CaMKII Mediates Digitalis-Induced Arrhythmias

Running title: Gonano et al.; CaMKII mediates digitalis toxicity but not inotropy

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Abstract:

**Background** - Digitalis-induced Na\(^+\) accumulation results in an increase in Ca\(^{2+}\) via the Na\(^+/\)Ca\(^{2+}\) exchanger leading to enhanced SR Ca\(^{2+}\) load, responsible for the positive inotropic and toxic arrhythmogenic effects of glycosides. Digitalis-induced increase in Ca\(^{2+}\) could also activate 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 electrical stimulation, ouabain promoted spontaneous contractile activity and Ca\(^{2+}\) waves. Ouabain activated CaMKII (P-CaMKII) which phosphorylated its downstream targets, phospholamban (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 analogue KN92. Similar results were obtained using the CaMKII inhibitor, AIP (1-2.5 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 Ca\(^{2+}\) content and Ca\(^{2+}\) spark frequency, indicative of enhanced SR Ca\(^{2+}\) leak. KN93 suppressed the ouabain-induced increase in Ca\(^{2+}\) spark frequency without affecting SR Ca\(^{2+}\) 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 arrhythmic/toxic effects. We suggest that CaMKII-dependent phosphorylation of the RyR, resulting in Ca\(^{2+}\) leak from the SR is the underlying mechanism involved.

**Key words:** Cardiotonic Steroids, Arrhythmias, CaMKII, Heart Failure.
Introduction

Cardiotonic glycosides selectively bind to and inhibit the sarcolemmal Na\(^+/K^+\)-ATPase (NKA) and cause an increase in intracellular Na\(^+\) which, in the heart, reduces Ca\(^{2+}\) extrusion and/or increases Ca\(^{2+}\) influx through the Na\(^+\)/Ca\(^{2+}\) exchanger (NCX). This increase in Ca\(^{2+}\)\(_i\) leads to an increase is sarcoplasmic reticulum (SR) Ca\(^{2+}\) load and to a positive inotropic effect which explains, at least in part, their therapeutic use for heart failure treatment\(^1\). However, these compounds have associated toxic/arrhythmic effects that conspire against their extensive use in the clinical practice\(^2\). The arrhythmic effects have been proposed to occur when SR Ca\(^{2+}\) storage capacity is exceeded so that oscillations of release-uptake cycles arise to reestablish the Ca\(^{2+}\) equilibrium between the cytosol and the SR. These transient increases in Ca\(^{2+}\)\(_i\) (Ca\(^{2+}\) waves) activate a transient inward (depolarizing) current (I\(_{\text{Iti}}\)), primarily mediated by the forward-mode NCX current. This I\(_{\text{Iti}}\) is responsible for the generation of delayed afterdepolarizations (DADs) which, if sufficiently large may achieve threshold and generate spontaneous action potentials leading to extrasystoles and ventricular arrhythmias\(^3\). However, several lines of evidence suggest that increased SR Ca\(^{2+}\) load in itself is not sufficient to promote diastolic spontaneous SR Ca\(^{2+}\) release. For example, PLN knockout mice which have a fully loaded SR, have not been shown to be prone to arrhythmias under basal conditions\(^4,5\).

Moreover, a recent report showed that ouabain-induced DAD’s could be prevented by using JTV-519, a putative ryanodine receptor (RyR) stabilizer\(^6\), suggesting that the underlying alteration responsible for ouabain-induced arrhythmias was at the level of the RyR rather than on SR Ca\(^{2+}\) load. Indeed, in addition to SR Ca\(^{2+}\) overload, an increase in
RyR open probability resulting in enhanced SR Ca$^{2+}$ leak is also a well known substrate for triggering Ca$^{2+}$ waves, I$\text{it}$, DADs and eventually arrhythmias$^7$.

In a previous study we showed that chronic treatment with low non toxic doses of the cardiotonic steroid, ouabain, can induce apoptosis through a mechanism that requires Ca$^{2+}$/calmodulin-dependent protein kinase II (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 (CTS)-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 (200-300 gr), 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 nonphosphorylatable form of phospholamban (PLN-DM), where the mutant PLN has alanine replacing both Ser-16 (PKA site) and Thr-17 (CaMKII site), (MMRC, mutant mice generation resource center, NCRR-NIH, Columbia, MO), were anaesthetised by intra-abdominal injection of sodium pentobarbitone (35 mg (Kg body weight)$^{-1}$). Central thoracotomy and heart excision was performed immediately after plane three of phase III of anaesthesia was reached, verified by the loss of the corneal reflex and appearance of slow deep diaphragmatic breathing.

Myocytes were isolated by enzymatic digestion, as previously described$^{10}$. Details are provided in Supplemental Material.

Indo-1 fluorescence and cell shortening measurements

Isolated myocytes were loaded with Indo-1/AM (17 μmol/l for 9 min)$^{10}$. Details of Indo-1 fluorescence and shortening methods are provided under Supplemental Material.

The propensity to develop DAD-like arrhythmias was estimated from the number of non stimulated contractile events (NSE). NSE were defined as spontaneous $\Delta$Ca$^{2+}$ increases with subsequent contractions of myocyte$^{11}$. These spontaneous $\Delta$Ca$^{2+}$ or Ca$^{2+}$ waves and the associated contraction can be used as a proximal direct index of triggered DAD-like arrhythmias$^{12}$.

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). Details for confocal imaging are given in Supplemental Material.

**Western Blot**

Homogenates, cytosolic fractions and SR membranes were prepared from the pulverized ventricular tissue from Langendorf perfused rat hearts as previously described\(^\text{10}\). Details are provided under Supplemental Material.

**Adenoviral gene transfer and transfection efficiency**

Two first-generation type 5 recombinant adenovirus, kindly supplied by Dr. Roger J. Hajjar (Mount Sinai School of Medicine, New York, NY, USA), were used. Ad.βgal, carrying the β-galactosidase and the green fluorescent protein (GFP) genes and Ad.CaMKII, carrying both the CaMKII\(^G\)C and the GFP genes, each under separate cytomegalovirus promoters. For details of the infection of myocytes with the adenovirus, see Supplemental Material.

**In vivo ECG measurements**

Surface ECG were recorded in Balb-c and SR-AIP mice using standard ECG electrodes for Powerlab/4st data acquisition system. Further details are provided in Supplemental Material.

**Ouabain Doses**

The concentration of ouabain used in this study (50 μmol/l) has been previously shown to be arrhythmogenic\(^\text{13}\). In the *in vivo* experiments 10 mg/Kg were injected IP. The rationale for using these doses is detailed in Supplemental Material.

**Statistical Analysis**
Unpaired Student t test, Mann-Whitney Rank sum test, Fisher’s exact test, One-way ANOVA or Kruskal Wallis One-way ANOVA were used for statistical comparisons when appropriate. Differences were considered significant at $p \leq 0.05$. Parametric and non-parametric 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 $\mu$mol/l ouabain on cell contraction and the associated intracellular Ca$^{2+}$ transient (CaiT) was tested in freshly isolated rat myocytes. The propensity for non-stimulated contractile events (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 $\mu$mol/l ouabain. After 20 min, stimulation was stopped and myocyte cell length and CaiT were monitored for an additional 10 min 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 non stimulated period compared to 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/10min.
We have previously demonstrated that chronic treatment with low dose of ouabain can activate CaMKII\(^8\). To evaluate whether CaMKII is also activated by acute ouabain administration, Langendorff perfused rat hearts were treated for 20 min with 50 \(\mu\)mol/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 non stimulated period. This ouabain-induced spontaneous activity is largely reduced when cells are pretreated with 2.5 \(\mu\)mol/l of the CaMKII inhibitor, KN93. Similar results were obtained in the presence of 1 \(\mu\)mol/l of the more specific CaMKII-inhibitory peptide, AIP (oua + AIP). In contrast, the number of NSE was not reduced by the inactive KN93 analogue, 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 prior to the administration of ouabain. The bar graph below (Panel B) 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 Oua (n=13
myocytes from 6 hearts) and 159.7 ± 13.3 % of control Oua + KN93 (n=14 myocytes from 6 hearts). To further confirm the central role played by CaMKII in ouabain-induced spontaneous activity by non-pharmacological means, two different strategies were followed: 1) we overexpressed CaMKIIδc (Adv.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 GFP, 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 (Adv.βgal). The representative tracings depicted in panel C 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 due to the prolonged culture period. As in fresh cells, ouabain increased spontaneous activity in both βgal and CaMKII overexpressing cells. However, the incidence of spontaneous contractile activity was significantly higher in AdCaMKII cells. In contrast, CaMKII overexpression did not affect the ouabain-induced positive inotropic effect. The bar graphs below 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.
Consistently, myocytes isolated from WT mice show a similar increase in the number of NSE in response to ouabain 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.**

Ca²⁺ 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. At least two factors have been shown to underlie spontaneous SR Ca²⁺ release or Ca²⁺ waves: 1) an increase in SR Ca²⁺ load and 2) an increase in the sensitivity of the RyR for Ca²⁺ release. CaMKII has been shown to enhance both these processes, suggesting its potential involvement in CTS-induced arrhythmias. However, whether CaMKII favours ouabain-induced arrhythmogenicity by affecting SR Ca²⁺ load, the sensitivity of the RyR for Ca²⁺ release or both these mechanisms, is unknown.

*a) Effect of CaMKII inhibition on ouabain-induced increase in SR Ca²⁺ load.*

Figure 5A shows the effect of ouabain on the phosphorylation of the CaMKII-dependent phospholamban residue, Thr17. Ouabain significantly increased Thr17 phosphorylation and this effect was prevented by 2.5 µmol/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 μmol/l KN93 (n=8 from 4 hearts). These results suggest that although ouabain enhances PLN phosphorylation, which would increase SERCA2a activity and favour 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 wild type and PLN-DM myocytes, indicating that targets other than PLN are involved in ouabain-induced, CaMKII-dependent spontaneous activity.

b) Effect of CaMKII on RyR phosphorylation, SR Ca$^{2+}$ leak and spontaneous Ca$^{2+}$ waves.

Figure 6A shows ouabain significantly increased the phosphorylation of the CaMKII-dependent RyR residue, Ser2814 and that this effect was prevented by 2.5 μmol/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$^{16}$. Using confocal imaging we assessed Ca$^{2+}$ spark frequency under resting conditions which reflects Ca$^{2+}$ leak from the SR$^{18}$. After 20 min 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). Both the ouabain-induced increase in Ca$^{2+}$ spark frequency and in Ca$^{2+}$ wave occurrence were prevented by KN93, indicating that CaMKII underlies these events.

**CaMKII inhibition prevents ouabain-induced arrhythmias in vivo.**

In the absence of ouabain, wild type 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 AV block due to 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; Fig 7B) and absent in SR-AIP mice (n=5; Fig 7C). Table 1 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. 9 out of 11 (18 % survival) mice treated with ouabain died after treatment whereas, 6 out 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.
CaMKII inhibition prevents digoxin-induced spontaneous activity

To examine whether the observed effect of ouabain on spontaneous activity was common to other related cardiotonic 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, non toxic but inotropic dose of Dig (10 \(\mu M\)) \(^{19}\) 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 \(\mu M\) 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 due to the presence of adverse toxic effects, characterized primarily by arrhythmias and as recently shown by us and others, by apoptosis\(^{2,8}\), 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\(^{15}\), could increase RyR open
probability and lead to spontaneous diastolic SR Ca\(^{2+}\) release that could activate a transient inward current (\(I_{\text{t}}\)), responsible for the generation of DAD’s, spontaneous action potentials and ventricular arrhythmias.  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\(^9,16\). 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 \textit{in vitro} and \textit{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.

\textit{Ouabain toxicity and CaMKII}

It is now generally accepted that ouabain increases intracellular Na\(^{+}\) through the inhibition of NKA. The resulting reduction of the transarcolemmal Na\(^{+}\) gradient favors the reverse mode of the NCX which increases intracellular Ca\(^{2+}\) load and results in a positive inotropic effect. It has been proposed that when this Ca\(^{2+}\) load exceeds the
capacity of the SR, abnormal Ca\(^{2+}\) release occurs, which in turn triggers abnormal electrical 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 & 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 analogue 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 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 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 et al. were the first to describe that CaMKII triggers a NCX-dependent arrhythmogenic transient inward current (I\textit{ti}) through its effect of SR Ca\(^{2+}\) load/release\(^20\). Said et al. 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 Ca\textsuperscript{2+} load and favoring DAD formation\textsuperscript{21}. Using a protocol similar to the one used in the present study, Curran et al. also implicated CaMKII in DAD-triggered arrhythmias of the failing heart\textsuperscript{12}. In this case, the authors concluded that CaMKII increases the sensitivity of the RyR, lowering the threshold for spontaneous Ca\textsuperscript{2+} release and thus providing the arrhythmogenic substrate. These studies clearly define the two main factors responsible for increasing the propensity for Ca\textsuperscript{2+} waves and subsequent DADs, increased SR Ca\textsuperscript{2+} load and/or increased Ca\textsuperscript{2+} sensitivity of the RyR. We showed that ouabain increased both SR Ca\textsuperscript{2+} load (figure 5) and the phosphorylation of the RyR, associated with SR Ca\textsuperscript{2+} leak (figure 6), indicative of increased Ca\textsuperscript{2+} sensitivity of the RyR. However, CaMKII inhibition, which prevented ouabain-induced arrhythmias, did not affect SR Ca\textsuperscript{2+} load whereas it reduced RyR phosphorylation, Ca\textsuperscript{2+} leak and Ca\textsuperscript{2+} wave propensity (Figure 6). Similar to the conclusion of Curran et al. these results suggest that CaMKII would primarily mediate ouabain-induced arrhythmias through increasing the sensitivity of the RyR to Ca\textsuperscript{2+}. 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 Ca\textsuperscript{2+} load, given that this phosphorylation is known to enhance SERCA2a activity and SR Ca\textsuperscript{2+} uptake. However, in the rat, a species with high resting SR Ca\textsuperscript{2+}\textsuperscript{2\textsuperscript{22}}, ouabain can fully load the SR through Ca\textsuperscript{2+} influx via the NXC and therefore CaMKII-dependent PLN phosphorylation would not further increase SR Ca\textsuperscript{2+} load. Our results showing that ouabain-induced spontaneous contractile activity was similar between PLN-DM myocytes and WT myocytes (fig 5) would support the contention that
CaMKII-dependent PLN phosphorylation is not involved in ouabain-induced arrhythmias. However, another possible interpretation to our results in 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 L-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 Coll. who elegantly showed that increasing RyR receptor open probability alone is not enough to produce arrhythmogenic diastolic Ca\(^{2+}\) release 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 (NCXm). These results obtained using
a different species and by inducing arrhythmias with ouabain plus isoproterenol 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,
NCXm 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.

Consistently with our findings, several studies have demonstrated that catecholaminergic
polymorphic ventricular tachycardia (CPVT), 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\(^6\). More importantly, CPVT 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 CPVT and digitalis-induced arrhythmias.

In summary, we have presented evidence indicating that cardiotonic 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\(^29\) has been reported. Finally, although the benefit
of glycoside therapy in patients with end stage 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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**Conflict of Interest Disclosures:** None

**References:**


24. Satoh H, Ginsburg KS, Qing K, Terada H, Hayashi H, Bers DM. KB-R7943 block of Ca(2+) influx via Na(+)/Ca(2+) exchange does not alter twitches or glycoside inotropy but prevents Ca(2+) overload in rat ventricular myocytes. Circulation. 2000;101:1441-1446.


27. Liu N, Ruan Y, Denegri M, Bachetti T, Li Y, Colombi B, Napolitano C, Coetzee WA, Priori SG. Calmodulin kinase II inhibition prevents arrhythmias in


Table 1. Frequency of arrhythmias in ouabain treated mice. Overall data showing the incidence of ventricular ectopic beats (premature or escape beats) and sustained ventricular tachycardia in Balb-c mice in the absence and presence of CaMKII inhibition (KN 93 and SR-AIP mice). Sustained ventricular tachycardia (VT) was defined as a run of > 10 ventricular ectopic beats.

<table>
<thead>
<tr>
<th>Number of mice</th>
<th>WT mice + Oua</th>
<th>WT mice + Oua + KN93</th>
<th>SR-AIP mice + Oua</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT mice + Oua</td>
<td>11 (100%)</td>
<td>9 (100%)</td>
<td>5 (100%)</td>
</tr>
<tr>
<td>AV Block</td>
<td></td>
<td></td>
<td></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>

Figure Legends:

Figure 1. Ouabain activates CaMKII and induces spontaneous Ca^{2+} release and contractile activity in rat myocytes. A: Representative continuous recordings of cell length and Ca^{2+} showing that ouabain produces a typical positive inotropic effect during pacing at 0.5Hz and a large number of spontaneous Ca^{2+} releases and contractile activity during the following non-stimulated period. Control cells in the absence of ouabain show stable
Ca²⁺-contraction cycles during the 20 min stimulation period and few spontaneous events in the absence of electrical stimulation.  

**B:** Representative blots and overall results showing that ouabain increases the activity of CaMKII (P-CaMKII) and its prevention with KN93. Data are expressed as means ± S.E.M. from 5 independent experiments from 5 hearts. (* p<0.05, One-way ANOVA-Newman Keuls).

**Figure 2.** CaMKII inhibition prevents ouabain-induced spontaneous contractile activity but does not affect its positive inotropic effect.  

**A:** Representative continuous chart recordings of cell length showing that KN93 does not prevent the ouabain-inotropy but reduces its spontaneous contractile activity. In contrast, the inactive analogue, KN92, did not prevent ouabain-induced NSE. Panel **B** shows average results of these experiments indicating that the number of ouabain-induced NSE in rat cells were significantly reduced by CaMKII inhibitors, KN93 and AIP, and by inhibiting SR function with Ry + TG but not by KN92. Data are medians ± percentiles for 6 to 14 cells from 6 hearts per group. (* p<0.05, Kruskal Wallis One way ANOVA-Dunn’s method).

**Figure 3.** Overexpression of CaMKII enhances ouabain-induced spontaneous activity.  

**A:** 24 hours after infection, coexpression of GFP demonstrates visually that β-galactosidase (left) and CaMKII (right) are being expressed in the cells.  

**B:** Representative blots and overall results of phospho-CaMKII and GAPDH confirmed the overexpression of CaMKII in Ad.CaMKII vs. Ad.βgal infected cells.  

**C:** Representative continuous chart recordings of cell length showing that CaMKII overexpression exacerbates ouabain-induced NSE. The bar graph below depicts overall
data showing that in β-galactosidase expressing cells, ouabain produces few NSE
during the non-stimulated period whereas in CaMKII overexpressing cells, the number
of NSE during the non-stimulated period is significantly exacerbated with respect to
Adv.βgal infected cells. CaMKII overexpression did no significantly affect ouabain-
inotropy. Data are medians ± percentiles for n=6 cells from 3 hearts per group. (*
p<0.05, Kruskal Wallis One way ANOVA-Dunn’s method).

**Figure 4.** SR targeted CaMKII inhibition prevents ouabain-induced spontaneous activity.

**A:** Representative continuous chart recordings of cell length showing that in the
presence of ouabain, WT mice myocytes present a large number of NSE whereas
myocytes from mice expressing the SR-targeted CaMKII inhibitor, AIP, have a reduced
number of events. The Bar graph in panel **B** depicts the overall data of these
experiments. Data are medians ± percentiles for n=6 cells from 3 hearts per group. (*
p<0.05, Mann Whitney rank sum test-). Panel **C** shows that SR-targeted AIP does no
significantly affect ouabain-inotropy. Data are means ± S.E.M for n=15 cells from 6
hearts.

**Figure 5.** Effect of ouabain on SR Ca²⁺ load and phospholamban phosphorylation. **A:**
Representative blots and overall results showing that ouabain increases the
phosphorylation of PLN at its Thr17 site and that KN93 prevents this increase. Data are
expressed as means ± S.E.M. from 5 independent experiments from 5 hearts. * p<0.05,
One-way ANOVA-Newman Keuls. **B:** Typical tracings and overall results of caffeine
pulses performed, to estimate SR Ca²⁺ 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 One-way ANOVA-Neumann Keuls n=8 cells from 4 hearts C: Overall results of the effect of ouabain on the number of non-stimulated 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-)

**Figure 6.** Effect of ouabain on RyR phosphorylation, Ca\(^{2+}\) spark and wave frequency.  
A: Representative blots and overall results showing that ouabain increases RyR Ser2814 phosphorylation and that KN93 prevents this increase. Data are expressed as means ± S.E.M. from 5 independent experiments form 5 hearts. * p<0.05 One-way ANOVA-Newman Keuls. B: Original confocal line-scan images of myocytes in the absence of ouabain or either in the presence of ouabain alone or ouabain + KN93. The bar graphs on the right indicate that ouabain significantly increased Ca\(^{2+}\) spark frequency and Ca\(^{2+}\) wave occurrence and that these effects were inhibited by KN93. Data are medians ± percentiles for 6 to 8 cells from 3 hearts per group. (* p<0.05, Kruskal Wallis One way ANOVA-Dunn’s method).

**Figure 7.** *In vivo* ECG of ouabain-induced arrhythmias in the absence and presence of CaMKII inhibition. Representative traces showing the progressive effects of ouabain on cardiac rhythm (A), in the presence of KN93 (B) and in SR-AIP mice (C). Figure 7A 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).

**Figure 8.** CaMKII inhibition reduces digoxin-induced increase in Ca$^{2+}$ spark and wave frequency and prevents spontaneous contractile activity.  

**A:** Overall results showing the effect of digoxin (Dig) in the presence and absence of KN93 on the number of non-stimulated events in rat myocytes. Data are medians ± percentiles from 5 cells from 3 hearts per group. (* $p<0.05$, Kruskal Wallis One way ANOVA-Dunn’s method)  

**B:** Overall results showing the effect of Dig in the presence and absence of KN93 on SR Ca$^{2+}$ load. Data are means ± S.E.M from 5 cells from 3 hearts per group. * $p<0.05$ One-way ANOVA-Newman Keuls  

**C:** Original confocal line-scan images of myocytes in the absence of Dig or either in the presence of 10 and 75 $\mu$M Dig or Dig 75 $\mu$M + KN93. The bar graphs on the right indicate that Dig significantly increased Ca$^{2+}$ spark frequency and Ca$^{2+}$ wave occurrence and that these effects were inhibited by KN93. Data are medians ± percentiles from 5 cells from 3 hearts per group. * $p<0.05$, Kruskal Wallis One way ANOVA-Dunn’s method.
A

Oua

5μm

0.5Hz

Oua

KN93

5μm

0.5Hz

Oua

KN92

4μm

0.5Hz

B

Number of NSE/10min

Cont  Oua  Oua+KN93  Oua+KN92  Oua+AIP  Oua+Rv&TG

*  *  *
CaMKII Mediates Digitalis-Induced Arrhythmias
Luis A. Gonano, Marisa Sepúlveda, Yanina Rico, Marcia Kaetzel, Carlos A. Valverde, John Dedman, Alicia Mattiazi and Martin Vila Petroff

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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 \(~2 \times 10^4\) 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 \(\text{Ca}^{2+}\). The SR \(\text{Ca}^{2+}\) content was evaluated by the amplitude of the \(\text{Ca}_i\) 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 (Bradilla, 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\(^7\). This dose is in the lower limit of toxicity in the rat heart, a species with extremely low sensitivity to ouabain\(^8\). 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\(^9\). 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 assessed 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.

References


4. Mundina-Weilenmann C, Vittone L, Ortale M, de Cingolani GC, Mattiazzi A. Immunodetection of phosphorylation sites gives new insights into the


