Calmodulin Kinase II Inhibition Shortens Action Potential Duration by Upregulation of $K^+$ Currents

Jingdong Li, Céline Marionneau, Rong Zhang, Vaibhavi Shah, Johannes W. Hell, Jeanne M. Nerbonne, Mark E. Anderson

Abstract—The multifunctional Ca$^{2+}$/calmodulin-dependent protein kinase II (CaMKII) is activated by elevated intracellular Ca$^{2+}$ (Ca$^{2+}$i), and mice with chronic myocardial CaMKII inhibition (Inh) resulting from transgenic expression of a CaMKII inhibitory peptide (AC3-I) unexpectedly showed action potential duration (APD) shortening. Inh mice exhibit increased L-type Ca$^{2+}$ current ($I_{Ca}$), because of upregulation of protein kinase A (PKA) activity, and decreased CaMKII-dependent phosphorylation of phospholamban (PLN). We hypothesized that CaMKII is a molecular signal linking Ca$^{2+}$i to repolarization. Whole cell voltage-clamp recordings revealed that the fast transient outward current ($I_{to}$) and the inward rectifier current ($I_{ki}$) were selectively upregulated in Inh, compared with wild-type (WT) and transgenic control, mice. Breeding Inh mice with mice lacking PLN returned $I_{to}$ and $I_{ki}$ to control levels and equalized the APD and QT intervals in Inh mice to control and WT levels. Dialysis of AC3-I into WT cells did not result in increased $I_{to}$ or $I_{ki}$, suggesting that enhanced cardiac repolarization in Inh mice is an adaptive response to chronic CaMKII inhibition rather than an acute effect of reduced CaMKII activity. Increasing PKA activity, by cell dialysis with cAMP, or inhibition of PKA did not affect $I_{ki}$ in WT cells. Dialysis of WT cells with cAMP also reduced $I_{to}$, suggesting that PKA upregulation does not increase repolarizing $K^+$ currents in Inh mice. These findings provide novel in vivo and cellular evidence that CaMKII links Ca$^{2+}$i to cardiac repolarization and suggest that PLN may be a critical CaMKII target for feedback regulation of APD in ventricular myocytes. (Circ Res. 2006;99:1092-1099.)

Key Words: Ca$^{2+}$/calmodulin-dependent protein kinase II ■ calcium signaling ■ repolarization

Cardiac contraction depends on a tightly orchestrated interplay between action potential duration (APD) and intracellular Ca$^{2+}$ (Ca$^{2+}$i). Increases in APD cause increases in Ca$^{2+}$i, leading to strengthened contraction.1 Changes in Ca$^{2+}$i, however, are also a source of feedback control for ionic currents that ultimately determine APD.2 The mechanisms for feedback regulation of APD by Ca$^{2+}$i remain to be elucidated, but we hypothesized that Ca$^{2+}$-activated signaling molecules may be important for regulating APD.

The multifunctional Ca$^{2+}$/calmodulin-dependent protein kinase II (CaMKII) is a serine/threonine kinase that is abundant in heart and targets key Ca$^{2+}$i homeostatic proteins.3 We studied mice with chronic cardiomyocyte-delimited CaMKII inhibition (Inh) by transgenic expression of a specific CaMKII inhibitory peptide (AC3-I). In addition to wild-type littermates (WT), we also used transgenic control mice (Con) with myocardial expression of an inactive, scrambled version of AC3-I (AC3-C). Inh mice have shortened optically recorded APDs, despite an increased inward L-type Ca$^{2+}$ current ($I_{Ca}$) that results from increased activity of protein kinase A (PKA).4 Because increased inward $I_{Ca}$ is unopposed by upregulation of other repolarizing currents, would cause APD lengthening rather than APD shortening, we hypothesized that CaMKII inhibition also increases repolarizing $K^+$ currents.

Most Ca$^{2+}$i for each heart beat is released from intracellular sarcoplasmic reticulum (SR) Ca$^{2+}$ stores.3 Overexpression of a SR Ca$^{2+}$ ATPase (SERCA) results in reductions in repolarizing $K^+$ currents and APD lengthening.2 On the other hand, Inh mice have reduced SR Ca$^{2+}$ content and APD abbreviation compared with Con or WT mice.3 These 2 findings suggested the hypothesis that SR Ca$^{2+}$ uptake is important for feedback regulation of APD. To test the potential role of CaMKII in this mechanism, we bred Inh (and Con) mice with mice null for phospholamban (PLN$^{-/-}$),6 a negative regulatory protein for SERCA and a phosphorylation target for CaMKII.7

Recordings from ventricular myocytes isolated from Inh mice revealed significant upregulation of 2 repolarizing $K^+$

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currents: the fast component of the transient outward current ($I_{to}$) and the inward rectifier current ($I_{k1}$), whereas other $K^+$ currents were unchanged compared with Con or WT cells. Increased $I_{to}$ and $I_{k1}$ appear to represent an adaptive response to chronic myocardial CaMKII inhibition and not an acute consequence of CaMKII inhibition, because neither $I_{to}$ nor $I_{k1}$ was increased in WT cells dialyzed with AC3-I. Furthermore, increased $I_{to}$ and $I_{k1}$ were not reversed by cell dialysis with a PKA inhibitory peptide in Inh cardiomyocytes, suggesting increased PKA activity is not involved in the feedback regulation of APD during CaMKII inhibition. In addition, quantitative real-time PCR (qRT PCR) and quantitative immunoblotting did not reveal correlative changes in the expression levels of several $K^+$ channel subunit genes/proteins, including subunits previously shown to contribute to $I_{to}$ and $I_{k1}$, in the ventricles of Inh mice. PLN ablation eliminated the differences in QT interval, APD, $I_{to}$ and $I_{k1}$ in Inh, Con and WT mice ex vivo and in vivo. These findings reveal that CaMKII is an important signal element for a previously unrecognized SR-dependent mechanism for feedback control of cardiac repolarization.

**Materials and Methods**

An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

**Mice With Myocardial CaMKII Inhibition**

The AC3-I (Inh) and AC3-C (Con) mice were generated by synthesis of a minigene based on the peptide sequence of AC3-I (KKAL-HRQEAVDCL) or AC3-C (KKALHAQERVDCL). This approach avoids the complications inherent in many previous studies using CaMKII inhibitory drugs that are known to also act as CaMKII-independent $K^+$ current antagonists. For some experiments, PLN-null mice (PLN$^{-/-}$) were bred with AC3-I and AC3-C mice for >4 generations.

**Cardiac Electrophysiology**

ECGs were recorded in vivo or ex vivo from Langendorff-perfused hearts. Total outward $K^+$ currents, $I_{k1}$, and $I_{k2}$ were recorded from isolated ventricular myocytes using previously described methods. Reagents for manipulating CaMKII and PKA activity were included in the pipette solution for some experiments: cAMP (1 mmol/L); protein kinase A inhibitory peptide (PKI) (50 μmol/L); CaMKII inhibitory peptide AC3-I and the inactive AC3-I congener peptide AC3-C (both at 30 μmol/L).

**K$^+$ Channel Subunit Protein Expression and mRNA Measurements**

Western blots and qRT PCR were performed on fractionated lysates prepared from the ventricles of 11- to 13-week-old male and female WT, Con, and Inh mice. Identical analyses were performed on hearts from PLN$^{-/-}$ and PLN$^{+/+}$ mice interbred with Con or Inh mice.

**Analysis of Ca$^{1.2}$ Phosphorylation**

Immunosignals for Cav1.2 were detected by chemiluminescence and quantified by densitometry as described.

**Data Analysis**

Data analysis was performed using SigmaStat. Values are means±SEM. Statistical significance was determined using ANOVA or Student’s $t$ test, as appropriate. Post hoc comparisons were assessed using Bonferroni’s correction for parametric data and Dunn’s test on ranks for nonparametric data. The null hypothesis was rejected for $P<0.05$.

**Results**

**Abbreviated Ventricular Repolarization in Hearts With Genetic CaMKII Inhibition**

The QT interval reflects the duration of ventricular repolarization. Inh mice had significantly shorter QT intervals compared with WT and Con mice in vivo and ex vivo (Figure 1). Spontaneous heart rates in isolated, Langendorff-perfused hearts were not different among Inh, WT, or Con hearts (supplemental Figure I), whereas PR intervals were prolonged in Inh hearts (supplemental Figure I), as occurs in vivo. The APDs at 90%, 75%, and 50% recovery to baseline from ventricular paced (RR=150 ms) WT (n=7), Inh (n=7), and Con (n=8) hearts. The vertical bars are arranged by group as in b and c. f, Representative surface ECG tracings. Summary data for QT90 (g) and QT90c (h) for WT (n=16), Inh (n=15), and Con (n=18) mice from surface ECGs. $^\dagger P<0.001$ and $^{**} P<0.01$ compared with WT and Con hearts.
Chronic CaMKII Inhibition

Increased I_{K1} or I_{to,f} in Inh mice. a, Representative I_{K1} currents recorded from Con (blue) and Inh (red) mice. Shown at left is I_{K1} in response to the voltage command ramp. The calibration bars are 1000 pA (vertical) and 1 second (horizontal). The right inset shows an expanded scale to illustrate the outward component of I_{K1} measured at −60 mV. The calibration bars are 50 pA (vertical) and 500 ms (horizontal). b and c, I_{K1} was measured at −100 mV (b) and −80 mV (c). The amplitude of I_{K1} is significantly greater in Inh compared with Con and WT at −100 mV (tP<0.001). c, I_{to,f} was measured at −80 mV (d). The slope conductance recorded at the calculated reversal potential for K^+ was significantly greater in Inh than Con or WT cardiomyocytes (tP=0.001).Vm indicates membrane potential; Erevo, reversal potential.

I_{K1} and I_{to,f} Are Selectively Increased in Mice With Chronic CaMKII Inhibition

Inh mice have increased I_{to,f} but an unopposed increase in L-type Ca^2+ current (I_{Ca}) should prolong the APD; therefore, we hypothesized that the mechanism for APD shortening by CaMKII inhibition was enhancement of repolarizing K^+ currents. We measured K^+ currents and found that peak outward K^+ current densities (at +80 mV) were significantly (P=0.05) higher in Inh (113±7 pA/pF; n=30), compared with WT (97±7 pA/pF; n=26) or Con (91±6 pA/pF; n=24) ventricular myocytes. Densities of I_{K1} (Figure 2) and I_{to,f} (Figure 3) were significantly upregulated in Inh, compared with Con and WT, cells. In contrast, other repolarizing K^+ currents were similar in Inh, Con, and WT ventricular myocytes (Figure 3). These findings show that with chronic in vivo CaMKII inhibition results in increased outward K^+ currents caused by a selective upregulation of 2 critical repolarizing currents and suggest that these currents are important for shortening repolarization in Inh mice.

Increases in I_{K1} and I_{to,f} did not appear to be part of an electrical remodeling program related to cardiomyocyte hypertrophy, because membrane capacitances were similar in WT (145.4±3.5 pF; n=27), Inh (138.6±5.1 pF; n=31), and Con (151.6±3.8 pF; n=27) myocytes, consistent with the similarities in Inh, Con, and WT hearts. We used qRT PCR to determine the expression levels of atrial natriuretic factor (ANF) and β-myosin heavy chain (β-MHC) as genetic markers of latent hypertrophy. ANF and β-MHC expression levels were not increased Inh (or Con), compared with WT hearts (supplemental Figure II). Taken together, these data suggest that upregulation of I_{K1} and I_{to,f} in Inh mice do not represent coordinate components of a hypertrophic signaling program.

Acute CaMKII Inhibition Does Not Increase I_{K1} or I_{to,f}

CaMKII activity is chronically suppressed in adult Inh mice because expression of the CaMKII antagonist peptide is driven by the α-MHC promoter that becomes active near the time of birth. However, the previous experiments do not provide insight into whether these changes are a direct result of CaMKII inhibition or, alternatively, reflect an indirect adaptive response to chronic CaMKII inhibition. We diazoyed ventricular myocytes isolated from WT mice with the Inh (AC3-I) peptide under conditions previously shown to prevent CaMKII actions on L-type Ca^2+ current. Acute (10-minute) exposure of ventricular myocytes to AC3-I failed to increase I_{K1} or I_{to,f} compared with WT cells without AC3-I or compared with WT cells diazoyed with the inactive control peptide AC3-C (supplemental Figure III). These findings do not support a connection between acute CaMKII inhibition and increased I_{K1} or I_{to,f} and, therefore, suggest that shortened repolarization observed in Inh mice is not directly related to reduced CaMKII-dependent phosphorylation of ion channel proteins.

Increased PKA Activity Directly Affects Ca_{1,2}

Upregulation of ventricular I_{to,f} in Inh mice is attributable to enhancement of PKA activity, without a change in expression of the pore-forming α1, Ca_{1,2} subunit. We used a phospho- and site-specific antibody to measure directly the phosphorylation state of α1 at Ser1928, a validated site for PKA phosphorylation. Western blot analyses revealed that phosphorylation of Ca_{1,2} at Ser1928 is significantly increased in Inh compared with Con hearts (Figure 4). There are 2 isoforms of α1, 1:2; a full-length 240-kDa form and a short form in which the C-terminal 300 residues have been cleaved off by the calcium-activated protease calpain. Because the short form is predicted to be more active than the
long form, we also evaluated the expression levels of the 2 isoforms separately. These experiments revealed no measurable differences in the expression level of either of the 2 Cav1.2 isoforms in Inh, compared with WT or Con, ventricles (Figure 4).

Increased PKA Activity Is Not a General Mechanism for Increasing \( I_{\text{K1}} \) and \( I_{\text{to,f}} \)

We next asked if enhanced PKA activity was a general compensatory response to chronic CaMKII inhibition and a mechanism for increasing \( I_{\text{K1}} \) and \( I_{\text{to,f}} \), similar to the PKA-dependent mechanism for \( I_{\text{Ca}} \) upregulation seen in Inh hearts. We dialyzed WT, Inh, and Con cells with the PKA activator cAMP or the PKA inhibitory peptide PKI for 10 minutes. We previously found that cAMP increased peak \( I_{\text{Ca}} \) in WT and Con myocytes to Inh levels, whereas PKI reduced \( I_{\text{Ca}} \) in Inh cells to WT and Con levels. In contrast, the increased \( I_{\text{K1}} \) in Inh, compared with Con and WT, ventricular myocytes was not consistently affected by dialysis with cAMP or PKI (Figure 5a and 5b), although PKI dialysis for 10 minutes increased peak \( I_{\text{K1}} \), recorded at \(-100\) mV, more in Inh than in WT or Con cells (Figure 5a). The larger inward component of \( I_{\text{K1}} \), recorded at \(-100\) mV, remained greater in Inh compared with WT or Con cells after cAMP (\( P=0.03 \)) or PKI (\( P=0.05 \)). These findings are not consistent with the concept that upregulation of PKA activity in Inh cardiomyocytes accounts for increases in \( I_{\text{K1}} \) in Inh cardiomyocytes.

Peak \( I_{\text{to,f}} \) was reduced significantly (\( P<0.001 \)) by cAMP in Inh, Con, and WT cells compared with cells dialyzed with PKI (Figure 5c). The pattern of relative increase in peak \( I_{\text{to,f}} \) recorded from Inh compared with WT and Con cardiomyocytes was maintained, but the differences among WT, Inh, and Con cells were no longer significantly different after 10 minutes of cAMP (\( P=0.129 \)) or PKI (\( P=0.199 \)). These findings suggest that \( I_{\text{to,f}} \) is responsive to PKA signaling but are most consistent with a model where PKA signaling reduces, rather than increases, peak \( I_{\text{to,f}} \). Taken together, these findings showed that upregulation of \( I_{\text{K1}} \) and \( I_{\text{to,f}} \) is not likely caused by increased PKA activity and suggest that PKA upregulation is not a general mechanism for cellular adaptation to chronic CaMKII inhibition.

Phospholamban Is Required for Enhanced Repolarization by CaMKII Inhibition

Inh mice have significantly reduced CaMKII-dependent PLN phosphorylation compared with Con and WT counterparts, and recent findings have shown that overexpression of SERCA1a markedly reduces the expression of repolarizing \( K^+ \) currents in heart. To test whether shortened repolarization observed in Inh mice was linked to the SR Ca\(^{2+} \)
regulation, we interbred the Inh and Con mice with mice lacking PLN (PLN−/−). PLN is a negative regulator of SR Ca2+ uptake that is phosphorylated by CaMKII. The reduction in SR Ca2+ content seen in Inh ventricular myocytes is prevented by PLN ablation, and ventricular myocytes from PLN−/− mice and PLN−/− mice expressing the CaMKII inhibitory peptide AC3-I (Inh/H11003) and expressing the inactive control peptide AC3-C (Con×PLN−/−) have equivalent SR Ca2+ content.6 Thus, if reduced SR Ca2+ content were part of the mechanism for enhanced repolarization in Inh mice, then repolarization should be equivalent in Inh and Con mice after breeding into a PLN−/− background, unless the interbred mice exhibited cardiac hypertrophy. The interbred mice did not show histological or morphometric evidence of hypertrophy.11 In addition, ANF and β-MHC mRNA were not increased in Inh×PLN−/− compared with WT and Con×PLN−/− hearts (supplemental Figure IV).

CaMKII inhibition–dependent effects on repolarization were prevented by ablation of PLN. Inh×PLN−/− and Con×PLN−/− mice had similar repolarization durations, ex vivo (Figure 6a through 6c) and in vivo (Figure 6d and 6e). In the absence of PLN, chronic CaMKII inhibition did not result in increased Ik1 (Figure 6f and 6g) or Inat (Figure 6h) compared with PLN−/− or Con×PLN−/− myocytes or compared with WT and Con myocytes (see Figures 2 and 3). PLN ablation, therefore, interrupts the effects of CaMKII inhibition on repolarization, consistent with the concept that the SR is a critical target for the observed remodeling of action potential repolarization by chronic myocardial CaMKII inhibition.

Remodeling of K+ Channel Subunit mRNA and Protein Expression Levels With CaMKII Inhibition and Deletion of PLN

CaMKII activity can regulate transcriptional activity and so a possible mechanism for increased Ik1 and Inat in Inh mice is upregulation of mRNAs encoding K+ channel proteins. We performed qRTP PCR using primer pairs specific for several K+ channel pore-forming subunit genes expressed in adult mouse ventricles (supplemental Table), including KCND2 and KCND3, which encode K,4,2 and K,4,3, respectively, and contribute to Ik1,8 KCNJ2 and KCNJ12, which encode Kir2.1 and Kir2.2, respectively, and contribute to Inat,9 These experiments revealed that the expression levels of KCND2, KCND3, KCNJ2, and KCNJ12, as well as of other K+ channel pore-forming subunit genes, including KCNA5 (K,1,5) and KCNB1 (K,2,1), are similar in Inh, Con, and WT ventricles (Figure 7a). Unexpectedly, these experiments also revealed markedly reduced expression of KCND2 (which encodes the K+ channel accessory subunit KChIP2) in Inh, compared with WT and Con, ventricles (Figure 7a). Expression levels of another accessory subunit gene, KCNAB1 (which encodes K,β1 subunits) were similar in Inh, Con, and WT ventricles. Similar results were obtained in the PLN−/− background (Figure 7b). These findings suggest that the increased Ik1 and Inat in Inh mice are not attributable to transcriptional upregulation of the genes encoding Ik1 and Inat channels.

![Figure 6](https://example.com/figure6.png)

**Figure 6.** PLN ablation prevents increased repolarization in Inh mice. a and b, Summary ECG interval data from spontaneously contracting Langendorff-perfused hearts from PLN−/− (n=11), Inh×PLN−/− (n=10), or Con×PLN−/− (n=12) mice. There were no significant differences among groups. QT90 indicates QT interval duration at 90% recovery to baseline; QT90C, QT90 corrected for heart rate. c, Monophasic APD at 50% (APD50), 75% (APD75), and 90% (APD90) are not different among PLN−/− (n=9), Inh×PLN−/− (n=8), and Con×PLN−/− (n=9) mice. There were no significant differences among groups. d and e, Summary surface (in vivo) ECG data from PLN−/− (n=11), Inh×PLN−/− (n=10), and Con×PLN−/− (n=9) mice. There were no significant differences among groups. f, g, and h, Summary APD data from WT, Inh, Con, and PLN−/− cardiomyocytes. There are no significant differences among groups for f, g, or h. The recordings in f through h are from the same cells, and the number of cells in each group is indicated by the numerals in parentheses.

The K,4,2, K,4,3, and KChIP2 proteins coassemble to produce Inat,8 whereas Kir2.1 is the critical pore-forming subunit for the generation of Ik1,9 and increases in these subunits could potentially explain the observed increases in Inat and Ik1 in Inh myocytes. Western blot analyses, however, revealed that the expression levels of K,4,2, K,4,3, and Kir2.1, as well as of other K+ channel subunits present in adult mouse ventricles, including K,1,5, K,2,1, Kir2.2, and K,β1, were similar in Inh, Con, and WT ventricles (Figure 8a through 8e). PLN ablation did not measurably affect the relative expression levels of these channel subunit proteins (Figure 8f). These experiments also revealed small, but statistically significant (P<0.05) differences in KChIP2 protein expression in Con, compared with WT and Inh, ventricles (Figure 8c and 8e), as well as in the ventricles of Inh...
amine toxicity, apoptosis, and myocardial dysfunction and ion conduction confers resistance to myocardial infarction, catechol-amine therapy or by cardiomyocyte-delimited AC3-I expression. CaMKII activity. CaMKII inhibition by chronic inhibitory inhibition are different from those to acute suppression of dialysis suggested that cellular responses to chronic CaMKII changes in Ca2+ have a role as a regulatory feedback signal for connecting processes including SR Ca2+ cycling. Our findings point to a potential connection between SR Ca2+ handling and CaMKII for sculpting action potential repolarization, because PLN ablation eliminated the effects of CaMKII inhibition on APD, \( I_{\text{ass}} \), and \( I_{\text{K1}} \). CaMKII activity is already known to be increased by the elevation in Ca2+ i, caused by APD prolongation, and the present findings suggest that CaMKII can also have a role as a regulatory feedback signal for connecting changes in Ca2+ i, to cardiac repolarization.

Model of Chronic In Vivo CaMKII Inhibition

One surprising finding was that upregulation of \( I_{\text{ass}} \) and \( I_{\text{K1}} \) did not appear to be caused by the acute effects of CaMKII-dependent phosphorylation, because neither current was increased by cellular dialysis with AC3-I. The finding that increases in \( I_{\text{ass}} \) and \( I_{\text{K1}} \) were not recapitulated by AC3-I dialysis suggested that cellular responses to chronic CaMKII inhibition are different from those to acute suppression of CaMKII activity. CaMKII inhibition by chronic inhibitory drug therapy or by cardiomyocyte-delimited AC3-I expression confers resistance to myocardial infarction, catecholamine toxicity, apoptosis, and myocardial dysfunction and arrhythmias in calcineurin cardiomyopathy; therefore, in-sight into the mechanisms of cellular compensation to chronic CaMKII inhibition could be important for developing future therapies.

CaMKII can directly increase \( I_{\text{ass}} \) by increasing the opening probability of L-type Ca2+ channels; therefore, we initially anticipated that \( I_{\text{ass}} \) would be less in Inh than in Con or WT mice. However, Inh mice have \( I_{\text{ass}} \) upregulation by increased PKA activity. Here we show direct evidence for enhanced phosphorylation of Ca,1.2 at Ser1928, but our experiments using cellular dialysis with cAMP and PKA failed to yield evidence that PKA activity contributes to increased \( I_{\text{ass}} \) or \( I_{\text{K1}} \) in Inh mice. PKA is typically associated with reduction in \( I_{\text{ass}} \) and cAMP reduced and PKI increased \( I_{\text{ass}} \) consistent with the findings of previous studies.

Discussion

CaMKII Regulates Myocardial Repolarization

This study identifies \( I_{\text{ass}} \) and \( I_{\text{K1}} \) as determinants for APD shortening in Inh mice with CaMKII inhibition. CaMKII is activated by Ca2+ i, and regulates key Ca2+ i, homeostatic processes including SR Ca2+ cycling. Our findings point to a potential connection between SR Ca2+ handling and CaMKII for sculpting action potential repolarization, because PLN ablation eliminated the effects of CaMKII inhibition on APD, \( I_{\text{ass}} \), and \( I_{\text{K1}} \). CaMKII activity is already known to be increased by the elevation in Ca2+ i, caused by APD prolongation, and the present findings suggest that CaMKII can also have a role as a regulatory feedback signal for connecting changes in Ca2+ i, to cardiac repolarization.

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with these reports. Our findings do not suggest that increased PKA activity is regulating \( I_{\text{cat}} \) in Inh mice, because \( I_{\text{cat}} \) is greater in Inh than in Con or WT animals. On the other hand, \( I_{\text{cat}} \) has been reported to increase\(^{24} \) and decrease\(^{25} \) in response to PKA stimulation. Cell dialysis with cAMP or PKI failed to increase \( I_{\text{K1}} \) in Inh, Con, or WT cells, suggesting that PKA was not a critical determinant of \( I_{\text{K1}} \) under our experimental conditions. Overall, our findings do not support a model in which increased PKA activity is important in the upregulation of \( I_{\text{cat}} \) or \( I_{\text{K1}} \) in Inh mice.

**SR Ca\(^{2+}\) Feedback Regulation of Repolarizing K\(^+\) Currents**

It is now established that prolongation of repolarization can elevate Ca\(^{2+}\)\(^{26} \) that contributes to increased cellular contraction\(^1 \) and arrhythmias.\(^{27} \) In some studies, increased APD results in hypertrophy,\(^{28} \) but hypertrophy is not present in other mouse models with action potential prolongation.\(^{29} \) Recent evidence also supports the concept that “retrograde” signaling from SR Ca\(^{2+}\)\(^{27} \) to ionic currents is important for regulating K\(^+\) channel synthesis and regulating APD.\(^3 \) Our results clearly reveal that targeting CaMKII activity selectively affects the functional expression of repolarizing cardiac K\(^+\) currents by a mechanism that requires PLN, without measurably affecting the expression levels of several subunit proteins that contribute to the formation of these channels.

Transgenic mice with enhanced myocardial SR Ca\(^{2+}\) uptake caused by overexpression of a skeletal muscle isoform of SERCA (SERCA1a) show APD prolongation and reduced expression of proteins underlying \( I_{\text{cat}} \) (KCNIP2, K,4.2, K,4.3) and \( I_{\text{Klow}} \) (K,2.1 and K,1.5).\(^2 \) Inh mice were previously shown to have reduced SR Ca\(^{2+}\) content, equivalent spark frequency, and markedly diminished PLN phosphorylation at Thr17 compared with Con mice.\(^{4,5} \) The increases in \( I_{\text{cat}} \) and \( I_{\text{K1}} \) in Inh mice are reversed to baseline by PLN ablation, a maneuver that eliminates CaMKII inhibition mediated reduction in SR Ca\(^{2+}\) content,\(^6 \) suggesting that PLN and SR Ca\(^{2+}\) are key components connecting CaMKII-dependent alterations in K\(^+\) currents to the physiology of myocardial repolarization. Taken together, these findings in Inh and SERCA1a-overexpressing mice point to an important relationship between SR Ca\(^{2+}\) uptake and action potential repolarization.

The mechanisms underlying the alterations in K\(^+\) current densities in the Inh and SERCA1a-overexpressing models are different. SERCA1a overexpressing mice have increased SR Ca\(^{2+}\) content and QT interval prolongation,\(^2 \) whereas Inh mice have reduced SR Ca\(^{2+}\) and show QT and APD shortening caused by increases in \( I_{\text{cat}} \) and \( I_{\text{K1}} \). SERCA1a-overexpressing mice show reduced \( I_{\text{cat}} \) and reduced slow (\( I_{\text{Kslow}} \)) and steady-state (\( I_{\text{Kss}} \)) currents, whereas \( I_{\text{K1}} \) was not reported.\(^3 \) The different \( I_{\text{Kslow}} \) responses in Inh and SERCA1a-overexpressing mice may indicate that individual repolarizing currents couple to distinct components of SR Ca\(^{2+}\) uptake regulation (eg, SERCA versus PLN). Although the feedback mechanisms linking SR Ca\(^{2+}\) content and cardiac repolarization remain incompletely understood in SERCA1a-overexpressing and Inh mice, neither model exhibited signs of cardiac hypertrophy. Thus, the feedback relationship between SR Ca\(^{2+}\) and cardiac repolarization seems to be operative even in the absence of underlying cardiac disease. There are similarities but also substantial differences in the molecular mechanisms for repolarization between mice and larger mammals, including humans.\(^30 \) An important future direction will be to determine whether feedback mechanisms among CaMKII, SR Ca\(^{2+}\) reuptake, and myocardial repolarization are similar in mice and in larger mammals.

**CaMKII Is a Dual Signal for Cardiac Hypertrophy and Electrical Remodeling**

APD prolongation is a stereotyped “electrical remodeling” response to structural heart disease from diverse causes that may contribute to arrhythmias in animal models and in patients with cardiac hypertrophy and heart failure.\(^15 \) The APD shortening in Inh mice suggests the possibility of a “reverse electrical remodeling” response to chronic CaMKII inhibition. Acute CaMKII inhibition can reduce arrhythmias in the setting of electrical remodeling and cardiac hypertrophy, but without changing APD.\(^31 \) On the other hand, chronic CaMKII inhibition can improve myocardial function, reduce mortality, and suppress ventricular arrhythmias in mice with calcineurin cardiomyopathy,\(^22 \) suggesting that distinct mechanisms control arrhythmic properties of acute and chronic CaMKII inhibition. Myocardial responses to chronic CaMKII inhibition are complex but are apparently adaptive and the present findings establish that they include a readjusting of repolarization that causes APD shortening by a mechanism that requires PLN.

**Acknowledgments**

The PLN-/- mice were a generous gift from Dr E. G. Kranias.

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**Disclosures**

None.

**References**


Calmodulin Kinase II Inhibition Shortens Action Potential Duration by Upregulation of \( K^+ \) Currents

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Li et al

Inh and Con mice
Ventricular myocytes were isolated from adult male and female mice (80 – 100 days old) by a previously described technique.\(^1\) CaMKII activity is reduced by ~40% in Inh, compared to Con or WT, cardiac homogenates, but the degree of CaMKII inhibition *in situ* is likely greater based on the near ablation of CaMKII-dependent phosphorylation of PLN at Thr\(^{17}\) in Inh ventricular myocytes.\(^2\) The genetic identity of PLN\(^{-/-}\) pups with transgenic expression of AC3-I or AC3-C was confirmed using PCR.\(^3,\,4\) The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Electrophysiology
Mice were anesthetized with Avertin (20 µl/g, i.p.) from a stock solution [10 g of tribromoethanol alcohol (Aldrich) + 10 ml of tert-amyl alcohol (Aldrich), stored at 4°C]. The QT interval at 90 percent recovery to baseline (QT\(_{90}\)) was corrected for heart rate (QT\(_{90}\)/RR)\(^{1/2}\). Ionic currents from ventricular myocytes were recorded using the whole-cell configuration of the patch–clamp technique.\(^6\) Tip resistance was 1–2 MΩ when pipettes were filled with intracellular solution, and series resistance compensation was routinely set at >85% in all experiments. All recordings were obtained at room temperature (22–26°C).

Outward K\(^+\) currents were dissected using the following expression: \(I(t) = I_{始} \exp(-t/\tau_{f}) + I_{K,slow1} \exp(-t/\tau_{slow1}) + I_{ss}\), where \(t\) is the time, \(\tau_{f}\) and \(\tau_{slow1}\) are time constants, \(I_{始}\) and \(I_{K,slow1}\) are corresponding amplitudes and \(I_{ss}\) (also called \(I_c\))\(^7\) is the amplitude of the steady-state component. The extracellular (bath) solution consisted of (mM): 137 NaCl, 5.4 KCl, 0.3 CaCl\(_2\), 0.5 MgCl\(_2\), 0.16 NaH\(_2\)PO\(_4\), 3 NaHCO\(_3\), 5 HEPES, 5 glucose and 2%BSA. The pH was adjusted to 7.35 (room temperature) with NaOH. The intracellular (pipette) solution consisted of (mM): 140 KCl, 10 K-EGTA, 10 K-HEPES, 5 K\(_2\)ATP, and 1 MgCl\(_2\), with pH adjusted to 7.35 with KOH.

\(K^+\) channel immunoblotting
Some experiments were completed on fractionated ventricular membrane proteins\(^8\) isolated from 11-13 week male WT (n = 4), Con (n = 4) and Inh (n = 4) mice. The monoclonal anti-Kv4.2 and anti-KChIP2 and the polyclonal anti-Kv4.3 antibodies used in these experiments have been described previously,\(^8,\,10\) and the anti-Kir2.1 antibody was obtained from Chemicon. For Western blot analyses, equal amounts of ventricular proteins (50µg) were fractionated on (8% or 12%) SDS-PAGE gels, transferred to PVDF membranes, followed by overnight incubation at 4°C with the anti-Kv4.2, anti-Kv4.3 or anti-KChIP2, or anti-Kir2.1 antibody (at 0.5 to 1µg/ml).\(^9,\,10\) In addition, each of these incubation solutions contained a monoclonal anti-β-actin antibody (at 1 µg/ml) (Sigma) as an internal control. Following washing, the membranes were incubated with an
alkaline phosphatase-conjugated goat-anti-mouse secondary antibody. The secondary incubation solution for membranes incubated with the polyclonal anti-Kv4.3 antibody also contained an alkaline phosphatase-conjugated goat-anti-rabbit antibody. Bound antibodies were detected using CPSD (Tropix), a chemiluminescent alkaline phosphatase substrate and, after washing to remove excess substrate, the membranes were exposed to x-ray film (Kodak). Films from individual experiments were scanned.

**CaV1.2 immunoblotting**

100 mg heart tissue was frozen and pulverized in a cylindrical steel mortar and pestle precooled with liquid nitrogen for immunoblotting, as described. The powdered heart tissue was resuspended and homogenized in 1 ml 1% Triton X 100 containing 20 mM EDTA, 10 mM EGTA, 10 mM Tris-Cl, pH 7.4. Protease inhibitors (1 µg/ml pepstatin A, 10 µg/ml leupeptin, 20 µg/ml aprotinin, 200 nM phenylmethanesulfonylfluoride, 8 µg/ml calpain inhibitor I, 8 µg/ml calpain inhibitor II) and phosphatase inhibitors (1 mM paranitrophenylphosphate, 2 µM microcystine LR, 25 mM NaF, 25 mM Na-pyrophosphate) were present throughout the procedure. After clearing the extracts from any non-soluble material by ultracentrifugation, Cav1.2 was immunoprecipitated with an antibody against loop II and analyzed by immunoblotting with the phosphospecific antibody against serine 1928 as described in more detail elsewhere.

**qRT PCR**

Hearts were excised from 10-12 week-old male and female WT (n = 6) and PLN-/- (n=6), as well as from mice expressing either the CaMKII Inh (ACS-I) or Con (ACS-C) peptide in the WT and PLN-/- backgrounds (n = 6 for each genotype). All animals were sacrificed by cervical dislocation, and the hearts were rapidly removed. Ventricles were dissected from each heart and flash-frozen in liquid nitrogen for further RNA isolation. Total RNA was isolated and DNase treated using the RNeasy Fibrrous Tissue Mini Kit (Qiagen). The quality of total RNA was assessed by gel electrophoresis. Genomic DNA contamination was assessed by PCR amplification of total RNA samples without prior cDNA synthesis; no genomic DNA was detected.

First strand cDNA was synthesized from 2 µg of total RNA using the High-Capacity cDNA Archive Kit (Applied Biosystems). The expression levels of genes encoding the K+ channel subunits Kv1.5, Kv2.1, Kv4.2, Kv4.3, Kir2.1, Kir2.2, KChIP2 and Kvβ1, as well as the gene encoding the hypertrophy markers atrial natriuretic factor (ANF) and β-myosin heavy chain (β-MHC), were determined by quantitative real-time PCR using 1 X SYBR Green PCR Master Mix (Applied Biosystems). PCR reactions were performed on 10 ng of cDNA using sequence specific primer pairs (Supplementary Table 1) on the ABI PRISM 7900HT Sequence Detection System. The cycling conditions included a hot start at 95°C for 10 min, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 min. All primer pairs were tested using mouse cDNA as the template, and templates giving 90-100% efficacy were chosen. In all cases, a single amplicon of the appropriate melting temperature or size was detected using the dissociation curve or gel electrophoresis, respectively.
Data were collected with instrument spectral compensations using the Applied Biosystems SDS 2.2.2 software and analyzed using the comparative threshold cycle (C_T) relative quantification method. The Hypoxanthine phosphoribosyl-transferase I (HPRT I) gene was used as an endogenous control to normalize the data. Individual sample measurements (n = 6) were averaged and 2^-ΔC_T values for each gene, corresponding to the relative expression level of that gene compared with HPRT, were calculated and are reported here. C_T values for all of the genes studied and analyzed here were < 32. Negative control experiments using RNA samples incubated without reverse transcriptase during cDNA synthesis showed no amplification.

Reference List


**Supplementary Figure 1 Heart rates and PR intervals**

Summary data for RR (a) and PR (b) intervals from Langendorff-perfused hearts shown in Fig 1b-c for WT, Inh and Con mice. †P<0.001. Summary data for RR (c) and PR (d) intervals from WT, Inh and Con mice recorded *in vivo* from mice shown in Fig 1g-h. *P<0.05.

**Supplementary Figure 2 qRT PCR for hypertrophy marker genes**

a. ANF and (b) β−MHC expression levels are not elevated in the ventricles of Inh (n = 9), compared with WT (n=9) and Con (n=9) ventricles.

**Supplementary Figure 3 Acute CaMKII inhibition does not increase I_{K1} or I_{tof}**

Amplitude of I_{K1} recorded at -60 mV (a) and -100 mV (b). c. The slope conductance of I_{K1} at the K+ reversal potential. d. Peak I_{tof} recorded at +80 mV. All data are from wild
type ventricular myocytes after 10 min dialysis without peptide, with the CaMKII inhibitory peptide AC3-I or the inactive control peptide AC3-C. The number of cells studied in each group are shown in parentheses in panel a. There were no significant differences between groups in panels a – d.

**Supplementary Figure 4 qRT PCR for hypertrophy marker genes**

a. ANF and (b) β–MHC expression levels are not significantly increased in the ventricles of Inh x PLN+/− (n = 6), compared with PLN+/− (n=6) and Con x PLN+/− (n=6) ventricles.
<table>
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<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<td>KCNA5 (Kv1.5)</td>
<td>5'-CCTGCGAAGGTCTCTGTATGC</td>
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<td>5'-TTCAACTTTCGCTCATCTTAGG</td>
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</table>
Supplementary Fig 3

(a) $I_{K1}$ (pA/pF) for $V_m = -60$ mV

(b) $I_{K1}$ (pA/pF) for $V_m = -100$ mV

(c) $I_{K1}$ slope (pS/pF) for $V_m = E_{rev}$

(d) $I_{o,f}$ (pA/pF) for $V_m = +80$ mV

(22) (17) (16)

No peptide, AC3-I, AC3-C