Calcium-Calmodulin Kinase II Mediates Digitalis-Induced Arrhythmias

Luis A. Gonano, MD; Marisa Sepúlveda, BS; Yanina Rico, BS; Marcia Kaetzel, PhD; Carlos A. Valverde, PhD; John Dedman, PhD; Alicia Mattiazzi, MD; Martin Vila Petroff, PhD

Background—Digitalis-induced Na^+ accumulation results in an increase in Ca^{2+}_i via the Na^+/Ca^{2+} exchanger, leading to enhanced sarcoplasmic reticulum (SR) Ca^{2+} load, responsible for the positive inotropic and toxic arrhythmogenic effects of glycosides. A digitalis-induced increase in Ca^{2+}_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 Ca^{2+} 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, autocamtide-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 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. **(*Circ Arrhythm Electrophysiol*. 2011;4:947-957.)**

Key Words: cardiotonic steroids ■ arrhythmias ■ CaMKII ■ heart failure

Cardiotonic glycosides selectively bind to and inhibit the sarcolemmal Na^+/K^+-ATPase 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 in 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 arrhythmic/toxic effects that conspire against their extensive use in the clinical practice.[2] The arrhythmic effects have been proposed to occur when the SR 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. These transient increases in Ca^{2+}_i (Ca^{2+} waves) activate a transient inward (depolarizing) current (I_{ti}), primarily mediated by the forward-mode NCX current. This I_{ti} 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[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, phospholamban (PLN) knock-out mice, which have a fully loaded SR, have not proven to be prone to arrhythmias under basal conditions.[4,5] Moreover, a recent report showed that ouabain-induced DADs could be prevented by using JTV-519, a putative RyR stabilizer,[6] suggesting that the underlying alteration responsible for ouabain-induced arrhythmias was at the level of the RyR rather than on the SR Ca^{2+} load. Indeed, in addition to SR Ca^{2+} overload, an increase in RyR open probability, resulting in enhanced SR

Clinical Perspective on p 957

Received April 8, 2011; accepted October 5, 2011.

From the Centro de Investigaciones Cardiovasculares (L.A.G., M.S., Y.R., C.A.V., A.M., M.V.P.), Conicet La Plata, Facultad de Ciencias Médicas, Universidad Nacional de La Plata, La Plata, Argentina; Department of Cancer and Cell Biology (M.K., J.D.), Genome Research Institute, University of Cincinnati Medical Center, Cincinnati, OH.

The online-only Data Supplement is available at http://circep.ahajournals.org/lookup/suppl/doi:10.1161/CIRCEP.111.964908/-/DC1.

Correspondence to Dr. Martin Vila Petroff, Centro de Investigaciones Cardiovasculares, Facultad de Ciencias Médicas, 60 y 120, La Plata 1900, Argentina. E-mail mvila@aetos.med.unlp.edu.ar

Ca^{2+} leak, is also a well-known substrate for triggering Ca^{2+} waves, I_{ti}, 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+}_i 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 arrythmias[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.[10] 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).[10] 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_iT increases with subsequent contractions of myocytes.[11] These spontaneous Ca_iT 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). 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 CaMKIIδ_C and the green fluorescent protein genes, each under separate cytomegalovirus 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.[13] 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 $P \leq 0.05$. 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.[8] 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).

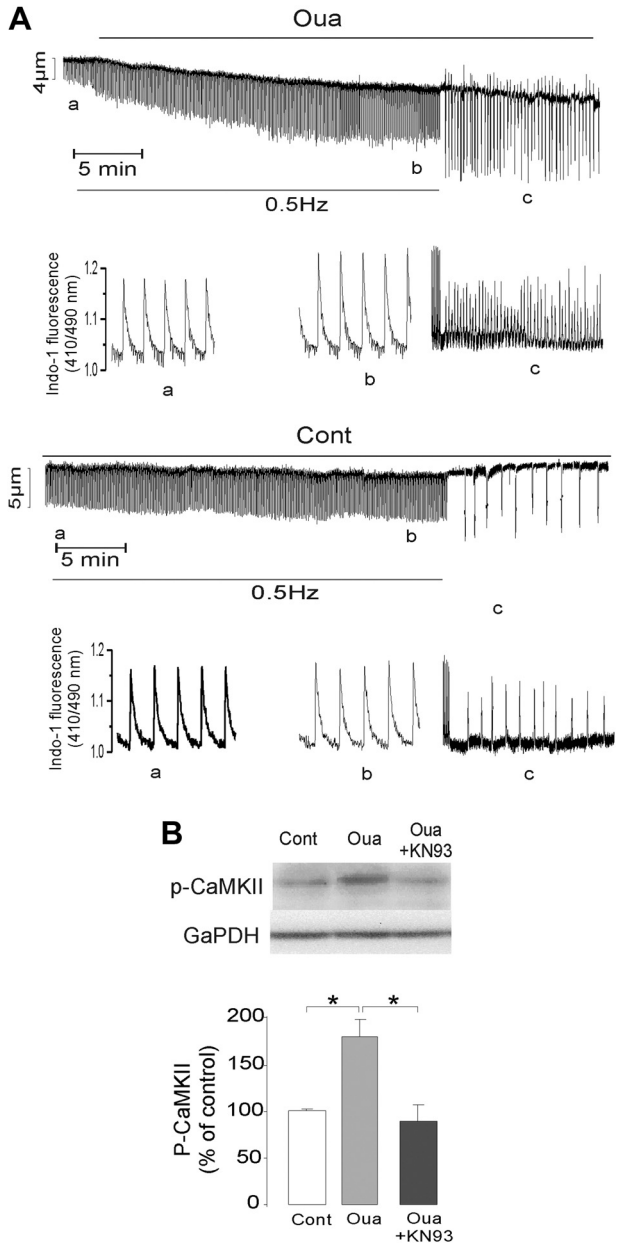

Figure 1. Ouabain activates calcium-calmodulin kinase II (CaMKII) and induces spontaneous Ca^{2+} release and contractile activity in rat myocytes. **A**, Representative continuous recordings of cell length and Ca^{2+}_i show that ouabain produces a typical positive inotropic effect during pacing at 0.5 Hz, and a large number of spontaneous Ca^{2+} releases and contractile activity during the following nonstimulated period. Control cells in the absence of ouabain show stable Ca^{2+} contraction cycles during the 20-minute stimulation period and few spontaneous events in the absence of electric stimulation. **B**, Representative blots and overall results show that ouabain increases the activity of CaMKII (p-CaMKII) and its prevention with KN93. Data are expressed as means±SEM from 5 independent experiments from 5 hearts. (*$P<0.05$, 1-way ANOVA, Newman-Keuls).

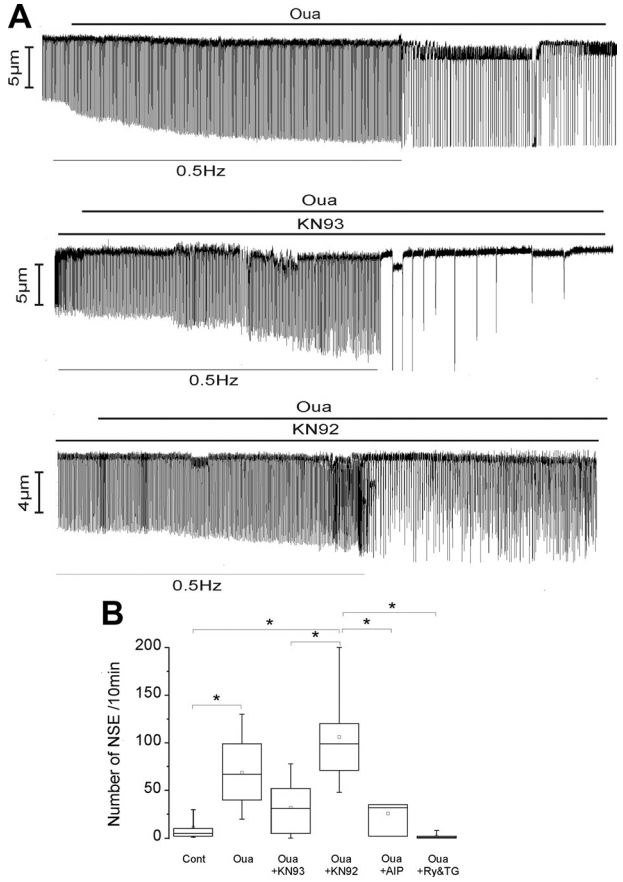

Figure 2. Calcium-calmodulin kinase II (CaMKII) inhibition prevents ouabain-induced spontaneous contractile activity but does not affect its positive inotropic effect. **A**, Representative continuous chart recordings of cell length show that KN93 does not prevent ouabain inotropy but reduces its spontaneous contractile activity. In contrast, the inactive analog, KN92, did not prevent ouabain-induced nonstimulated contractile events (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 sarcoplasmic reticulum (SR) function with ryanodine and thapsigargin but not by KN92. Data are medians±percentiles for 6 to 14 cells from 6 hearts per group (*$P<0.05$, Kruskal-Wallis 1-way ANOVA, Dunn method).

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

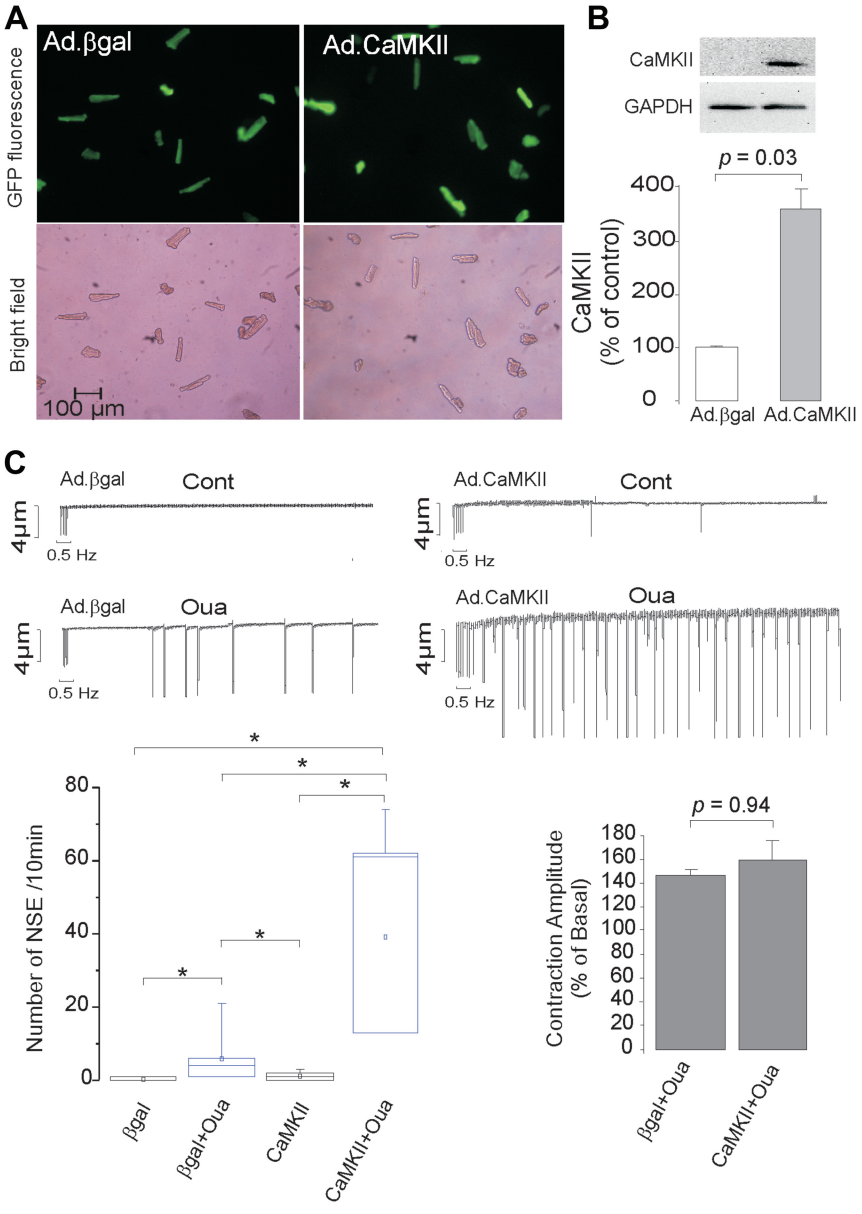

Figure 3. Overexpression of calcium-calmodulin kinase II (CaMKII) enhances ouabain-induced spontaneous activity. **A**, Twenty-four hours after infection, coexpression of green fluorescent protein demonstrates visually that β-galactosidase (left) and CaMKII (right) are being expressed in the cells. **B**, Representative blots and overall results of phospho-CaMKII and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) confirmed the overexpression of CaMKII in Ad.CaMKII vs Ad.β-galactosidase-infected cells. **C**, Representative continuous chart recordings of cell length show that CaMKII overexpression exacerbates ouabain-induced nonstimulated contractile events (NSE). The bar graph below depicts overall data showing that in β-galactosidase-expressing cells, ouabain produces few NSE during the nonstimulated period, whereas in CaMKII-overexpressing cells, the number of NSE during the nonstimulated period is significantly exacerbated with respect to Adv.β-galactosidase-infected cells. CaMKII overexpression did not significantly affect ouabain inotropy. Data are medians± percentiles for n=6 cells from 3 hearts per group (*$P<0.05$, Kruskal-Wallis 1-way ANOVA, Dunn method).

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 CaMKIIδc (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 activity in both β-galactosidase and CaMKII overexpressing 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.

Consistently, myocytes isolated from WT mice show a similar increase in the number of NSE in response to ouabain

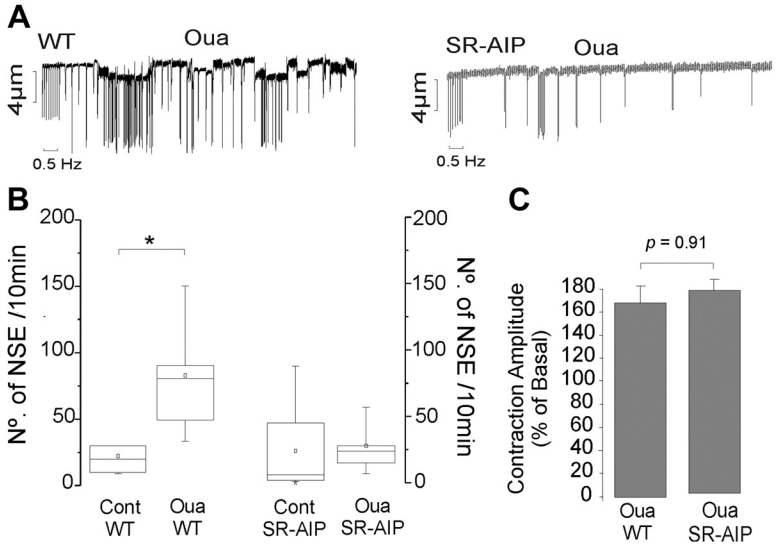

Figure 4. Sarcoplasmic reticulum (SR)-targeted calcium-calmodulin kinase II (CaMKII) inhibition prevents ouabain-induced spontaneous activity. **A**, Representative continuous chart recordings of cell length show that in the presence of ouabain, wild-type mice myocytes present a large number of nonstimulated contractile events, whereas myocytes from mice expressing the SR-targeted CaMKII inhibitor AIP have a reduced number of events. The bar graph in **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). **C** shows that SR-targeted AIP does not significantly affect ouabain inotropy. Data are means±SEM for n=15 cells from 6 hearts.

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+} load 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 cardiotonic 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 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 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 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 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

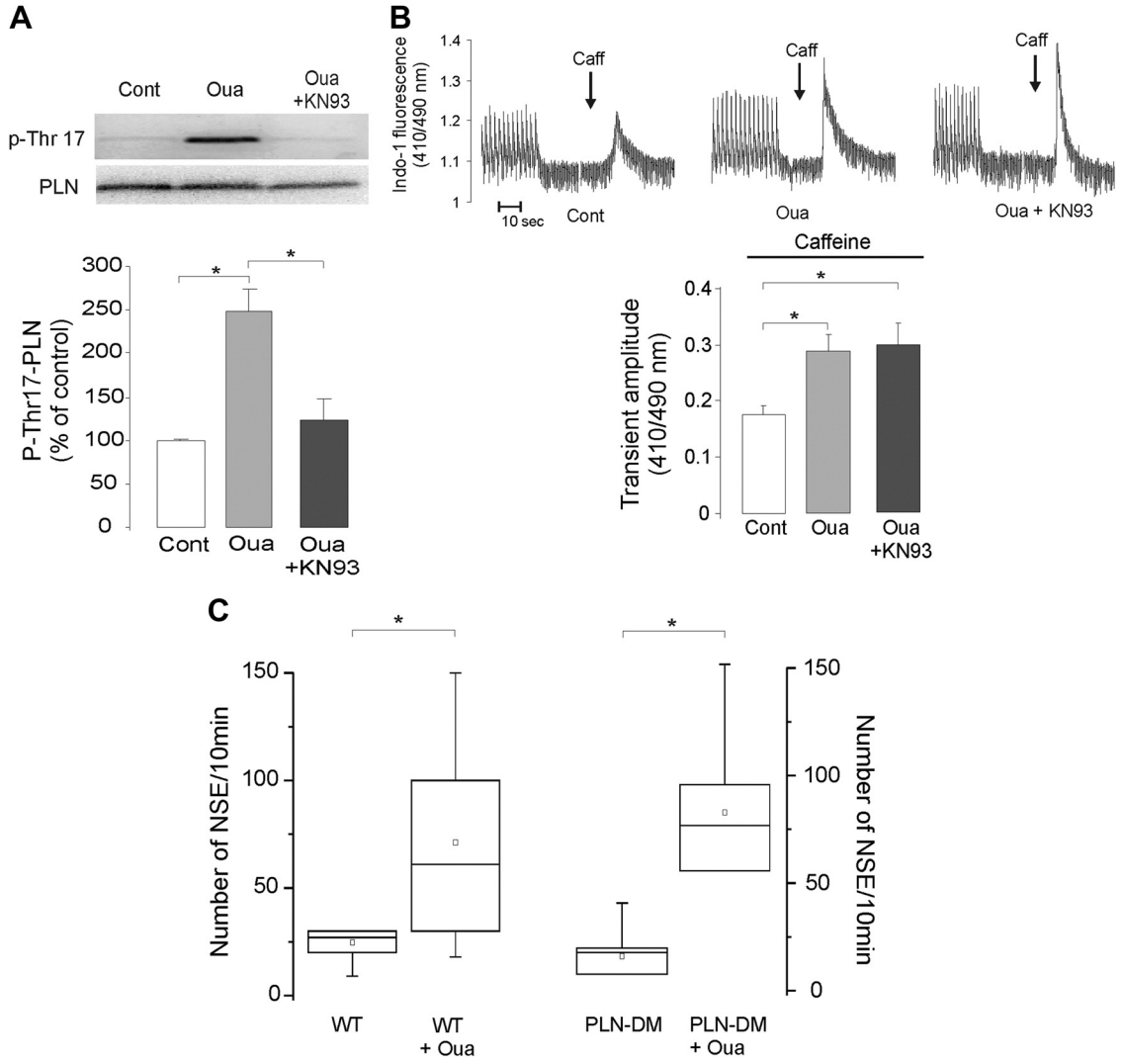

Figure 5. Effect of ouabain on sarcoplasmic reticulum (SR) Ca^{2+} load and phospholamban phosphorylation. **A**, Representative blots and overall results show that ouabain increases the phosphorylation of PLN at its Thr17 site and that KN93 prevents this increase. Data are expressed as means±SEM from 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).

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.

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, 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-

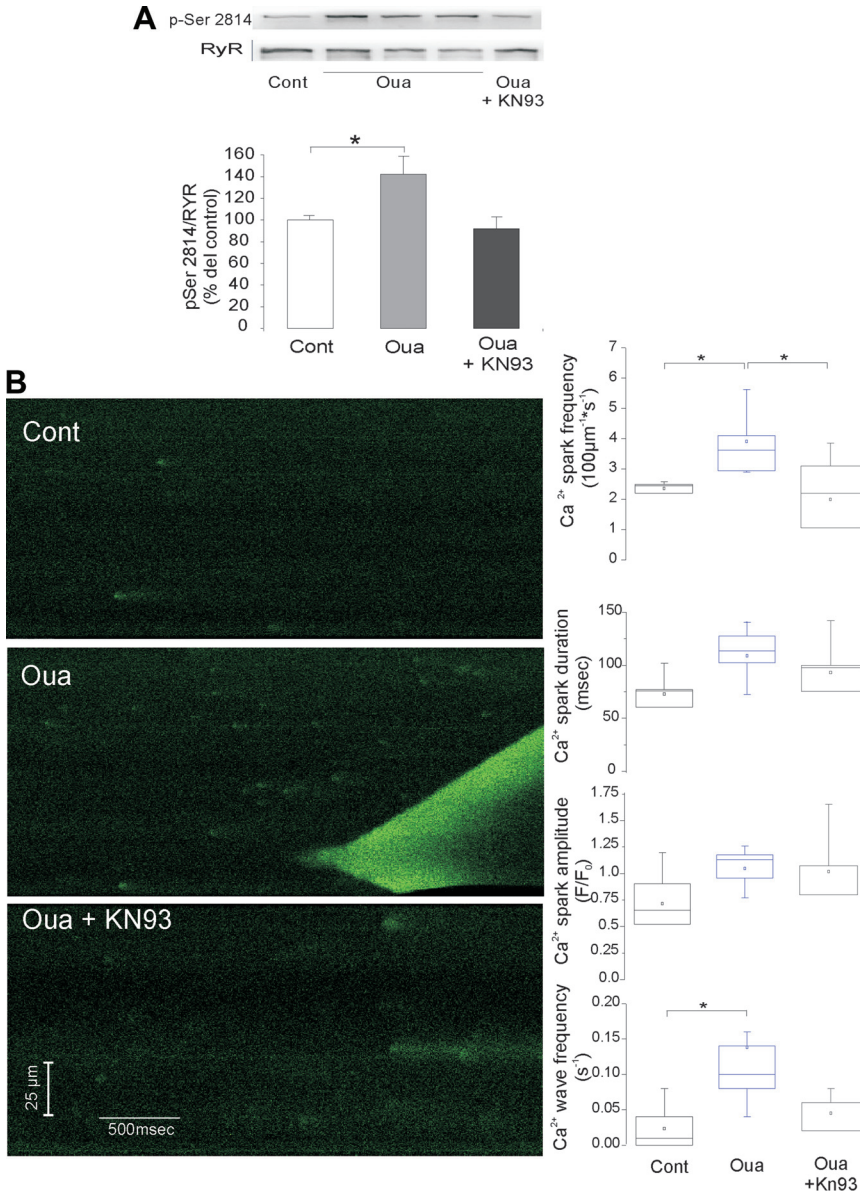

Figure 6. Effect of ouabain on ryanodine receptor (RyR) phosphorylation, Ca^{2+} spark and wave frequency. **A**, Representative blots and overall results show that ouabain increases RyR Ser2814 phosphorylation and that KN93 prevents this increase. Data are expressed as means±SEM from 5 independent experiments from 5 hearts (*$P<0.05$, 1-way ANOVA, Newman-Keuls) **B**, 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 1-way ANOVA, Dunn method).

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,[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 I_{ti}, responsible for the generation of DADs, spontaneous action potentials, and ventricular arrhythmias. A digitalis-induced increase in $Ca^{2+}{}_i$ 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 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.

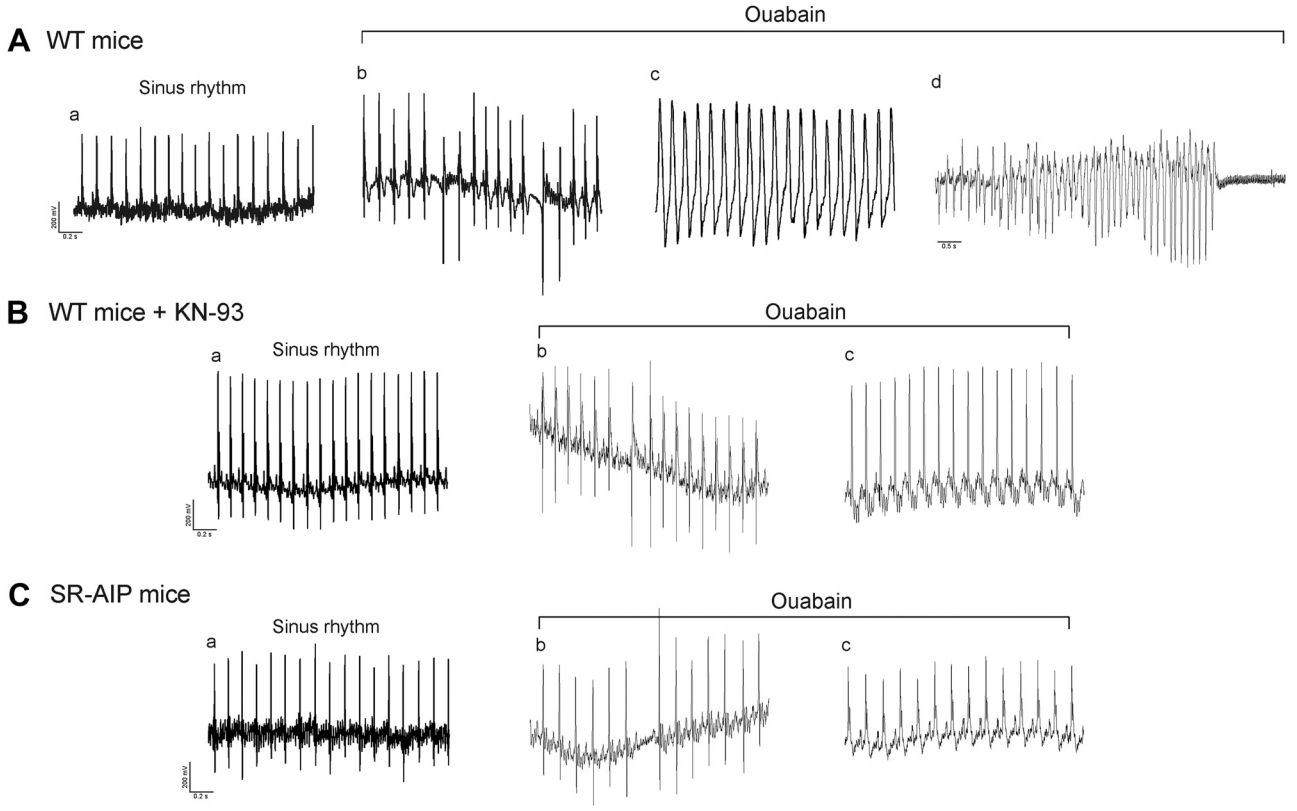

Figure 7. In vivo ECG of ouabain-induced arrhythmias in the absence and presence of calcium-calmodulin kinase II (CaMKII) inhibition. Representative traces show the progressive effects of ouabain on cardiac rhythm (**A**), in the presence of KN93 (**B**), and in SR-AIP mice (**C**). **A** shows that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

Ouabain Toxicity and CaMKII

It is now generally accepted that ouabain increases intracellular Na^+ through the inhibition of Na^+/K^+-ATPase. 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 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.

Table. Frequency of Arrhythmias in Ouabain-Treated Mice

	WT Mice +Ouabain	WT Mice +Ouabain+KN93	SR-AIP Mice +Ouabain
No. of mice	11	9	5
Sinus bradycardia/AV block	11 (100%)	9 (100%)	5 (100%)
Ventricular ectopic beats (>1/h)	9 (81.2%)	3 (33.3%)	1 (20%)
Sustained VT	9 (81.2%)	2 (22.2%)	0 (0%)

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.

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 and colleagues were the first to describe that CaMKII triggers an NCX-dependent arrhythmogenic transient I_{ti} through its effect of SR Ca^{2+} load/release.[20] Said

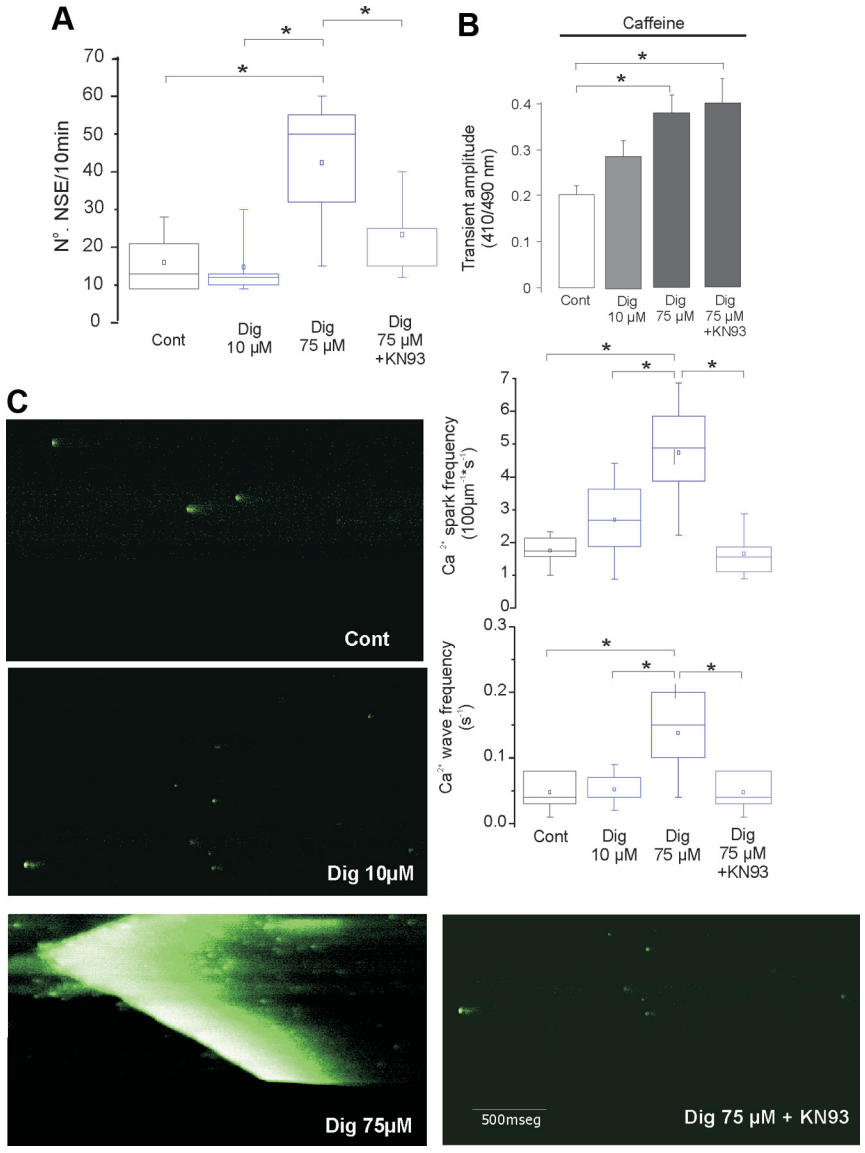

Figure 8. Calcium-calmodulin kinase II (CaMKII) inhibition reduces digoxin (DIG)-induced increase in Ca^{2+} spark and wave frequency and prevents spontaneous contractile activity. **A**, Overall results show the effect of DIG in the presence and absence of KN93 on the number of nonstimulated events in rat myocytes. Data are medians±percentiles from 5 cells from 3 hearts per group (*$P<0.05$, Kruskal-Wallis 1-way ANOVA, Dunn method). **B**, Overall results show the effect of DIG in the presence and absence of KN93 on sarcoplasmic reticulum (SR) Ca^{2+} load. Data are means±SEM from 5 cells from 3 hearts per group (*$P<0.05$, 1-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 μmol/L DIG or DIG 75 μmol/L + 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 1-way ANOVA-Dunn method).

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 Ca^{2+} 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 Ca^{2+} release and thus providing the arrhythmogenic substrate. These studies clearly define the 2 main factors responsible for increasing the propensity for Ca^{2+} waves and subsequent DADs increased SR Ca^{2+} load, and/or increased Ca^{2+} sensitivity of the RyR. We showed that ouabain increased both SR Ca^{2+} load (Figure 5) and the phosphorylation of the RyR associated with SR Ca^{2+} leak (Figure 6), indicative of increased Ca^{2+} sensitivity of the RyR; however, CaMKII inhibition, which prevented ouabain-induced arrhythmias, did not affect SR Ca^{2+} load, whereas it reduced RyR phosphorylation, Ca^{2+} leak, and Ca^{2+} 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 Ca^{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^{2+} load, given that this phosphorylation is known to enhance SERCA2a activity and SR Ca^{2+} uptake; however, in the rat, a species with high resting SR Ca^{2+},[22] ouabain can fully load the SR through Ca^{2+} influx via the NXC, and, therefore, CaMKII-dependent PLN phosphorylation would not further increase SR Ca^{2+} 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 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 colleagues, 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. 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, 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.[6] 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 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] 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.

Acknowledgments

We gratefully acknowledge the technical support of Mónica Rando and veterinarian Pablo Stringa and confocal imaging assistance of Vanina Perez. We also wish to thank Roger Hajjar for his generous gift of CaMKIIδc adenoviruses.

Sources of Funding

This study was supported by grants PICT 1727 from Agencia Nacional de Promoción Científica y Técnica (Agencia) and PIP 1448 from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) to M.V.P. and PICT 26117 (Agencia), PIP 2139 (CONICET) and Fogarty Grant # RO3TW07713 from NIH to A.M. and PICT 1770 to C.A.V.

Disclosures

None.

References

1. Altamirano J, Li Y, DeSantiago J, Piacentino V III, Houser SR, Bers DM. The inotropic effect of cardioactive glycosides in ventricular myocytes requires Na+-Ca2+ exchanger function. *J Physiol*. 2006;575:845–854.
2. Rathore SS, Curtis JP, Wang Y, Bristow MR, Krumholz HM. Association of serum digoxin concentration and outcomes in patients with heart failure. *JAMA*. 2003;289:871–878.
3. Ferrier GR. Digitalis arrhythmias: role of oscillatory afterpotentials. *Prog Cardiovasc Dis*. 1977;19:459–474.
4. Santana LF, Kranias EG, Lederer WJ. Calcium sparks and excitation-contraction coupling in phospholamban-deficient mouse ventricular myocytes. *J Physiol*. 1997;503:21–29.
5. Zhang T, Guo T, Mishra S, Dalton ND, Kranias EG, Peterson KL, Bers DM, Brown JH. Phospholamban ablation rescues sarcoplasmic reticulum Ca(2+) handling but exacerbates cardiac dysfunction in CaMKIIdelta(C) transgenic mice. *Circ Res*. 2010;106:354–362.
6. Sedej S, Heinzel FR, Walther S, Dybkova N, Wakula P, Groborz J, Gronau P, Maier LS, Vos MA, Lai FA, Napolitano C, Priori SG, Kockskämper J, Pieske B. Na+-dependent SR Ca2+ overload induces arrhythmogenic events in mouse cardiomyocytes with a human CPVT mutation. *Cardiovasc Res*. 2010;87:50–59.
7. Venetucci LA, Trafford AW, O'Neill SC, Eisner DA. The sarcoplasmic reticulum and arrhythmogenic calcium release. *Cardiovasc Res*. 2008;77:285–292.
8. Sapia L, Palomeque J, Mattiazzi A, Petroff MV. Na+/K+-ATPase inhibition by ouabain induces CaMKII-dependent apoptosis in adult rat cardiac myocytes. *J Mol Cell Cardiol*. 2010;49:459–468.
9. Erickson JR, Anderson ME. CaMKII and its role in cardiac arrhythmia. *J Cardiovasc Electrophysiol*. 2008;19:1332–1336.
10. Vila-Petroff M, Salas MA, Said M, Valverde CA, Sapia L, Portiansky E, Hajjar RJ, Kranias EG, Mundina-Weilenmann C, Mattiazzi A. CaMKII inhibition protects against necrosis and apoptosis in irreversible ischemia-reperfusion injury. *Cardiovasc Res*. 2007;73:689–698.
11. Sag CM, Wadsack DP, Khabbazzadeh S, Abesser M, Grefe C, Neumann K, Opiela MK, Backs J, Olson EN, Brown JH, Neef S, Maier SK, Maier LS. Calcium/calmodulin-dependent protein kinase II contributes to cardiac arrhythmogenesis in heart failure. *Circ Heart Fail*. 2009;2:664–675.
12. Curran J, Brown KH, Santiago DJ, Pogwizd S, Bers DM, Shannon TR. Spontaneous Ca waves in ventricular myocytes from failing hearts depend on Ca(2+)-calmodulin-dependent protein kinase II. *J Mol Cell Cardiol*. 2010;49:25–32.

13. Lotan CS, Miller SK, Pohost GM, Elgavish GA. Amiloride in ouabain-induced acidification, inotropy and arrhythmia: 23Na & 31P NMR in perfused hearts. *J Mol Cell Cardiol*. 1992;24:243–257.
14. Venetucci LA, Trafford AW, Eisner DA. Increasing ryanodine receptor open probability alone does not produce arrhythmogenic calcium waves: threshold sarcoplasmic reticulum calcium content is required. *Circ Res*. 2007;100:105–111.
15. Bers DM. *Excitation-contraction coupling and cardiac contractile force.* Dordrecht/Boston/London: Kluwer Academic Publishers; 2001.
16. Ai X, Curran JW, Shannon TR, Bers DM, Pogwizd SM. Ca2+/calmodulin-dependent protein kinase modulates cardiac ryanodine receptor phosphorylation and sarcoplasmic reticulum Ca2+ leak in heart failure. *Circ Res*. 2005;97:1314–1322.
17. Davis BA, Schwartz A, Samaha FJ, Kranias EG. Regulation of cardiac sarcoplasmic reticulum calcium transport by calcium-calmodulin-dependent phosphorylation. *J Biol Chem*. 1983;258:13587–13591.
18. Cheng H, Lederer WJ, Cannell MB. Calcium sparks: elementary events underlying excitation-contraction coupling in heart muscle. *Science*. 1993;262:740–744.
19. Alemanni M, Rocchetti M, Re D, Zaza A. Role and mechanism of subcellular Ca2+ distribution in the action of two inotropic agents with different toxicity. *J Mol Cell Cardiol*. 2011;50:910–918.
20. Wu Y, Roden DM, Anderson ME. Calmodulin kinase inhibition prevents development of the arrhythmogenic transient inward current. *Circ Res*. 1999;84:906–912.
21. Said M, Becerra R, Palomeque J, Rinaldi G, Kaetzel MA, Diaz-Sylvester PL, Copello JA, Dedman JR, Mundiña-Weilenmann C, Vittone L, Mattiazzi A. Increased intracellular Ca2+ and SR Ca2+ load contribute to arrhythmias after acidosis in rat heart. Role of Ca2+/calmodulin-dependent protein kinase II. *Am J Physiol Heart Circ Physiol*. 2008;295:H1669–H1683.
22. Shattock MJ, Bers DM. Rat vs. rabbit ventricle: Ca flux and intracellular Na assessed by ion-selective microelectrodes. *Am J Physiol*. 1989;256:C813–C822.
23. Brittsan AG, Ginsburg KS, Chu G, Yatani A, Wolska BM, Schmidt AG, Asahi M, MacLennan DH, Bers DM, Kranias EG. Chronic SR Ca2+-ATPase inhibition causes adaptive changes in cellular Ca2+transport. *Circ Res*. 2003;92:769–776.
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.
25. Tanaka H, Shimada H, Namekata I, Kawanishi T, Iida-Tanaka N, Shigenobu K. Involvement of the Na+/Ca2+ exchanger in ouabain-induced inotropy and arrhythmogenesis in guinea-pig myocardium as revealed by SEA0400. *J Pharmacol Sci*. 2007;103:241–246.
26. Liu T, Brown DA, O'Rourke B. Role of mitochondrial dysfunction in cardiac glycoside toxicity. *J Mol Cell Cardiol*. 2010;49:728–736.
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 RyR2(R4496C+/−) mice with catecholaminergic polymorphic ventricular tachycardia. *J Mol Cell Cardiol*. 2011;50:214–222.
28. Gottlieb SS, Rogowski AC, Weinberg M, Krichten CM, Hamilton BP, Hamlyn JM. Elevated concentrations of endogenous ouabain in patients with congestive heart failure. *Circulation*. 1992;86:420–425.
29. Hoch B, Meyer R, Hetzer R, Krause EG, Karczewski P. Identification and expression of delta-isoforms of the multifunctional Ca2+/calmodulin-dependent protein kinase in failing and nonfailing human myocardium. *Circ Res*. 1999;84:713–721.

CLINICAL PERSPECTIVE

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.