Myocardial KChIP2 Expression in Guinea Pig Resolves an Expanded Electrophysiologic Role

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Abstract

Cardiac ion channels and their respective accessory subunits are critical in maintaining proper electrical activity of the heart. Studies have indicated that the K⁺ channel interacting protein 2 (KChIP2), originally identified as an auxiliary subunit for the channel Kv4, a component of the transient outward K⁺ channel (I_{to}), is a Ca²⁺ binding protein whose regulatory function does not appear restricted to Kv4 modulation. Indeed, the guinea pig myocardium does not express Kv4, yet we show that it still maintains expression of KChIP2, suggesting roles for KChIP2 beyond this canonical auxiliary interaction with Kv4 to modulate I_{to}. In this study, we capitalize on the guinea pig as a system for investigating how KChIP2 influences the cardiac action potential, independent of effects otherwise attributed to I_{to}, given the endogenous absence of the current in this species. By performing whole cell patch clamp recordings on isolated adult guinea pig myocytes, we observe that knock down of KChIP2 significantly prolongs the cardiac action potential. This prolongation was not attributed to compromised repolarizing currents, as I_{Kr} and I_{Ks} were unchanged, but was the result of enhanced L-type Ca²⁺ current due to an increase in Cav1.2 protein. In addition, cells with reduced KChIP2 also displayed lowered I_{Na} from reduced Nav1.5 protein. Historically, rodent models have been used to investigate the role of KChIP2, where dramatic changes to the primary repolarizing current I_{to} may mask more subtle effects of KChIP2. Evaluation in the guinea pig where I_{to} is absent, has unveiled additional functions for KChIP2 beyond its canonical regulation of I_{to}, which defines KChIP2 as a master regulator of cardiac repolarization and depolarization.

Introduction

K⁺ channel interacting proteins (KChIPs) represent a class of highly diverse Ca²⁺-sensors originally discovered for their interaction with the cytoplasmic N-terminus of the Kv4 family of potassium channels [1]. The assembly of these two proteins creates the native current known as A-type current (I_{A}) in neuronal tissue and the fast-inactivating transient-outward potassium current (I_{to}) in the heart [2–6]. While the expression of Kv4 alone is sufficient to observe this current, co-expression with KChIP results in currents with slowed inactivation, faster recovery...
from inactivation, and increased current densities, effectively creating native Kv4 current [1, 7].

In total, there are four KChIP genes (KChIP1-4) [1, 8]. While all four KChIPs can be observed in the heart, the brain is seemingly simplified by expressing only KChIP2. Together, these proteins are characterized by a highly conserved C-terminal domain containing 4 EF-hand motifs and a highly variable N-terminus which is thought to provide both altered localization and activity [9, 10]. Indeed, the KChIP family has become the most diverse of the Ca\(^{2+}\)-sensing proteins [11, 12] not just in numbers, but in their breadth of function. In addition to serving as K\(^+\)-channel subunits, KChIP3 and 4 were discovered in the brain to both regulate sensing proteins [11, 12] not just in numbers, but in their breadth of function. In addition to

The authors have declared that no competing interests exist.

Competing Interests:

Abbreviations: KChIP, K\(^+\) channel interacting protein; APD, Action Potential Duration.
5′-GGCATTTGAAGAGAAAGTGGCA-3′, human gapdh:F 5′-TCCTCTGACTTCAACACGC GA-3′, human gapdh:R 5′-GGGTCTTACTCTTTGAGGC-3′

Viral Constructs

cDNA of the identified guinea pig KChIP2 antisense sequence was created and cloned into an adenoviral vector under the regulation of a CMV promoter (KChIP2-KD). The vector coexpressed GFP through an internal ribosomal entry site. A control adenoviral vector was used that omitted the antisense sequence and expressed GFP alone.

Immunoblotting

Freshly isolated guinea pig ventricular myocytes were cultured for 24 h at 37°C in M199 medium under control conditions or with KChIP2 antisense virus. Upon collection, cardiac myocytes were washed in ice-cold PBS and resuspended in a RIPA lysis buffer (150 mM sodium chloride, 1.0% NP-40 or Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS (sodium dodecyl sulphate), 50 mM Tris, pH 8.0, plus Roche Inhibitor tablet) and then sonicated on ice to disrupt cell membranes. 30–40 μg of whole cell extract was loaded into SDS-PAGE gels, transferred to nitrocellulose membrane, and Western blotting performed using KChIP2 mouse monoclonal antibody (UC Davis NeuroMab 75–017) at a 1:500 dilution, beta-actin mouse monoclonal antibody (Sigma-Aldrich, A1978) at a 1:1000 dilution, Cav1.2 mouse monoclonal antibody (UC Davis NeuroMab 75–257) at a 1:500 dilution, Nav1.5 mouse monoclonal antibody (Sigma S8809) at a 1:800 dilution, and pan-cadherin rabbit monoclonal antibody (Cell Signaling 4068S) at a 1:1000 dilution. Protein concentrations were determined by the BCA method (Pierce).

Guinea Pig Ventricular Myocytes: Isolation and Short-term Culture

Single ventricular myocytes were isolated from adult guinea pigs as described previously [21]. Briefly, guinea pigs were anesthetized by injection of fatal plus. Hearts were quickly removed and perfused via the aorta with a physiological salt solution (PSS) containing (in mmol/L) NaCl 140, KCl 5.4, MgCl₂ 2.5, CaCl₂ 1.5, glucose 11, and HEPES 5.5 (pH 7.4). After 5 minutes, perfusate was switched to a nominally calcium-free PSS with collagenase (Roche, 0.5 mg/mL) being added after an additional 5 minutes. After 20 to 35 minutes of digestion, hearts were perfused with a high K⁺ solution containing (in mmol/L) potassium glutamate 110, KH₂PO₄ 10, KCl 25, MgSO₄ 2, taurine 20, creatine 5, EGTA 0.5, glucose 20, and HEPES 5 (pH 7.4). Ventricles were minced in high K⁺ solution, and single myocytes were obtained by filtering through a 115-μm nylon mesh. Myocytes were left to settle for 2 hours at room temperature before being collected in a low-speed spin. Cell pellets were resuspended in M199 medium supplemented with antibiotic and plated on uncoated 10 cm dishes. Cultures were left untreated or they were treated with GFP adenovirus or adenovirus with a kcnip2 (KChIP2) mRNA antisense coding sequence in IRES with GFP. Cultures were then incubated in 5% CO₂ at 37°C for 24 hrs. Untreated and GFP treated cells were found to have no significant differences across the studies conducted, and therefore the datasets between these two groups were combined to comprise our control group.

Cellular Electrophysiology

$I_{K1}, I_{Ks}, I_{Kr}$, and action potentials were recorded in isolated ventricular guinea pig myocytes cultured overnight in M199 medium using the following intracellular solution: 119 mM potassium gluconate, 15 mM KCl, 3.75 mM MgCl₂, 5 mM EGTA, 5 mM HEPES, 4 mM K-ATP, 14 mM
phosphocreatine, 0.3 mM Tris-GTP, and 50 U/ml creatine phosphokinase, pH 7.2. The extra-
cellular solution was 132 mM NaCl, 4.5 mM KCl, 1.2 mM MgCl₂, 1.8 mM CaCl₂, 10 mM Glu-
cose, and 5 mM HEPES, pH 7.4. \( I_{Ks} \) currents were elicited from a holding potential of -40 mV
with depolarizing voltage pulses from -100 mV to 40 mV. \( I_{Ks} \) currents were elicited from a
holding potential of -40 mV with depolarizing voltage pulses from -30 mV to 60 mV for 2.5 s
and then return to -40 mV to generate outward tail currents in the presence of 5 uM E4031 to
block \( I_{Kr} \). \( I_{Kr} \) currents were isolated as E4031 sensitive current. \( I_{Ca,L} \) were recorded with an
intracellular solution of 130 mM CsMES, 20 mM TEA Cl, 1 mM MgCl₂, 10 mM HEPES, 10 mM EGTA, 0.3 mM TRIS GTP, 14 mM Phosphocreatine, 4 mM Mg ATP, 2 mM Creatine
phosphokinase and brought to a pH of 7.3. Myocytes were placed in the solution containing
137 mM NaCl, 5.4 mM CsCl, 1.8 mM MgCl₂, 2 mM CaCl₂, 10 mM glucose, 10 mM HEPES,
PH 7.3. \( I_{Ca,L} \) were elicited from a holding potential of -40 mV with depolarizing voltage pulses from
-30 mV to 60 mV for 300 ms. \( I_{Na} \) was recorded in the solution containing 20 mM NaCl,
120 mM N-methyl D-glucamine, 5.4 mM CsCl, 1 mM MgCl₂, 10 mM glucose, 10 mM HEPES,
PH 7.3. 1 uM of nisodipine was used to block L-type Ca currents. \( I_{Na} \) were elicited from a hold-
ing potential of -120 mV with depolarizing voltage pulses from -80 mV to 60 mV for 16 ms.
Ionic current density (pA/pF) was calculated from the ratio of current amplitude to cell capaci-
tance. pClamp software (Molecular Devices) was used for generation of voltage-clamp proto-
cols and data acquisition. All experiments were performed at 35°C except \( I_{Na} \) (room
temperature).

Statistical Analysis

The experimental data were expressed as mean ± SEM. A Student’s \( t \)-test or a paired Student’s
\( t \)-test (for Western blot analysis) was performed. All tests were two-sided and a significance
level of \( p < 0.05 \) was defined as statistically significant (SPSS 18.0 software, SPSS, Chicago, IL)

Ethics Statement and Tissue Acquisition

This study was carried out in strict accordance with the recommendations in the Guide for the
Care and Use of Laboratory Animals of the National Institutes of Health. The protocol for tis-
sue isolation from adult guinea pig (Protocol Number: 2012–0175), adult and neonatal rat
(Protocol Number: 2013–0015), and canine (Protocol Number: 2014–0026) samples were
approved by the Committee on the Ethics of Animal Experiments of Case Western Reserve
University. 14 Hartley guinea pigs, 3 adult Sprague Dawley rats, and 3 neonatal hearts from 3
separate litters were provided from Charles River to conduct our studies. 3 separate hearts of
canine left ventricular tissue were sourced from purpose bred canines from Marshall Farms.
Tissue from the left ventricular free wall of 3 non-failing, human samples were acquired from
the Cleveland Clinic Foundation (CCF) tissue repository from unmatched organ donors. All
protocols were approved by the CCF Institutional Review Board (IRB# 2378). Samples were
received coded and no identifying metrics were documented for the study.

Results

We began our investigation by first identifying the maintained expression of KChIP2 in the
guinea pig myocardium. Sequence alignment of the identified transcript shows significant
sequence homology compared to other mammalian species which possess Kv4 encoded \( I_{to} \) (Fig
1A). Variability exists, however, in the relative levels of \( kcnip2 \) (KChIP2) mRNA expression.
Left ventricular tissue isolated from guinea pig expresses approximately 35-fold less KChIP2
than the ventricular tissue from an adult rat (Fig 1B), making the level of KChIP2 mRNA
expression comparable to neonatal rat ventricular myocytes. Previous work has established the
degree of KChIP2 expression directly correlates with the amount of $I_{to}$ density [22–24]. Indeed, adult rat which has high levels of KChIP2 has the most prominent $I_{to}$ density of the animals shown, while neonatal rats which express much less KChIP2 has a smaller $I_{to}$ density which reflects the lower expression [25]. An important distinction, however, is that neonatal rat myocytes display measurable $I_{to}$, while the guinea pig myocardium, while maintaining comparable KChIP2 levels expresses no $I_{to}$, suggesting a functional consequence of KChIP2 presence beyond the modulation of $I_{to}$.

To begin to address if guinea pig cardiac KChIP2 contributes to cardiac excitability and modulation of other ionic currents, we evaluated the guinea pig action potential following acute changes to KChIP2 expression. Ventricular cells isolated from the adult guinea pig heart were left untreated or were treated for 24 hrs with an adenosine expressing GFP or an mRNA antisense sequence for KChIP2 to acutely silence KChIP2. Evaluation by Western blot shows we were able to significantly reduce KChIP2 protein in KChIP2 antisense treated cells compared to control cells (Fig 2A). Critically, the consequence of this reduction was significant prolongation of the cardiac action potential, extending APD$_{90}$ from 211±10.8 ms in control cells to 262±14.6 ms in anti-sense treated cells (Fig 2B). This prolongation was significant at both 90% and 50% of repolarization and occurred at multiple pacing frequencies (Fig 2C and 2D). The overlap at phase 1 of the action potential traces between both groups is reflective of the absence of $I_{to}$ or any other comparable repolarizing current that KChIP2 has been shown to effect. This data implicates a clear influence of KChIP2 on factors beyond $I_{to}$.

We next sought to identify the currents responsible for the prolonged repolarization observed following KChIP2 silencing. Given the observed prolongation of the plateau phase of the guinea pig action potential, we focused on changes to $I_{kr}$, $I_{ks}$, and $I_{Ca,L}$. While there is no history for KChIP2 regulation on either of the delayed rectifier currents, we measured their activity to determine if these currents might be responsible for the delayed repolarization.
However, assessment of the tail current densities for $I_{Kr}$ and $I_{Ks}$ displayed no significant change to either current (Fig 4A and 4B). Overall, the loss of KChIP2 does not appear to affect outward repolarizing currents in the guinea pig myocardium. Therefore, we next looked at potential changes to $I_{Ca,L}$, to determine if enhancement of this depolarizing current might explain a longer APD. Indeed, when looking at the changes to L-type Ca$^{2+}$ current we see that reduction of KChIP2 produced an increase in current density across multiple potentials when compared to control cells (Fig 5A and 5B). Peak current density at 10 mV was enhanced from -5.43±0.73 pA/pF in control cells to -10.68±1.46 pA/pF in KChIP2 anti-sense treated cells, nearly doubling the current density. Analysis of channel activation and voltage-dependent inactivation revealed that the kinetics of $I_{Ca,L}$ were unaltered by KChIP2 KD and could not account for the increase in current density (Fig 5C and 5D). However, assessment of Cav1.2 protein expression showed that KChIP2 KD led to a significant increase in expression compared to control cells (1.85 ± 0.05 fold change, Fig 5E). Given that current kinetics were left unaltered, this suggests $I_{Ca,L}$ augmentation occurs through an increase in channel expression following the loss of KChIP2.

Lastly, to evaluate the full panel of currents relevant in the guinea pig cardiac action potential, changes to $I_{Na}$ was assessed. Evaluation of $I_{Na}$ density showed a decrease in the peak...
current from -24.51 ± 1.70 pA/pF in control cells, to -19.14 ± 2.09 pA/pF in KChIP2 knockdown treated cells (Fig 6A and 6B). To understand this loss in current, Nav1.5 protein was assessed, which showed a mild but significant decrease following KChIP2 loss (0.71 ± 0.07 fold change, Fig 6C), reflecting the change in current density. This suggests that KChIP2 not only has influence over the rate of repolarization, but may potentially have regulation over cardiac excitability as well.

Discussion

This study sought to characterize the influence of KChIP2 expression in the guinea pig myocardium. The species was specifically chosen for its unique, endogenous absence of $I_{to}$ despite our observation of maintained KChIP2 expression, suggesting an alternative significance for this protein. Additionally, this model provided a means of evaluating the electrophysiological consequences of KChIP2 loss without the influence of diminished $I_{to}$ density. Indeed, when we silenced KChIP2, we observed a significantly longer plateau phase of the action potential, resulting in a prolonged APD. This prolongation was due to enhanced L-type Ca$^{2+}$ current density, with no change to the repolarizing currents $I_{Kr}$ and $I_{Ks}$. Additionally, we measured significant reduction to $I_{Na}$ density. Together, these data show that KChIP2 is indeed a multi-modal effector of cardiac ionic currents.

In rodents, $I_{to}$ serves as the primary repolarizing current maintaining a strong influence over APD [26]. In response to cardiac stressors like myocardial infarction and pressure...
overload, both KChIP2 and Kv4 experience reduced expression, significantly contributing to APD prolongation [27–29]. It was found that maintaining KChIP2 expression even in the presence of cardiac stress could normalize the APD back towards baseline levels [30]. This had the therapeutic effect of mitigating the hypertrophic response by minimizing increases in the Ca\textsuperscript{2+} transient and turning off Ca\textsuperscript{2+} activated hypertrophic signaling pathways. In larger mammals, however, this relationship is less straightforward as \textit{I}_\text{to} is comparatively smaller and therefore does not fully repolarize the membrane [31], which means decreased \textit{I}_\text{to} does not necessarily translate to prolonged APD. In fact, data suggests reductions in \textit{I}_\text{to} can actually lead to APD shortening by altering the driving force for Ca\textsuperscript{2+} entry [31][32]. Yet, independent of species, cardiac pathologies almost invariably result in diminished KChIP2 expression and a prolonged APD [33–35]. Therefore, the observations in the guinea pig of KChIP2 loss directly augmenting \textit{I}_{\text{Ca,L}} through increases in Cav1.2 protein (Fig 5) is highly significant in fully understanding its contributions to disease pathogenesis. It shows that independent of its effect on \textit{I}_\text{to}, KChIP2 depletion can still prolong the cardiac action potential (Fig 2) and possibly drive disease progression through Ca\textsuperscript{2+} dependent signaling cascades.

This is not the first instance showing that KChIP2 is capable of causing changes to L-type Ca\textsuperscript{2+} current. Thomsen \textit{et al} previously identified in a mouse KChIP2 knockout model a significantly reduced \textit{I}_{\text{Ca,L}} in the absence of KChIP2 [17], the opposite regulation measured in the guinea pig here. This regulation was discovered to be the consequence of KChIP2 interaction with an N-terminal auto-inhibitory (NTI) domain on Cav1.2, which was interrupted when KChIP2 was bound, resulting in an increased open probability for the channel. However, when

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**Fig 4.** APD prolongation observed following KChIP2 silencing is not defined by compromised repolarization. (A) Upper panel shows representative current traces for \textit{I}_\text{Ks} for both control and KChIP2 KD cardiomyocytes following 24 hrs incubation. Currents were generated from a holding potential of -40 mV with depolarizing voltage pulses from -30 mV to 60 mV, and then a return to -40 mV to generate outward tail currents in the presence of E4031. Lower panel shows the resulting I/V curve summary data between control (n = 19) and KChIP2 KD (n = 10) yielding no significant difference between treatment groups. (B) Upper panel shows representative current traces for \textit{I}_\text{Kr} for both control and KChIP2 KD cardiomyocytes following 24 hrs incubation. Currents were isolated as E4031 sensitive. Lower panel shows the resulting I/V curve summary data for tail currents between control (n = 9) and KChIP2 KD (n = 4). There was no significant difference between the two groups. Data presented as mean ± SEM; two-tailed Student’s t-test performed.

doi:10.1371/journal.pone.0146561.g004
identifying this NTI domain in the guinea pig, alignment with the mouse shows an incomplete conservation of the amino acid residues, suggesting that the KChIP2 interaction site within the NTI domain may be absent in guinea pig. Without this mode of regulation present, this may explain why we do not observe a decrease in L-type Ca\textsuperscript{2+} current density, and instead may even be unmasking a secondary mode of regulation by KChIP2 on Cav1.2. Indeed, the KChIP2 null...
mice were observed to have increased Cav1.2 protein expression, despite the diminished current density [17]. This reinforces the observations here in the guinea pig where increased Cav1.2 protein expression results in a corresponding increase in $I_{\text{Ca,L}}$. Notably, alternative splicing to the N-terminus of Cav1.2 can produce transcripts that omit this NTI domain [36]. While these variants predominate in endothelial cells, it does suggest KChIP2 may in fact have differential regulation on Cav1.2, depending on which variant is being expressed. That we see

![Image](https://example.com/image.png)

**Fig 6.** KChIP2 knock down attenuates $I_{\text{Na}}$ density. (A) Representative traces for $I_{\text{Na}}$ in cardiomyocytes in control and KChIP2 KD cells following 24 hrs incubation. (B) Summary data of the I/V curve for control (n = 13) and KChIP2 KD (n = 12) cardiomyocytes, showing reduced current density in response to KChIP2 loss. (C) Left panel shows representative immunoblots for Nav1.5 protein from whole cell lysates for control and KChIP2 KD treated myocytes. Protein expression was normalized to pan-cadherin expression. Right panel shows the average fold change from KChIP2 KD treated cardiomyocytes from control, which resulted in significantly decreased expression following KChIP2 KD (n = 3). Data presented as mean ± SEM; *P < 0.05 two-tailed Student’s t-test performed for I/V plot, paired two-tailed Student’s t-test performed for Western blot.

doi:10.1371/journal.pone.0146561.g006
increased current density in the guinea pig suggests KChIP2 regulation of $I_{Ca,L}$ has additional regulatory pathways yet to be determined.

The implications of cardiac KChIP2 expression grows even further when we consider its impact on $I_{Na}$. We have previously shown that silencing KChIP2 in rat myocytes resulted in loss of $I_{to}$ and $I_{Na}$ [19]. Immunoprecipitation studies suggested that the subunits comprising the currents interacted to form a structural and functional subunit complex. The data we show here takes this further to say that the regulation by KChIP2 on $I_{Na}$ occurs independently of $I_{to}$ and can influence $I_{Na}$ without the need to be part of a larger channel complex. This is supported by the observation that KChIP2 can coimmunoprecipitate with Nav1.5 [19]. Notably, our studies were conducted on dissociated myocytes and therefore take away the ability to study impulse propagation within native tissue, but it is intriguing to consider the influence this might have in an intact tissue.

Ultimately, our goal in this study was to characterize the changes in the cardiac action potential following KChIP2 loss, independent of any influences by $I_{to}$. Indeed, we were able to reveal a more profound significance for KChIP2 by identifying regulation on $I_{Ca,L}$ and $I_{Na}$. These influences are especially critical in cardiac pathologies where KChIP2 loss is so common and where these currents are also modified. There are numerous electrical remodeling events in the diseased heart, and the more we understand of the multiple roles of KChIP2, the more it seems KChIP2 might be responsible in mediating those changes. Together, this establishes KChIP2 as essential in the maintenance of cardiac repolarization and depolarization, even independently from its role as a potassium channel interacting protein.

Supporting Information

S1 Table. Individual $kcnip2$ expression levels across species portrayed in Fig 1. (XLSX)

S2 Table. Individual APD$_{90}$ and APD$_{50}$ measurements at 1000 ms and 400 ms cycle lengths as portrayed in Fig 2. (XLSX)

S3 Table. Individual values underlying action potential amplitude (Fig 3A), resting membrane potential (Fig 3B), and IK1 density (Fig 3D). (XLSX)

S4 Table. Individual values underlying the current-voltage relationship for $I_{Ks}$ (Fig 4A) and $I_{Kr}$ (Fig 4B). (XLSX)

S5 Table. Individual values underlying the current-voltage relationship for $I_{Ca,L}$ (Fig 5B), normalized conductance measurements (Fig 5C), $I_{Ca,L}$ decay kinetics (Fig 5D), and the normalized densitometry values for Cav1.2 (Fig 5E). (XLSX)

S6 Table. Individual values underlying the current-voltage relationship for $I_{Na}$ (Fig 6B), and the normalized densitometry values for Nav1.5 (Fig 6C). (XLSX)

Acknowledgments

The authors would like to thank Christine S. Moravec and Lance Wilson for their generous contributions of human and canine cardiac tissue samples, respectively.
Author Contributions
Conceived and designed the experiments: DMN XW ID. Performed the experiments: DMN XW HL. Analyzed the data: DMN XW. Contributed reagents/materials/analysis tools: XW HL ID. Wrote the paper: DMN XW ID.

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