however, one explanation that could reconcile the apparent discrepancies with our results is that, in the context of ouabain challenge, mitochondrial NCX activation could provide an additional source of Ca\(^{2+}\) for loading the SR and for CaMKII activation, thus its blockade would reduce these processes and prevent arrhythmogenesis.

Consistent with our findings, several studies have demonstrated that catecholaminergic polymorphic ventricular tachycardia (an arrhythmic condition which resembles that of digitalis-toxicity) is also linked to Ca\(^{2+}\) leak from the SR and can be triggered by ouabain.\(^{9}\) More importantly, catecholaminergic polymorphic ventricular tachycardia can be prevented by CaMKII inhibition.\(^{27}\) These results not only demonstrate the fundamental role of CaMKII in these types of arrhythmias but also highlight the potential use of CaMKII inhibition as a valid therapeutic option for both catecholaminergic polymorphic ventricular tachycardia and digitalis-induced arrhythmias.

In summary, we have presented evidence indicating that cardiotoxic steroids activate CaMKII, which increases the Ca\(^{2+}\) sensitivity of the RyR, 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\(^{28}\) and CaMKII expression\(^{29}\) have been reported. Finally, although the benefit of glycoside therapy in patients with endstage heart failure is widely acknowledged, the finding that CaMKII inhibition prevents ouabain-induced arrhythmias, without affecting its positive inotropic effect, suggests the potential use of CaMKII inhibitors as an adjunct to digitalis treatment for cardiovascular disease.

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Disclosures

None.

References

Cardiac glycosides have been used for the treatment of congestive heart failure for more than 200 years; however, these compounds have a narrow therapeutic window because of the presence of adverse toxic effects, including arrhythmias, which limit their extensive use in the clinical practice. The arrhythmic effects have been proposed to occur when sarcoplasmic reticulum (SR) Ca\(^{2+}\) storage capacity is exceeded and spontaneous SR Ca\(^{2+}\) release (Ca\(^{2+}\) waves) arise and activate a depolarizing current, which, if sufficiently large, may achieve threshold and generate spontaneous action potentials and ventricular arrhythmias. In the present study, we show that cardiac glycoside activates calcium-calmodulin kinase II (CaMKII), which phosphorylates the ryanodine receptor-favoring spontaneous SR Ca\(^{2+}\) release, predisposing the heart for delayed after depolarization-triggered arrhythmias. Our results also reveal that CaMKII inhibition prevents digitalis-induced arrhythmias without affecting its positive inotropic effect, suggesting the potential use of CaMKII inhibitors as an adjunct to digitalis for the treatment of heart failure. Thus, our findings could not only help to widen the therapeutic window of cardiac glycosides but also 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.
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**Supplemental Material**

*Myocyte isolation and culture*

Myocytes were isolated by enzymatic digestion\(^1\) and kept in a HEPES buffered solution at room temperature (20-24 °C), until use. Unless otherwise specified, experiments were performed at room temperature. For culture, isolated cells were resuspended in DMEM medium containing (in g/l) 0.017 ascorbic acid, 0.4 L-carnitine, 0.66 creatine, 0.62 taurine, 50U/ml penicillin, and 50 U/ml streptomycin, and counted. Myocytes were plated at a density of ~2 X 10\(^4\) rod-shaped cells/ml into culture dishes for 1h to allow cell attachment. After this period, the culture media was changed for a fresh one and infected with viral particles according to the experiments performed (see results). After 24 h of culture, the cells were photographed to assess transfection efficiency and collected to perform functional experiments.

*Indo-1 fluorescence and cell shortening measurements*

Cells were placed on the stage of an inverted microscope (Nikon Diaphot 200) adapted for epifluorescence, continuously superfused with HEPES buffered solution (pH 7.4) at a constant flow of 1 ml/min and field stimulated via two platinum electrodes on either side of the bath, at 0.5 Hz. The ratio of the Indo-1 emission (410 and 490 nm) was taken as an index of Ca\(^{2+}\). The SR Ca\(^{2+}\) content was evaluated by the amplitude of the Ca\(_i\)T induced by 15 mM caffeine.

Resting cell length and cell shortening were measured by a video-based motion detector (Crescent electronics, UT, USA) and stored by software for an off-line analysis (PowerLab/400 ADInstruments).

*Confocal imaging of intact cardiac myocytes*
Confocal images of Ca\textsuperscript{2+} sparks were taken in the line scan mode\textsuperscript{2}. Cells were exited with the 488nm line of an argon laser and fluorescence was collected at >515nm. Each image consisted of 512 line scans obtained at 4 ms intervals. Data were visualized using Leica Application Suite and Ca\textsuperscript{2+} sparks were measured using the “Sparkmaster” plugin for ImageJ\textsuperscript{3}. Sparks were obtained in quiescent cells after 20 min stimulation at 0.5 Hz either in the presence or absence of ouabain 50 µmol/l.

**Western Blot**

Protein was measured by the Bradford method using BSA as standard. Lysates (~90 µg of total protein per gel line) were seeded in a 10% SDS polyacrylamide gel\textsuperscript{4} and transferred to polyvinylidene difluoride membranes. Blots were probed overnight with the antibodies raised against phospholamban (PLN) (ABR, California, USA) Phospho-Thr17-PLN (Badrilla, Leeds, UK) CaMKII (Chemicon International, Lake Placid, NY) phospho-Thr286-CaMKII, (Badrilla, Leeds, UK) phospho-Ser2814-RyR (Badrilla, Leeds, UK) and Anti GAPDH (Santa Cruz Biotechnology, Santa Cruz, USA) for normalization. Immunoreactivity was visualized by a peroxidase-based chemiluminescence detection kit (Amersham Biosciences) using a Chemidoc Imaging System. The signal intensity of the bands in the immunoblots was quantified by densitometry using Image J software (NIH).

**Adenoviral Infection**

Plated rat myocytes were infected with adenoviruses at a multiplicity of infection (MOI) of 100 and cultured for 24 hrs. The verification of the transgene expression was monitored by western blot and GFP fluorescence at an excitation wavelength of 480 nm after the culture period\textsuperscript{1}. 
In vivo ECG measurements

ECGs were acquired at 40 KHz sampling rate. Recordings were performed after 20 min intraperitoneal injection of 10mg/kg ouabain and for a period of 60 min. Arrhythmias were assessed by quantifying the incidence of ventricular ectopic beats (premature or ventricular escape beats) and sustained ventricular tachycardia during the 60 min recording period. Similar experiments were conducted in mice pretreated with the CaMKII inhibitor KN 93 (30 µmol/Kg IP). This dose of KN 93 has been shown to prevent arrhythmic processes of different etiology\textsuperscript{5,6}.

To avoid restraining mice during the 60 minutes ECG recording period a more physiologic ECG recording system in which the mice can move freely around the cage was designed and used in a group of mice. As shown in the photograph, we made a cloth vest holding the ECG electrodes connected through very thin cables to the acquisition system. This vest was placed on the mouse’s chest and ECG was recorded during one hour in the absence and presence of ouabain and ouabain + KN93. Disconnected vests were placed on the mice for 24 hrs prior to the experiment for their habituation. Using this more physiological approach, we obtained similar results to those using the retrained animal.
Ouabain Doses

The therapeutic and toxic levels of both ouabain and digoxin are more than 100 times higher in rodents than in humans. The dose of ouabain used in this study (50 µmol/l) has been previously shown to be arrhythmogenic. This dose is in the lower limit of toxicity in the rat heart, a species with extremely low sensitivity to ouabain. We chose this dose of ouabain because in control experiments (not shown) we observed that it was able to systematically and reproducibly produce arrhythmias and utilized this as a model to study the underlying subcellular mechanisms involved in ouabain-induced arrhythmias.

The dose of ouabain used in our in vivo experiments was 10 mg/Kg I.P. In control experiments we observed that ouabain at an order of magnitude below the dose used, did not promote arrhythmias within the first hour following administration, whereas ouabain at an order of magnitude above the one used, killed the animals during the first hour. Furthermore, the dose of ouabain recognized as the i.p. lethal dose 50 (LD50) in the mouse is 11 mg/kg. Thus, for our in vivo ECG measurements we used a similar dose (10 mg/Kg).

Data Analysis:

Data sets resulting from the quantification of random events (NSE, Ca2+ spark characteristics and wave frequency), were summarized in box and whisker plots. The horizontal lines in the box denote the 25th, 50th, and 75th percentile values. The error bars denote the 5th and 95th percentile values. The square symbol in the box denotes the mean of the data. Non parametric statistical tests were used to assed whether differences between groups were statistical significant. Kruskal Wallis one-way ANOVA analysis of variance followed by Dunn’s method was used
for multiple comparisons and Mann-Whitney Rank sum test was used for two sample groups.

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