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Protein Phosphatase 2A Regulates Cardiac Na^+ Channels

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Abstract

Rationale: Voltage-gated Na^+ channel (I_{Na}) function is critical for normal cardiac excitability. However, the Na^+ channel "late" component ($I_{Na,L}$) is directly associated with potentially fatal forms of congenital and acquired human arrhythmia. Ca^{2+}/calmodulin-dependent kinase II (CaMKII) enhances $I_{Na,L}$ in response to increased adrenergic tone. However, the pathways that negatively regulate the CaMKII/$Na_v1.5$ axis are unknown and essential for the design of new therapies to regulate the pathogenic $I_{Na,L}$.

Objective: To define phosphatase pathways that regulate $I_{Na,L}$ in vivo.

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DISCLOSURES

XHTW is a founding partner of Elex Biotech, a start-up company that developed drug molecules that target ryanodine receptors for the treatment of cardiac arrhythmia disorders. Other authors have no conflicts.

Methods and Results: A mouse model lacking a key regulatory subunit (B56α) of the protein phosphatase 2A (PP2A) holoenzyme displayed aberrant action potentials following adrenergic stimulation. Unbiased computational modeling of B56α knockout (KO) mouse myocyte action potentials revealed an unexpected role of PP2A in $I_{Na,L}$ regulation that was confirmed by direct $I_{Na,L}$ recordings from B56α KO myocytes. Further, B56α KO myocytes display decreased sensitivity to isoproterenol-induced induction of arrhythmogenic $I_{Na,L}$, and reduced CaMKII-dependent phosphorylation of $Na_v1.5$. At the molecular level, PP2A/B56α complex was found to localize and co-immunoprecipitate with the primary cardiac Na_v channel, $Na_v1.5$.

Conclusions: PP2A regulates $Na_v1.5$ activity in mouse cardiomyocytes. This regulation is critical for pathogenic $Na_v1.5$ "late" current and requires PP2A-B56α. Our study supports B56α as a novel target for the treatment of arrhythmia.

Subject Terms:

Animal Models of Human Disease; Arrhythmia; Basic Science Research; Calcium Cycling/ Excitation-Contraction Coupling; Cell Signaling/Signal Transduction

Keywords

PP2A; ankyrin; CaMKII; $Na_v1.5$; arrhythmia (mechanisms); physiology/function; phosphorylation; phosphatase

INTRODUCTION

Voltage-gated Na^+ channels (Na_v) play a critical role in regulation of myocyte membrane excitability and cardiac function. Na_v channel current (I_{Na}) is a large amplitude, short duration inward current that is regulated by rapid channel activation and immediate inactivation.[1, 2] However, a small "late" component of I_{Na} ($I_{Na,L}$) is present at baseline. This $I_{Na,L}$ increases in response to heightened adrenergic conditions and has been directly associated with potentially fatal forms of congenital and acquired human arrhythmia.[3–6] Thus, the mechanisms underlying $I_{Na,L}$ regulation have been a critical area of translational research both in academics and industry.[7–10]

Ca^{2+}/calmodulin-dependent kinase II (CaMKII)-dependent phosphorylation of $Na_v1.5$ is directly linked with increased $I_{Na,L}$. Increased CaMKII-dependent phosphorylation $Na_v1.5$ (pS571; Nav1.5 phosphorylation at amino acid 571) is now a signature of adrenergic imbalance in human heart disease and animal models of cardiovascular disease.[4, 11–16] Despite the clear impact of the $Na_v1.5$/CaMKII regulatory axis in human disease, we are unaware of any in vitro or in vivo data on the molecular mechanisms that negatively regulate the CaMKII/$Na_v1.5$ axis. We expect these data to advance the design of new therapies aimed at regulating arrhythmogenic $I_{Na,L}$.

There are three major protein phosphatase (PP) families: tyrosine, serine-threonine, and dual specificity phosphatases, with serine-threonine phosphatases (e.g. PP1, PP2A and PP2B) responsible for approximately 90% of de-phosphorylation events in the heart. While both PP1 and PP2B have been tightly linked with cardiac signaling and disease, PP2A function

has been relatively less explored. Unlike many phosphatase enzymes, the PP2A holoenzyme is formed from three subunits. The core enzyme has constitutive activity and is comprised of a catalytic (C) and scaffolding (A) subunit, each of which are encoded by two genes.[17] Unique to the PP2A class of phosphatases is the third, regulatory (B) subunit family with thirteen members each encoded by a separate gene to regulate PP2A tissue- and cell-expression, activity, and subcellular distribution.[18]

Here, we utilized an unbiased approach using the cardiac action potential of B56α deficient mice to define novel roles of PP2A in cardiac excitability. Our data implicate an unexpected and key role of PP2A, and more specifically PP2A/B56α, in the regulation of $Na_v1.5$ phosphorylation and $I_{Na,L}$. Specifically, action potentials (APs) recorded from B56α knockout (KO) mice (show increased myocyte PP2A activity) displayed decreased sensitivity to APD prolongation in response to isoproterenol treatment. Computational modeling supported a potential role for $I_{Na,L}$ in AP phenotypes and direct recording of $I_{Na,L}$ from B56α KO myocytes illustrated that B56α KO mice are insensitive to isoproterenol-induced arrhythmogenic $I_{Na,L}$. Mechanistically, we identify that PP2A/B56α complex is localized at the intercalated disc with the primary cardiac Na_v channel, $Na_v1.5$. We illustrate that the B56α-associated PP2A complex is critical for Na_v channel activity as $Na_v1.5$ pS571 is reduced in mice lacking B56α. Finally, we illustrate that the B56α/PP2A is a *bona fide* regulatory molecule for $Na_v1.5/I_{Na,L}$ in myocytes, as B56α KO myocytes are insensitive to isoproterenol-induced augmentation of $I_{Na,L}$. In summary, our findings define a pathway that regulates CaMKII-dependent phosphorylation in the heart, and identify a potential novel target to suppress arrhythmogenic activity of I_{Na} in heart.

METHODS

The data that support the findings of this study are available from the corresponding author on reasonable request.

Animals.

All animal procedures were approved and in accordance with institutional guidelines (Institutional Animal Care and Use Committee; The Ohio State University). All mice used were age-matched (male and female littermates) and were housed in the same facility (temperature and humidity), consumed the same diet, provided water *ad libitum*, and kept on identical 12-h light/dark cycles. The investigation conformed to the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health. B56α KO mice (backcrossed >7 generations for this study) were created as described.[19]

Detailed descriptions on the materials and methods used in this study are provided in the Online Supplement.

RESULTS

B56α knockout mice display altered cardiomyocyte excitability.

Our group previously identified the B56α class of PP2A regulatory subunits as key regulatory proteins in heart. Work from our group and others implicate this pathway as

essential for regulation of RyR2 phosphorylation and cardiac calcium signaling.[19] Finally, B56α KO mice illustrate that this subunit behaves as an auto-inhibitor of PP2A activity in heart and B56α KO hearts display increased PP2A activity and reduced spontaneous calcium release in response to increased sympathetic activity.[19]

To test the impact of B56α deficiency on cardiomyocyte excitability, APs were recorded from isolated cardiac myocytes of WT mice or mice lacking B56α (B56α KO) at 0.5 and 1.0 Hz. B56α KO myocytes displayed significantly shortened AP duration (APD) at 50%, 75% and 95% repolarization (APD_{50}, APD_{75}, and APD_{95}) compared with WT myocytes. Specifically, APD_{50}, APD_{75}, and APD_{95} were reduced 53%, 46%, and 32% respectively, in B56α KO ventricular myocytes paced at 1-Hz (Figure 1A–B; $p<0.05$). Action potential amplitude (APA) and maximum upstroke velocity (dv/dt_{max}) demonstrated a downward trend in B56α KO myocytes, but did not achieve statistical significance (Figure 1C–D). While we previously implicated B56α and PP2A with the regulation of RyR2 in myocytes[19], these APD data support potential new roles of PP2A-dependent regulation in myocyte excitability.

B56α KO mice display decreased sensitivity to adrenergic stimulation.

Based on the role of PP2A in autonomic regulation of the heart, we tested the impact of beta-adrenergic stimulation on myocyte excitability in WT and B56α KO myocytes. When exposed to 100nM isoproterenol (Iso), WT myocytes exhibited a 42% and 47% prolongation of APD_{95} at 0.5 Hz and 1.0 Hz pacing respectively (Figure 2A,C; 1 Hz). In contrast, 100nM Iso did not alter APD_{95} in B56α KO myocytes at either pacing frequency (Figure 2B, D, 1Hz). Most notably, B56α KO myocytes displayed nearly identical repolarization profiles late in the AP. No significant changes in APA or dv/dt_{max} were identified between WT or B56α KO myocytes following Iso treatment (Figure 2E–H). Together, these new data support key roles of PP2A in regulation of cardiac excitability at baseline and following adrenergic stimulation. Importantly, these findings support new roles of PP2A in the regulation of late phases of the cardiac action potential.

Identification of Nav1.5 as PP2A target in heart.

To define potential new targets of the PP2A/B56α complex in heart, computational modeling was performed. Briefly, partial least-squares regression analysis was performed using the Hund-Rudy AP model to identify sets of parameters that produced the best-fit to experimental data from WT and B56α KO AP measurements (APD, APA, and dv/dt_{max}; representative regression coefficients are shown for APD in Figure 3A).[20–23] This unbiased approach supported work from our group and others linking PP2A with cardiac ion channels and transporters important for intracellular calcium handling (Figure 3A; $Ca_v1.2$, NCX, SERCA2a).[19, 24] However, this analysis predicted a new link between PP2A/B56α and I_{Na} regulation. While not the focus of this manuscript, the findings also suggest a potential role in $I_{Ca,L}$ regulation. Specifically, the simulations predicted an important role for altered $I_{Na,L}$, but not $I_{Na,peak}$, in abnormal cell membrane excitability observed in the setting of B56α deficiency. In fact, the unbiased computational analysis identified a likely 60–70% decrease in $I_{Na,L}$ as a mechanistic determinant of altered repolarization in B56α KO myocytes (Figure 3B–C).

Identification of Nav1.5 as PP2A target in heart.

Based on computational predictions, we tested the impact of B56α deficiency on I_{Na} properties in isolated mouse cardiomyocytes. As shown in Figure 4A–C, we observed no difference in the current-voltage relationship or peak I_{Na} at baseline between WT and B56α KO myocytes (p=N.S.). Further, we observed no difference in I_{Na} voltage-dependent activation, steady-state voltage-dependent inactivation, or time-dependent recovery between WT and B56α KO myocytes (Figure 4D–E; p=N.S.). Detailed analysis of these properties by gender did not identify a significant difference in whole cell I_{Na} properties or cell capacitance between male or female mice (Online Figure I; p=N.S.). However, direct recording of $I_{Na,L}$ illustrated that integration of I_{Na} from 50 to 100 msec was ~40 % lower at baseline in B56α KO myocytes compared to WT myocytes (Figure 4F–G; WT 9.65 ±1.87 vs. 5.82±1.06 nA.msec in B56α KO; $p<0.05$; no statistical difference in cell capacitance, Figure 4H). In summary, $I_{Na,L}$ is reduced in B56α KO mouse cardiomyocytes at baseline compared with WT myocytes.

B56α/PP2A complex is localized with CaMKII at the myocyte intercalated disc.

As prior work has focused on the link between PP2A and transverse-tubule/sarcoplasmic reticulum calcium handling machinery[19, 25–27], we next investigated the predicted association of PP2A with the primary cardiac Na_v channel, $Na_v1.5$ (Online Figure IIA–J). While present at multiple membrane domains[2829], $Na_v1.5$ is enriched at the cardiac intercalated disc (Online Figure IIA).[30, 31] In support of a regulatory role of B56α/PP2A for $I_{Na,L}$ regulation, populations of B56α as well as PP2A catalytic and scaffolding subunits were also localized to the intercalated disc of WT myocytes (Online Figure IIC, E,F,G,). In the heart, CaMKIIδ phosphorylation of $Na_v1.5$ is critical for regulation of I_{Na} availability and $I_{Na,L}$.[13, 32] As previously described[13], and in support of a role for dual I_{Na} adrenergic regulation by CaMKII/PP2A, we observed localization of CaMKIIδ at the intercalated disc (Online Figure IID). Finally, the adapter protein ankyrin-G, localized primarily to the intercalated disc (Online Figure IIB) is critical for both the targeting and regulation of $Na_v1.5$.[3331, 34] Specifically, ankyrin-G directly interacts with $Na_v1.5$ and β_{IV}-spectrin, a CaMKII-scaffolding protein at the intercalated disc (Online Figure IIH).[13] In our studies, we observed significant expression of CaMKIIδ and ankyrin-G at sites co-localized in triple-labeling experiments with PP2A subunits at the intercalated disc (Online Figure III–J). In summary, B56α and PP2A core enzyme subunits are localized with the CaMKII regulatory complex. However, it is important to note that while enriched at the intercalated disc, these components, as well as $Na_v1.5$ populations, are present at other membrane domains as previously demonstrated by multiple groups[2829]. As expected, the localization of ankyrin-G, CaMKIIδ, and PP2A-C were independent of the B56α expression (Online Figure III).

PP2A/B56α regulatory complex associates with membrane complex.

Based on our new data, we performed co-immunoprecipitation experiments using detergent-soluble lysates of mouse left ventricle to determine whether the PP2A holoenzyme associates with the $Na_v1.5$ regulatory complex. We observed association of ankyrin-G and CaMKIIδ with $Na_v1.5$ (Figure 5A–B). Further, we demonstrated association of the PP2A catalytic subunit (PP2A-C, Figure 5C), the PP2A B56α regulatory subunit (Figure 5D), and

the PP2A scaffolding subunit (PP2A-A) with $Na_v1.5$ by co-immunoprecipitation (Figure 5E). Ankyrin-G also associated with PP2A-C by co-immunoprecipitation (Figure 5F). Finally, we confirmed our findings in human heart, through co-immunoprecipitation of Nav1.5 with PP2A subunits from detergent-soluble lysates of non-failing human left ventricle (Figure 5G). In summary, the PP2A holoenzyme exists in complex with the $Na_v1.5$ complex in heart.

We previously identified an interaction of ankyrin-B (unique gene product from ankyrin-G) with B56α in heart.[35] Ankyrin-B directly associates with the C-terminus of B56α and is necessary for the targeting of B56α to the cardiac dyad to modulate CaMKII-dependent regulation of RyR2.[19, 25, 36, 37] Based on our new findings, we tested a potential interaction of ankyrin-G with B56α. Purified GST-AnkG, but not GST- alone associated with B56α from detergent-soluble lysates from mouse heart (Figure 5H). Further GST-ankyrin-G, but not GST associated with PP2A catalytic and scaffolding subunits, supporting the interaction of ankyrin-G with the full holoenzyme (Figure 5I–J). This interaction was specific, as we did not observe association of ankyrin-G with the structurally similar (but lacks the ankyrin-binding motif) PP2A B56α regulatory subunit (Figure 5K). Thus, ankyrin-G (and thus $Na_v1.5$) associates with PP2A via the B56α regulatory subunit.

B56α KO mice display aberrant CaMKII-dependent $Na_v1.5$ phosphorylation.

$Na_v1.5$ pS571 phosphorylation is increased in both ischemic and non-ischemic human heart disease, as well as in canine and mouse models of disease.[4] Finally, mice harboring $Na_v1.5$ phosphomimetic S571E display increased $I_{Na,L}$, altered myocyte electrical regulation and arrhythmia phenotypes.[12] To test the impact of B56α-associated PP2A on cardiac $Na_v1.5$, we evaluated $Na_v1.5$ phosphorylation using affinity-purified Ig directed against pS571 (Figure 6A–G). As expected, we did not observe a significant increase in total $Na_v1.5$ protein levels between WT and B56α KO mice following isoproterenol treatment (Figure 6A,E). Additionally, we observed no difference in total CaMKIIδ between WT and B56α KO mice (Figure 6A, D). However, compared with WT mouse hearts, we observed a significant decrease in cardiac $Na_v1.5$ pS571 levels in hearts of B56α KO mice (Figure 6A,F). This change was significant when normalizing for total Nav1.5 protein expression levels (Figure 6F). Notably, total PP2A-C levels were unchanged between WT and B56α KO mice (Figure 6A, C). In summary, our findings support a role of B56α-linked PP2A in regulation of $Na_v1.5$ phosphorylation.

B56α is required for $I_{Na,L}$ regulation.

To test the role of B56α for I_{Na} and $I_{Na,L}$ regulation, we evaluated peak and late I_{Na} in WTand B56α KO myocytes ± isoproterenol treatment to mimic increased adrenergic tone. As expected, isoproterenol treatment induced an increase in both peak and $I_{Na,L}$ in WT myocytes (Figure 7A, C-D; $p<0.05$). In contrast, consistent with a critical role of B56α in $I_{Na,L}$ regulation, we observed no significant difference of peak I_{Na} or $I_{Na,L}$ in B56α KO myocytes±isoproterenol treatment (Figure 7B–D; p=N.S.; no significant difference in cell capacitance between genotypes). In fact, at baseline, we observed a reduction in $I_{Na,L}$ between WT and B56α KO myocytes likely representing the basal increase in PP2A-dependent phosphatase activity in the B56α KO heart.[19, 38, 3940] In summary, myocytes

lacking B56α have reduced sensitivity to isoproterenol-mediated increases in $I_{Na,L}$ and importantly arrhythmogenic $I_{Na,L}$.

DISCUSSION

In vertebrates, cellular activity is tightly governed by highly evolved protein signaling platforms. Key to this regulation is the control of protein function via reversible protein phosphorylation. In the heart, protein phosphorylation is a central signaling axis with implications for excitation-contraction coupling, transcriptional regulation and metabolism.[41, 42] Alterations in protein phosphorylation are present in both congenital and acquired forms of heart disease including fatal arrhythmias and heart failure.[43–45] While the protein kinase axis has received appropriate attention and has been vital in specific cardiovascular therapies, the removal of phosphate groups by phosphatases is relatively less studied, although mounting data support important roles for PP1 (protein phosphatase type 1) and PP2A in cardiovascular disease.[46–49] For example, we and others have illustrated that B56α is critical for targeting key populations of PP2A to the cardiac T-tubule/SR through the interaction of B56α with ankyrin-B.[35] Mice lacking ankyrin-B display a loss of T-tubule/SR B56α targeting and alterations in CaMKII/PP2A signaling, contributing to increased adrenergic tone and substrate for the development of catecholamine-based arrhythmias.[35, 36] Further, PP2A activity has been linked to a number of key ion channel activities including the L-type calcium-channel ($Ca_v1.2$)[24], ryanodine receptor 2 (RyR2)[19], Na^+/Ca^+ exchanger (NCX)[50], and Na^+/K^+ ATPase.[35, 51]

Work from our group and others has defined a critical role of CaMKII in $Na_v1.5$ regulation for normal cardiac electrical function and in disease. Specifically, CaMKII-dependent phosphorylation of $Na_v1.5$ at sites including serine 571 have been highly associated with decreased $Na_v1.5$ channel availability and increased $I_{Na,L}$.[11] In fact, mice harboring a phosphomimetic S/E residue at Ser571 display increased $I_{Na,L}$ and increased arrhythmia susceptibility, whereas ablation of the serine site by S/A substitution abrogates effects of isoproterenol on $I_{Na,L}$ and burden of arrhythmia.[12] Notably, CaMKII is tightly linked with its effector molecule $Na_v1.5$ via interaction with the β_{IV}-spectrin/ankyrin-G scaffold.[13] While other Nav channel subunits are present in heart[52, 53,54] we did not observe interaction of either $Na_v1.6$ or Nav β subunits with the PP2A complex in co-immunoprecipitation assays (Online Figure IVA–B). However, future experiments will be necessary to definitively define the role of PP2A for $Na_v1.5$-independent I_{Na} in heart.

Our new data expand the ankyrin-G/$Na_v1.5$/CaMKIIδ/β_{IV}-spectrin protein complex and define the specific signaling pathway underlying negative/inhibitory modulation of $Na_v1.5$ and $I_{Na,L}$, as well as other potential membrane and submembrane effectors at the cardiac intercalated disc (Figure 8). Specifically, our new data implicate PP2A via interaction with B56α, as critical for AP duration, both at baseline and following adrenergic stimulation. Our new findings illustrate key potential downstream membrane complexes as targets for PP2A/B56α including $I_{Na,L}$. We demonstrate that B56α is localized with $Na_v1.5$ and CaMKII, as well as ankyrin-G and β_{IV}-spectrin at the intercalated disc, and biochemical experiments show both co-immunoprecipitation of the full PP2A holoenzyme (catalytic, regulatory, scaffolding subunits) and interaction of B56α with ankyrin-G, and $Na_v1.5$. Notably, in line

with prior findings illustrating elevated $Na_v1.5$ pSer571 phosphorylation and elevated $I_{Na,L}$ in the presence of heightened adrenergic state, B56α KO hearts displayed reduced $Na_v1.5$ pSer571 phosphorylation and $I_{Na,L}$ in response to isoproterenol treatment. In summary, our new findings define a key nodal regulatory complex in heart, as well as a new molecular target for the regulation of $Na_v1.5$, and specifically the pathogenic $I_{Na,L}$.

This is the first study evaluating the role of PP2A in the negative regulation of CaMKII/$Na_V1.5$; thus there are limitations. First, it is important to note that although our data support PP2A as a central regulatory molecule for $Na_v1.5$ and $I_{Na,L}$, our study does not preclude secondary mechanisms. Certainly, I_{Na} regulation is complex and relies on multiple phosphorylation and secondary post-translational modifications.[55–57] Second, PP1 is a dominant phosphatase, particularly in heart. Thus, we cannot rule out key roles of PP1 and even other non-traditional cardiac protein kinases and phosphatases for $Na_v1.5$ regulation.[58, 59] Third, while our studies were performed in vivo, it will be critical for expansion of these studies into large animal and even human models. Fourth, while B56α KO mice have served as an excellent first-generation model to understand PP2A signaling in vivo, secondary small molecule inhibitors will be the next logical step to ensure appropriate and selective acute or chronic B56α inhibition depending on the pathology. Nonetheless, these studies provide compelling new evidence supporting a critical role of local signaling complexes for ion channel regulation in health and disease. Finally, while prior data supports that reduced basal $I_{Na,L}$ in B56α KO myocytes is a function of increased basal phosphatase activity in B56α hearts, future experiments will be necessary to define the direct and indirect (compensatory) changes that result in altered $I_{Na,L}$ in the chronic absence of B56α.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Nonstandard Abbreviations and Acronyms:

Aps	Action potentials
APA	Action potential amplitude
APD	Action potential duration
CaMKII	Ca^{2+}/calmodulin-dependent kinase II
dv/dt_{max}	Maximum upstroke velocity
I_{Na}	Na_v channel current

$I_{Na,L}$	late component of I_{Na}
KO	Knockout
Nav1.5	voltage-gated Na^+ channel 1.5
PP2A	Protein phosphatase 2A
PP1	Protein phosphatase 1
RyR2	Ryanodine receptor 2

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NOVELTY AND SIGNIFICANCE

What Is Known?

- Cardiac function depends on the coordinated activity of voltage-gated Na^+ channels (Na_v). Na_v channel current (I_{Na}) is a short-duration inward current caused by rapid channel activation and immediate inactivation.
- Na^+ channel "late" component ($I_{Na,L}$) is present at baseline and increases in response to adrenergic stimulation. This late component is directly associated with potentially fatal forms of congenital and acquired human arrhythmia.
- Ca^{2+}/calmodulin-dependent kinase II (CaMKII) enhances $I_{Na,L}$ in response to increased adrenergic tone. CaMKII-dependent phosphorylation $Na_v1.5$ (Nav1.5 phosphorylation at amino acid 571) is a hallmark of adrenergic imbalance in human heart disease and animal models of cardiovascular disease.

What New Information Does This Article Contribute?

- We define a pathway that negatively regulates CaMKII-dependent phosphorylation in the heart.
- The PP2A holoenzyme exists in complex with the $Na_v1.5$ complex in heart. These findings support a regulatory role for PP2A-B56α in $Na_v1.5$ phosphorylation.

Although critical for normal cardiac excitability, defects in the activity of the primary voltage-gated Nav channel, Nav1.5 are linked with both congenital and acquired forms of cardiac arrhythmia. More specifically, the Na^+ channel "late" component ($I_{Na,L}$) is directly associated with potentially fatal forms of arrhythmia. Ca^{2+}/calmodulin-dependent kinase II (CaMKII) enhances $I_{Na,L}$ in response to elevated adrenergic tone and this current may support arrhythmia. Nevertheless, the molecular mechanisms that negatively regulate the CaMKII/$Na_v1.5$ pathway are not well known. Our findings delineate a role of PP2A/B56α in the modulation of $I_{Na,L}$. This work illustrates that PP2A-based pathways regulate CaMKII-dependent phosphorylation in the heart and support a potential target to suppress the arrhythmogenic activity of Nav1.5.

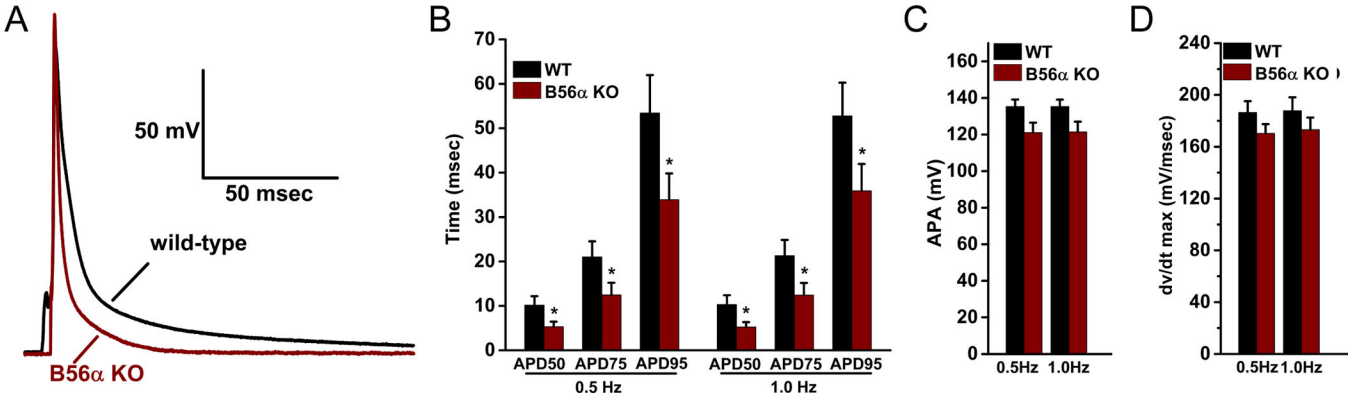

Figure 1. B56α KO mice display aberrant cardiomyocyte excitability.

(A-B) Representative action potential (APs, 1.0 Hz pacing) and summary of APD at 50%, 75% and 95% repolarization for 0.5 and 1.0 Hz pacing in WT and B56α KO myocytes. (C-D) AP amplitudes (APA) and maximum upstroke velocity (dv/dt_{max}) in WT and B56α KO myocytes. Results are shown for 0.5 and 1.0 Hz pacing frequencies (for B-D, WT, N=3; n= 9 and B56α KO, N=3; n=8; *p<0.05).

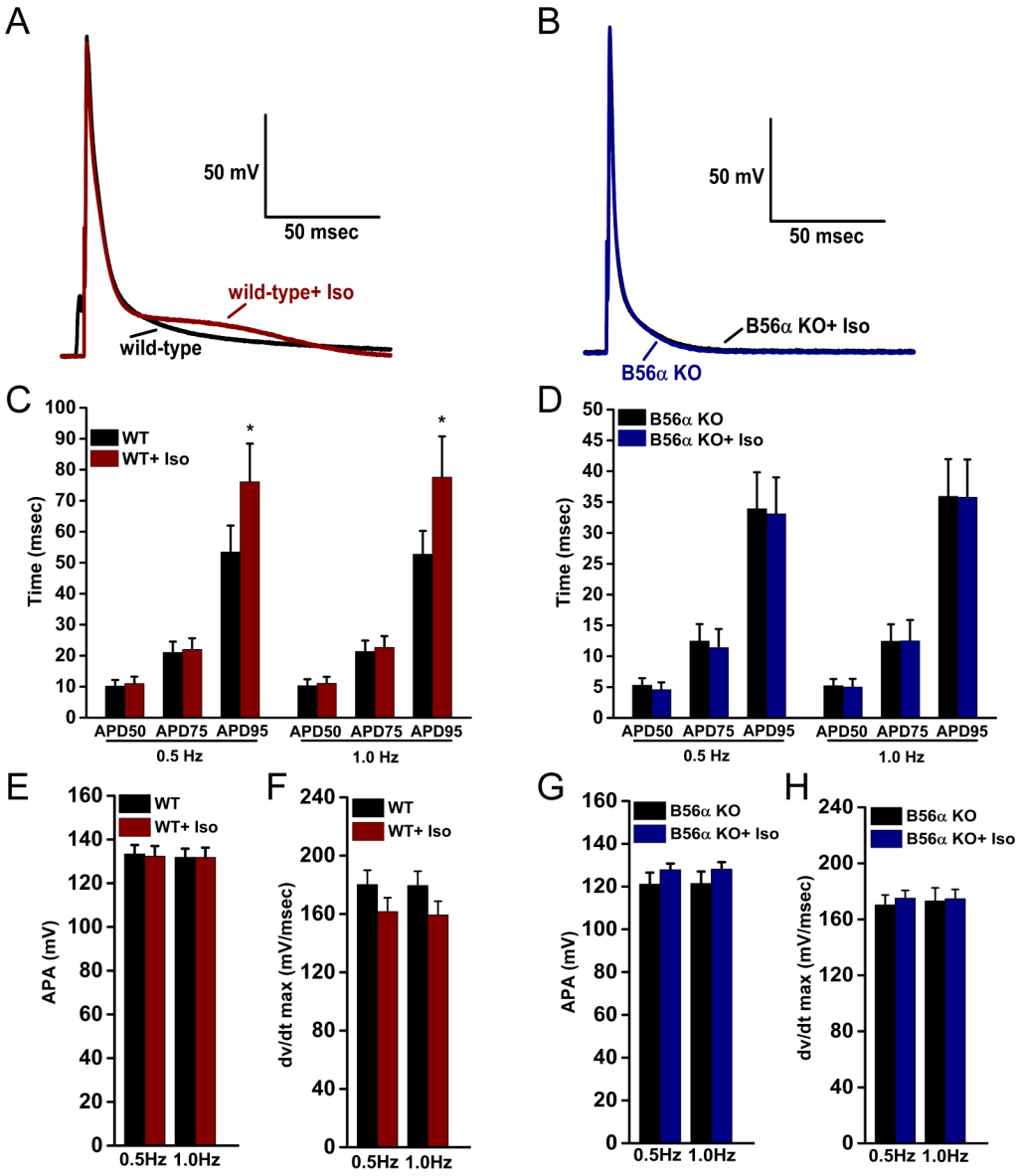

Figure 2. B56α KO ventricular myocytes display decreased sensitivity to isoproterenol-induced APD prolongation.

(A-D) Representative APs (1.0 Hz pacing) and summary of APD at 50%, 75% and 95% repolarization at 0.5 and 1.0 Hz pacing in WT and B56α KO myocytes ±100nM Iso. (E-H) Action potential amplitudes (APA) and maximum upstroke velocity (dv/dt_{max}) in WT and B56α KO myocytes ±Iso. Results are shown for 0.5 and 1.0 Hz pacing frequencies (WT, N=3; n=9 and B56α KO, N=3; n=8 *p<0.05).

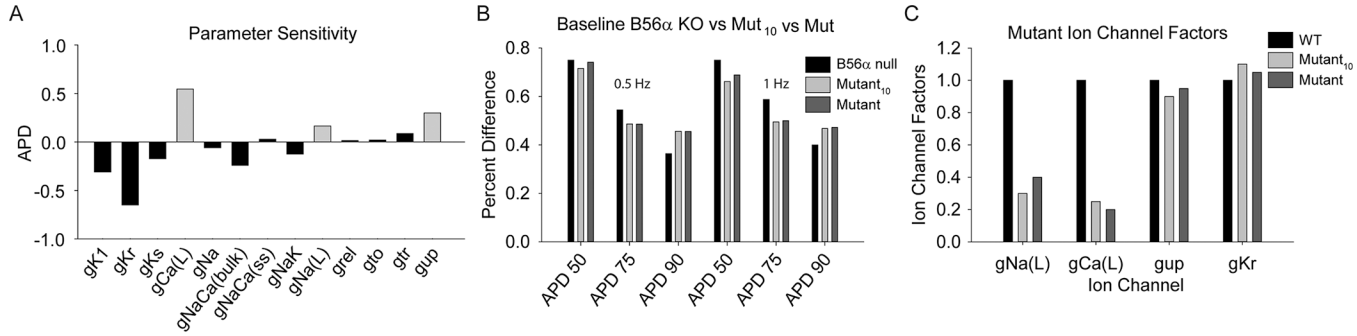

Figure 3. Identification of Nav1.5 as PP2A target.

Computer simulations to predict the mechanism underlying changes in cell excitability in the setting of B56α-deficiency. (A) Regression coefficients showing relative impact of changes in ion channel conductance/transport rates on action potential duration at 90% repolarization (APD) in the Hund-Rudy dynamic cell model. Abbreviations are as follows: inwardly rectifying K current (g_{K1}), rapid delayed rectifier K^+ current (g_{Kr}), slow delayed rectifier K^+ current (g_{Ks}), L-type Ca^{2+} current ($g_{Ca(L)}$), fast inward Na^+ current (g_{Na}), subspace and bulk Na^+/Ca^{2+} exchanger ($g_{NaCa(ss)}$ and $g_{NaCa(bulk)}$, respectively), Na^+/K^+ ATPase (g_{Nak}), late Na^+ current ($g_{Na,L}$), Ca^{2+} release from SR (g_{rel}), transient outward K^+ current (g_{to}), Ca^{2+} translocation from network to junctional SR (g_{tr}) and Ca^{2+} uptake into SR (g_{up}). (B) APD_{90} (expressed relative to WT) in experiment (B56α KO), and in the following computational models: 1) model with parameter values identified in the regression analysis with smallest sum-of-squared error compared to experiment (Mutant), and 2) model with parameter values selected as average of 10 best solutions ($Mutant_{10}$). (C) Corresponding model parameters (expressed relative to WT) for Mutant and $Mutant_{10}$ computational models.

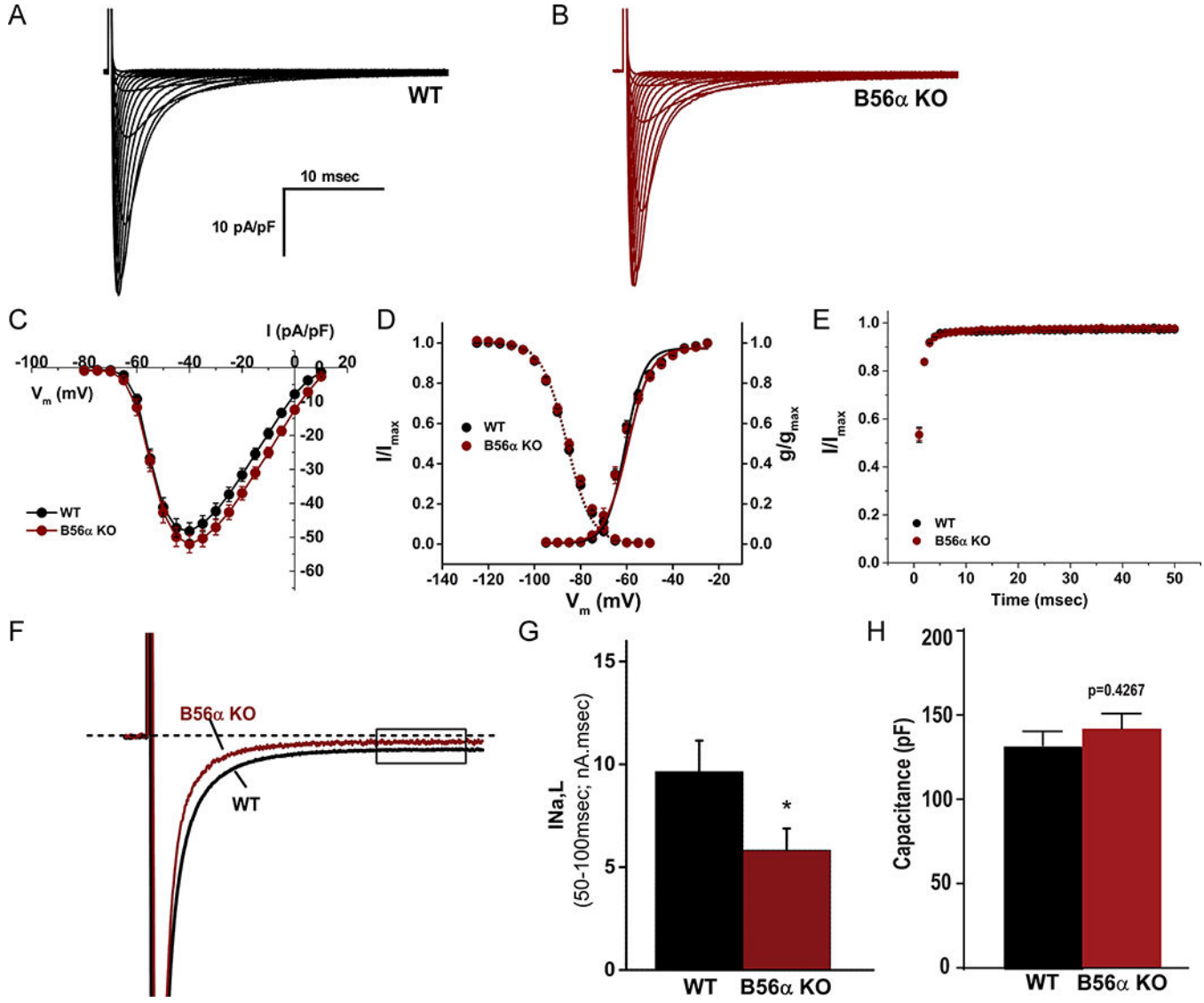

Figure 4. B56α KO myocytes display decreased $I_{Na,L}$.

(A-B) Representative recordings of whole cell I_{Na} from WT and B56α KO ventricular myocytes. (C) Current-voltage relationship, (D) voltage-dependent activation and voltage-dependent inactivation curves, and (E) time-dependent recovery of I_{Na} in WT and B56α KO ventricular myocytes. No significant difference was observed in peak I_{Na} at experimental voltages ranging from −60 to −15mV (p=N.S.), in $V_{½}$ as determined by Boltzmann fits of the steady-state voltage-dependent inactivation (p=N.S.) and time-dependent recovery (p=N.S.; N=5,5, n=22,21 combined genders). (F) Representative $I_{Na,L}$ traces from WT and B56α KO ventricular myocytes. (G) Quantification of $I_{Na,L}$ from WT and B56α KO ventricular myocytes at baseline (WT, N=4; n=10 and B56α KO, N=4; n=9; *p<0.05). (H) No difference in cell capacitance was observed between WT and B56α KO myocytes (p=N.S.; shown for $I_{Na,L}$ and I_{Na} experiments).

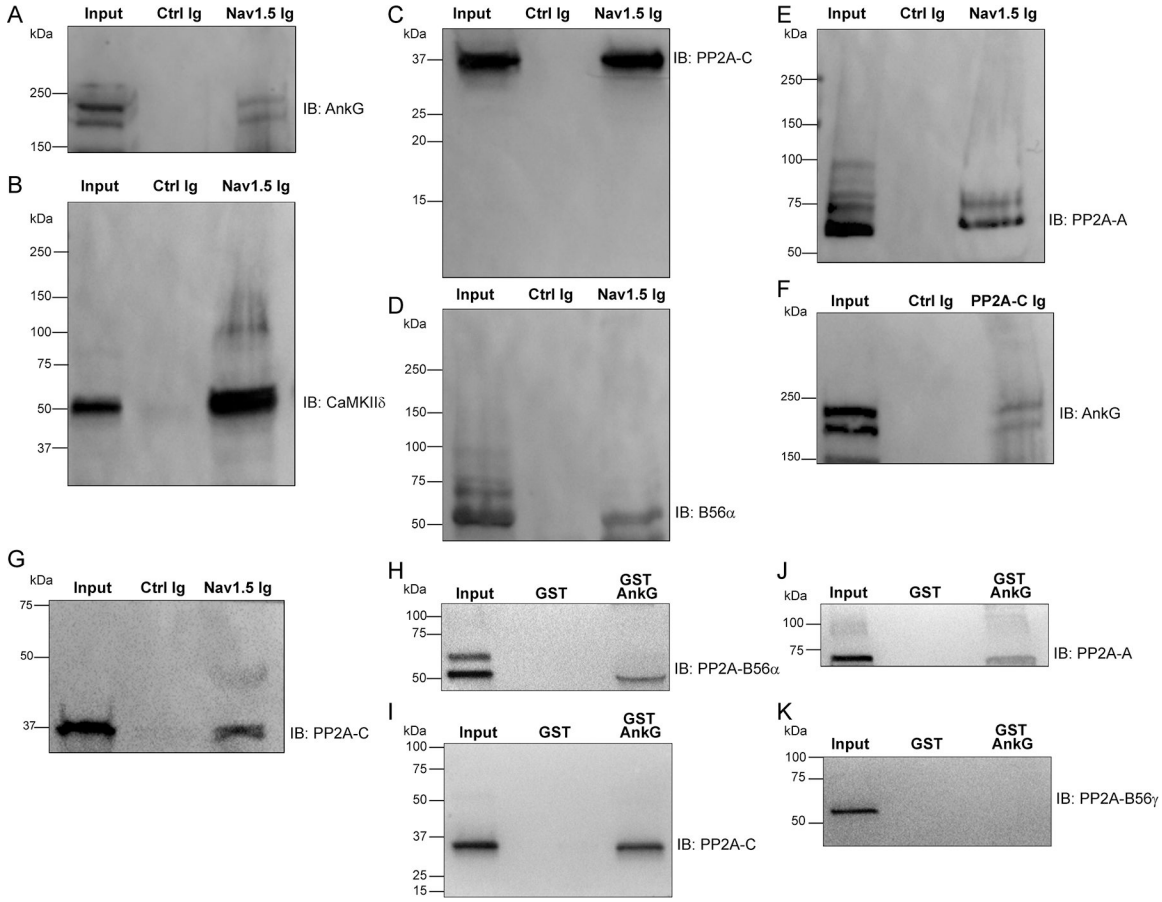

Figure 5. PP2A/B56α complex associates with $Na_v1.5$ regulatory complex.

(A-E) Co-immunoprecipitation experiments were performed from detergent-soluble lysates of adult mouse hearts using beads conjugated to $Na_v1.5$ Ig or control Ig. Bound protein was eluted and immunoblotted with ankyrin-G (A), CaMKIIδ (B), PP2A-C (C), PP2A-B56α (D) or PP2A-A (E). Data are representative of experiments repeated three times. (F) Co-immunoprecipitation experiments were performed from detergent-soluble lysates of adult mouse hearts using beads conjugated to PP2A-C Ig or control Ig. Bound protein was immunoblotted with ankyrin-G. (G) Co-immunoprecipitation experiments were performed from human left ventricular lysates (non-failing hearts) using beads conjugated to $Na_v1.5$Ig or control Ig. Bound protein was immunoblotted with PP2A-C. Experiments were repeated three times. (H-K) Pull-down experiments were performed using detergent-soluble extracts of WT mouse hearts using glutathione S-transferase (GST) or GST-AnkG. Bound protein was analyzed using antibodies specific for PP2A-B56α (H), PP2A-C (I), PP2A-A (J) or PP2A-B56γ (L). Data are representative of experiments repeated three times.

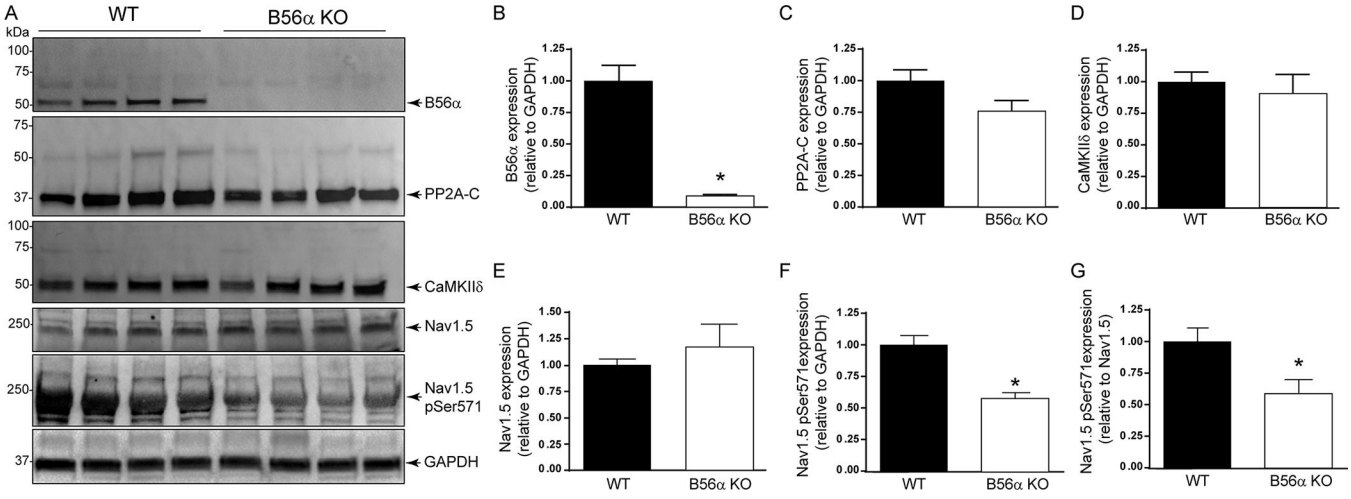

Figure 6. B56α KO mice display decreased Nav1.5 Ser571 phosphorylation.

(A-G) Immunoblots and quantitative analysis of PP2A-B56α (WT, N=5; B56α KO, N=7; $p<0.05$), PP2A-C (WT, N=7; B56α KO, N=7; p=N.S.), CaMKIIδ (WT, N=7; B56α KO, N=7; p=N.S.) Nav1.5 (WT, N=11; B56α KO, N=11; p=N.S.), Nav1.5 pSer571 (WT, N=11; B56α KO, N=10; $p<0.05$) and GAPDH expression in WT and B56α KO mouse hearts following isoproterenol treatment. B56α KO mice showed a significant decrease in both B56α expression and Nav1.5 Ser571 phosphorylation, as well as the ratio of Nav1.5 Ser571 phosphorylation to Nav1.5 expression (WT, N=11; B56α KO, N=10; $p<0.05$) following isoproterenol treatment. For $Na_v1.5$pS571/total $Na_v1.5$ratio, data is expressed based on normalized protein expression versus GAPDH as shown in E and F. We observed no difference in GAPDH expression between genotypes (WT, N=11; B56α KO, N=10; p=N.S.).

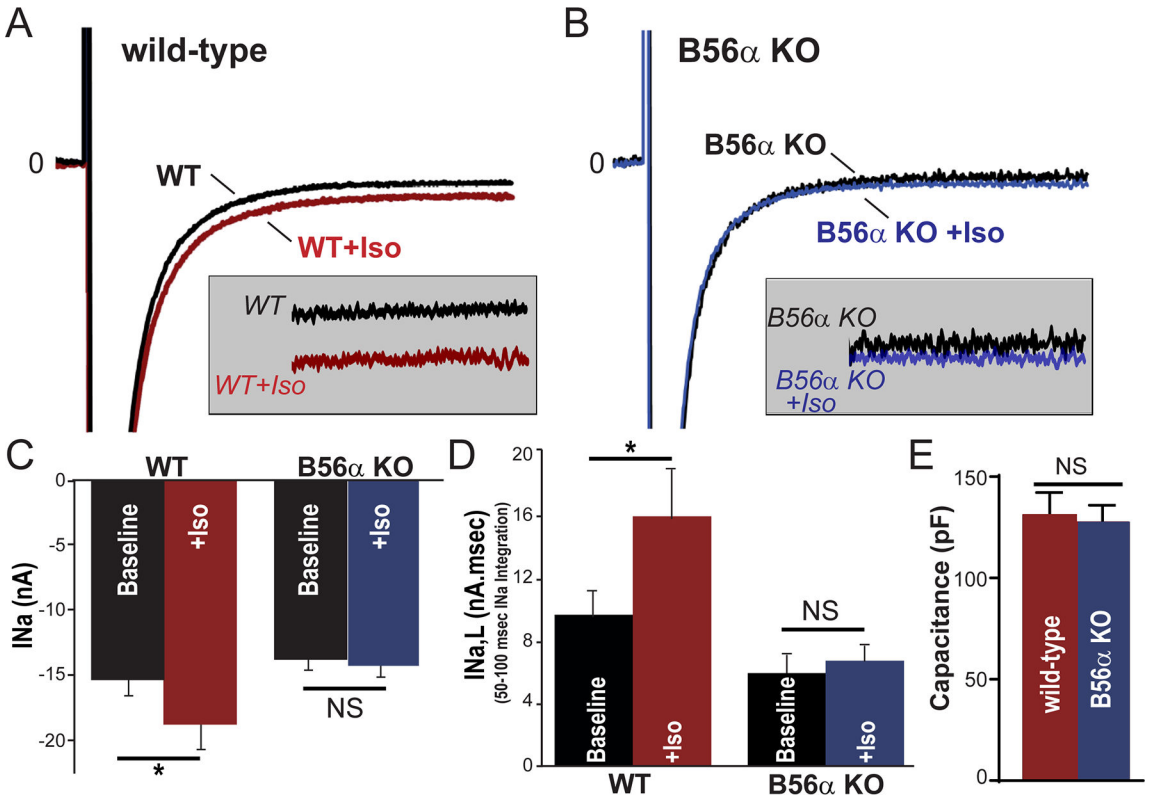

Figure 7. B56α KO myocytes are insensitive to isoproterenol-induced increases of $I_{Na,L}$.

(A-B) Representative voltage-gated Na^+ current (I_{Na}) traces from WT and B56α KO ventricular myocytes ± isoproterenol (100nM). (C-D) Summary data (mean ± SEM) for peak I_{Na} (at −30 mV), and $I_{Na,L}$ in response to 100nM Iso (*p<0.05). (E) Cell capacitance was not different between groups (p=N.S., WT, WT Iso N=5, 4; n=10, 9 and B56α KO, B56α KO Iso N=4,4; n= 9,7).

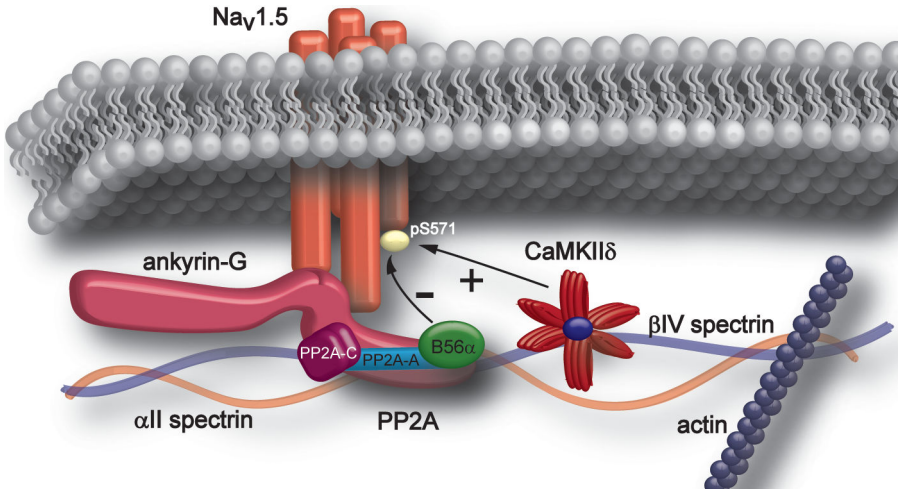

Figure 8. Intercalated disc Na$_V$1.5 macromolecular signaling complex.

Via direct interaction of Nav1.5 with ankyrin-G and βIV spectrin, CaMKIIδ resides in close proximity with its target molecule, Nav1.5. Our new findings implicate ankyrin-G in targeting the PP2A complex via B56α to the Nav1.5 intercalated disc complex to balance CaMKII-dependent phosphorylation. Together, CaMKIIδ and PP2A regulate $I_{Na,L}$ in heart.