A β$_{IV}$-spectrin/CaMKII signaling complex is essential for membrane excitability in mice

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Ion channel function is fundamental to the existence of life. In metazoans, the coordinate activities of voltage-gated Na$^+$ channels underlie cellular excitability and control neuronal communication, cardiac excitation-contraction coupling, and skeletal muscle function. However, despite decades of research and linkage of Na$^+$ channel dysfunction with arrhythmia, epilepsy, and myotonia, little progress has been made toward understanding the fundamental processes that regulate this family of proteins. Here, we have identified β$_{IV}$-spectrin as a multifunctional regulatory platform for Na$^+$ channels in mice. We found that β$_{IV}$-spectrin targeted critical structural and regulatory proteins to excitable membranes in the heart and brain. Animal models harboring mutant β$_{IV}$-spectrin alleles displayed aberrant cellular excitability and whole animal physiology. Moreover, we identified a regulatory mechanism for Na$^+$ channels, via direct phosphorylation by β$_{IV}$-spectrin–targeted calcium/calmodulin-dependent kinase II (CaMKII). Collectively, our data define an unexpected but indispensable molecular platform that determines membrane excitability in the mouse heart and brain.

Introduction

Membrane excitability requires the coordinate function of precisely synchronized signaling networks. Inherent to this precision are evolved molecular pathways that compartmentalize specific signaling molecules to ensure tight spatial and temporal coupling between plasma membrane and intracellular receptors, effector proteins, and target molecules. Members of the calcium/calmodulin-dependent protein kinase II (CaMKII) family — CaMKIIα, CaMKIIβ, CaMKIIγ, and CaMKIIδ — are multifunctional serine/threonine kinases with critical roles in both excitable and non-excitable cells. CaMKII regulates diverse cellular functions, including ion channel biophysics, organelle transport, metabolism, and transcription, to modulate synaptic plasticity, cardiac excitation-contraction coupling, and hormone secretion (1–5). Furthermore, CaMKII signaling has been linked to specific disease phenotypes (5–13), including human heart failure and cognitive defects (14, 15), through its effects on a host of diverse and spatially distinct target proteins including ion channels and transporters (i.e., voltage-gated Na$^+$ channels; refs. 16–18), transcription factors, and cell death pathways (19–22). Finally, CaMKII inhibition has shown exciting promise for the treatment of excitable cell disease (5, 23–26). Collectively, these data strongly support the notion that local CaMKII/effector signaling nodes represent key cellular rheostats to translate local alterations in the cellular environment to global changes in membrane excitability and organism function.

Here, we define what we believe to be a novel signaling platform for the regulation of membrane excitability. Specifically, we identify β$_{IV}$-spectrin, a key structural component required for ion channel clustering (including voltage-gated Na$^+$ channels) in the nervous system (27–34), as a multifunctional regulatory stage for

Na$^+$ channel signaling in excitable cells. β$_{IV}$-spectrin targets critical structural and regulatory proteins to excitable membranes in heart and brain, and animal models harboring mutant β$_{IV}$-spectrin alleles display aberrant cellular excitability and whole-animal physiology. Here we show a fundamental, but unanticipated, requirement for β$_{IV}$-spectrin-dependent targeting of CaMKII to a controlling phosphorylation site, S571, on the dominant cardiac Na$^+$ channel (Na$_v$1.5). Moreover, our findings provide evidence for a similar targeting and regulatory complex in neurons. Collectively, our data define an unexpected yet commanding molecular platform that determines vertebrate membrane excitability.

Results

Identification of novel CaMKII-binding proteins. We screened the human genome for novel CaMKII-binding proteins using an algorithm derived from the CaMKII autoregulatory domain sequence and identified 32 candidates. Putative CaMKII-binding molecules included nuclear, cytosolic, and mitochondrial proteins with a host of disparate roles, including cell metabolism, cytoskeletal dynamics, and signaling (Figure 1, A and B). All CaMKII gene products (α, β, γ, and δ) were recognized by the screen; notably, only 1 known CaMKII-binding partner was identified (35). All candidates were cloned from human tissue (brain or heart), and CaMKII-binding activity was assessed by in vitro binding assays using radiolabeled target proteins and activated CaMKII (CaMKII T287D). Of 32 candidates containing the consensus CaMKII autoregulatory domain consensus motif, only 12 clones displayed remarkable CaMKII-binding activity in vitro (see Supplemental Figure 1 for examples; supplemental material available online with this article; doi:10.1172/JCI43621DS1). Positive targets included critical signaling molecules (PKCε, phospholipase A2 zeta; ref. 36), cytoskeletal-associated proteins (*SPTBN4* and *NEDD1*, ref. 37; girdin, ref. 38), and ion channel regulatory molecules. Notably, targets that shared the highest
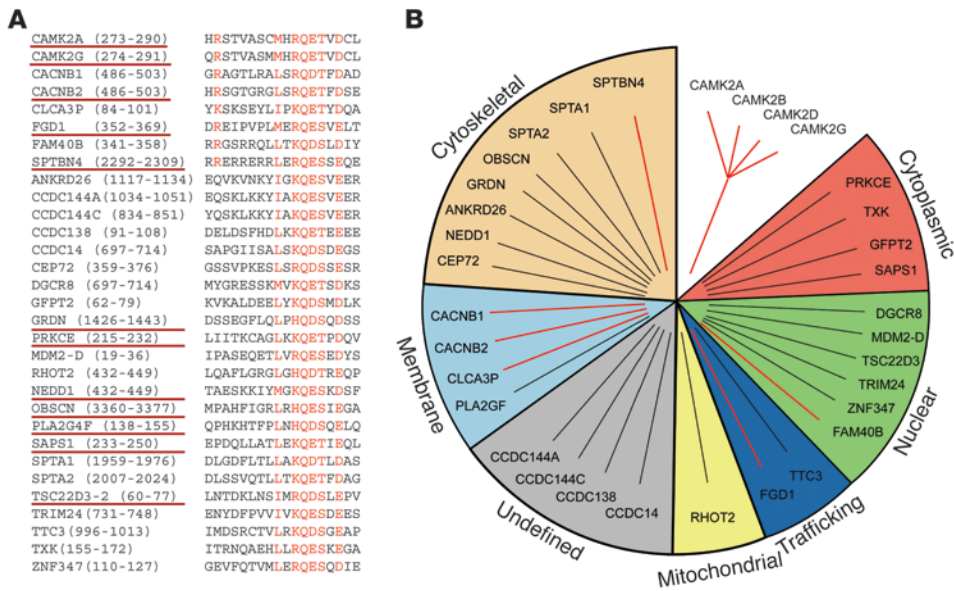
Figure 1
Identification of putative new CaMKII-binding proteins. (**A**) Putative CaMKII-targeting proteins identified through a screen of the human genome using a sequence from the CaMKII autoregulatory domain as bait. Candidates with the highest homology to CaMKII autoregulatory domain are listed first. (**B**) Candidate targeting molecules included cytoskeletal, nuclear, cytoplasmic, and mitochondrial proteins with identified roles in cell metabolism, cytoskeletal dynamics, and signaling. All CaMKII gene products (α, β, γ, and δ) were recognized by the screen; notably, only 1 known CaMKII-binding partner was identified (β2a). Candidates were cloned from human cDNA, and CaMKII-binding activity was assessed by in vitro binding assays using radiolabeled target proteins and activated CaMKII (CaMKII T287D). Notably, only clones underlined in red in **A** showed robust binding for CaMKII.

sequence identity with the CaMKII autoregulatory domain (i.e., *SPTBN4*) retained the most consistent kinase binding activity (Figure 1A and Supplemental Figure 1).

β$_{IV}$-spectrin associates with CaMKII. SPTBN4 encodes β$_{IV}$-spectrin (Figure 2A), an actin-associated protein with roles in nervous system membrane biogenesis and maintenance as well as in ion channel clustering (39, 40). Importantly, all β$_{IV}$-spectrin orthologs harbored sequences nearly identical to the CaMKII autoregulatory motif (Figure 2B). Based on these characteristics, we hypothesized that β$_{IV}$-spectrin may target CaMKII in excitable cells. We first verified the β$_{IV}$-spectrin/CaMKII association using radiolabeled CaMKIIδ and a biotinylated peptide mimicking the putative kinase-binding domain in human β$_{IV}$-spectrin (CTP-P; residues 2,292–2,317). Consistent with a specific interaction, CTP-P, but not scrambled peptide control (CTP-C), bound radiolabeled CaMKII (Figure 2C).

β$_{IV}$-spectrin is expressed in the heart and associates with CaMKII in vivo. Based on the critical role of CaMKII in regulating cardiac membrane excitability (5), we tested human heart for β$_{IV}$-spectrin expression. Notably, both brain and heart expressed β$_{IV}$-spectrin mRNA (Figure 2D). Moreover, β$_{IV}$-spectrin Σ1 and Σ6 isoforms were readily detected in the left ventricle of multiple species by immunoblot, albeit at lower levels than were observed in brain (Figure 2E). In agreement with peptide binding experiments using radiolabeled CaMKII, CTP-P, but not CTP-C, interacted with endogenous CaMKIIδ from heart (Figure 2F). Moreover, consistent with an in vivo interaction, endogenous β$_{IV}$-spectrin associated with endogenous CaMKIIδ in coimmunoprecipitation experiments (Figure 2G).

At the cellular level, β$_{IV}$-spectrin was concentrated at the intercalated disc of cardiomyocytes (colocalized with N-cadherin; Figure 2, H, I, K, and L, and Supplemental Figure 2), an excitable membrane domain critical for cardiac action potential (AP) initiation and impulse conduction. We identified 2 distinct subcellular distributions of CaMKII in myocytes. Consistent with a potential in vivo interaction, CaMKIIδ (the primary CaMKII isoform in heart; ref. 41) was coexpressed with β$_{IV}$-spectrin at the intercalated disc (Figure 2, J and M). A second population of CaMKIIδ was localized to the myocyte transverse-tubule network, apparently independent of β$_{IV}$-spectrin (Figure 2J, yellow arrowheads). Together, our data identify β$_{IV}$-spectrin as a cardiac protein that associates with endogenous CaMKII.

β$_{IV}$-spectrin organizes a macromolecular signaling complex in the heart. In the central nervous system, β$_{IV}$-spectrin and ankyrin-G are highly enriched at axon initial segments (AISs) and nodes of Ranvier, and the clustering of Na$^+$ channels at these sites depends on their interaction with ankyrin-G (31, 42). Furthermore, mice deficient in β$_{IV}$-spectrin display defects in neuronal Na$^+$ channel clustering and function (29). In heart, ankyrin-G targets voltage-gated Na$^+$ channels (Na$_v$1.5) to the intercalated disc (43, 44), and cardiac Na$^+$ channel membrane regulation has been associated with CaMKII activity (16). We therefore tested whether the β$_{IV}$-spectrin/CaMKII complex formed a macromolecular complex with ankyrin-G and Na$_v$1.5 in heart. Similar to β$_{IV}$-spectrin and CaMKIIδ, ankyrin-G and Na$_v$1.5 were enriched at the myocyte intercalated disc (Figure 2, N–Q). Moreover, ankyrin-G and Na$_v$1.5 associated with both β$_{IV}$-spectrin and CaMKIIδ by coimmunoprecipitation (Figure 2, R–U). Thus, β$_{IV}$-spectrin coordinates an in vivo cardiac macromolecular complex, effectively linking a critical signaling protein (CaMKIIδ) with its target substrate (Na$_v$1.5) at a specialized cellular domain.

β$_{IV}$-spectrin targets CaMKII to cardiomyocyte intercalated disc. We evaluated the role of β$_{IV}$-spectrin for CaMKII targeting in vivo using primary cardiomyocytes and heart tissue from the *qv^{3J}* mouse strain (45). *qv^{3J}* mice harbor a nucleotide insertion (InsT6786) in the β$_{IV}$-spectrin gene immediately proximal to the CaMKII-binding motif, resulting in a premature C-terminal truncation (Figure 3A and ref. 45). In contrast to WT β$_{IV}$-spectrin, the mutant *qv^{3J}* polypeptide lacked cardiac CaMKIIδ-binding activity (Figure 3, B–D), as predicted by our peptide binding studies (Figure 2, C and F). However, both WT and mutant proteins retained binding activity for ankyrin-G (Figure 3E), actin, and α-spectrin (data not shown). Consistent with the location of the *qv^{3J}* mutation, we observed association of ankyrin-G with CaMKIIδ
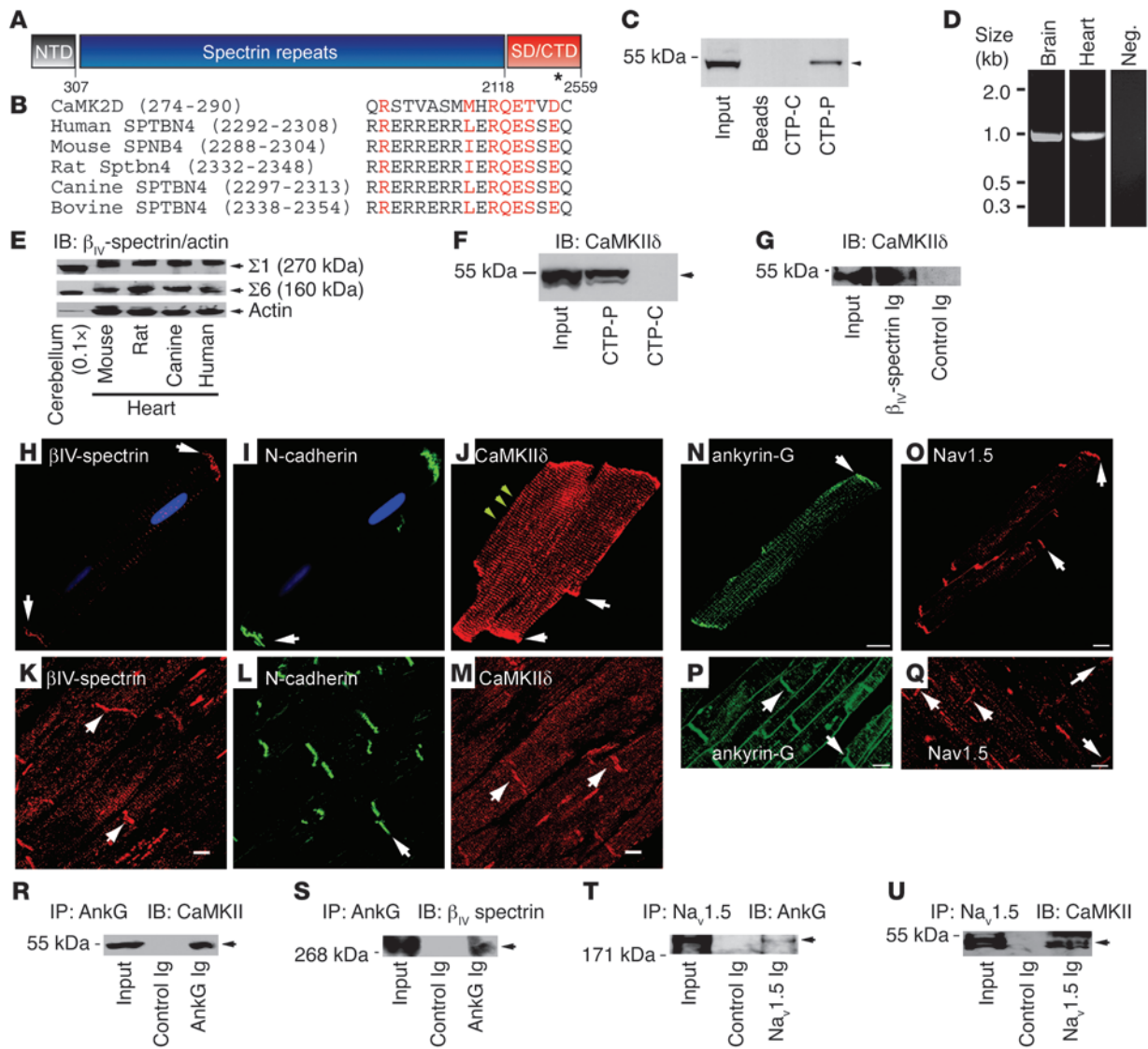
Figure 2
β_{IV}-spectrin is a CaMKII-binding protein in heart. (**A**) β_{IV}-spectrin contains an N-terminal actin-binding domain (NTD), 17 spectrin repeats, and specific and C-terminal domains (SD/CTD). The putative CaMKII-binding site is denoted by an asterisk. (**B**) The putative CaMKII-binding domain in β_{IV}-spectrin was homologous to a CaMKIIδ autoregulatory domain motif and conserved across orthologs. (**C**) CTP-P bound radiolabeled CaMKIIδ; CTP-C and GST beads alone lacked binding. (**D**) β_{IV}-spectrin RNA levels in adult rat brain and heart. (**E**) β_{IV}-spectrin ($\Sigma 1$ and $\Sigma 6$) in ventricular lysates from multiple species. β_{IV}-spectrin was expressed approximately 8–10 fold higher in cerebellum than in heart. Cardiac β_{IV}-spectrin migrated approximately 4 kDa larger than did cerebellar β_{IV}-spectrin. (**F**) CTP-P, but not CTP-C, associated with CaMKIIδ from rat heart. (**G**) Endogenous CaMKIIδ and β_{IV}-spectrin coimmunoprecipitated from adult heart lysate. (**H** and **I**) β_{IV}-spectrin and N-cadherin in rat cardiomyocytes. Nuclei are shown by Topro-3 dye (blue). (**J**) CaMKIIδ localization in adult rat myocytes. CaMKIIδ localized to the intercalated disc (white arrows) here and to a second population at transverse-tubules (yellow arrows). Ventricular sections stained for (**K**) β_{IV}-spectrin, (**L**) N-cadherin, and (**M**) CaMKIIδ showed coexpression of these proteins at the intercalated disc (white arrows). (**N** and **P**) Ankyrin-G and (**O** and **Q**) Na$_v$1.5 were also found at the intercalated disc (white arrows) in rat myocytes and tissue sections. (**R–U**) Coimmunoprecipitation studies demonstrate cardiac complex of β_{IV}-spectrin, CaMKIIδ, ankyrin-G (AnkG), and Na$_v$1.5. Scale bars: 10 μm (**H–Q**).

from WT but not qv^{3J} heart (Figure 3F). Thus, the β_{IV}-spectrin qv^{3J} mouse represented an ideal in vivo system to directly test the role of β_{IV}-spectrin for CaMKII targeting.

Consistent with the location of the qv^{3J} mutation, qv^{3J} mouse cardiomyocytes displayed normal expression of β_{IV}-spectrin, ankyrin-G, and Na$_v$1.5 as well as normal levels of intercalated disc proteins N-cadherin and β-catenin (Figure 3, G, H, K, and L, and Supplemental Figures 3 and 4). However, CaMKIIδ expression levels were

modestly reduced in qv^{3J} hearts (Figure 3G and Supplemental Figure 3). Parallel immunostaining experiments revealed a striking absence of CaMKIIδ from the myocyte intercalated disc (Figure 3, I and N, and Supplemental Figure 5). In contrast, CaMKIIδ localization at transverse-tubule membranes was unaffected (Figure 3, I and N, and Supplemental Figure 5), indicative of a β_{IV}-spectrin–independent pathway that localizes CaMKII to transverse-tubules. Notably, we also observed dramatic reduction of acti-
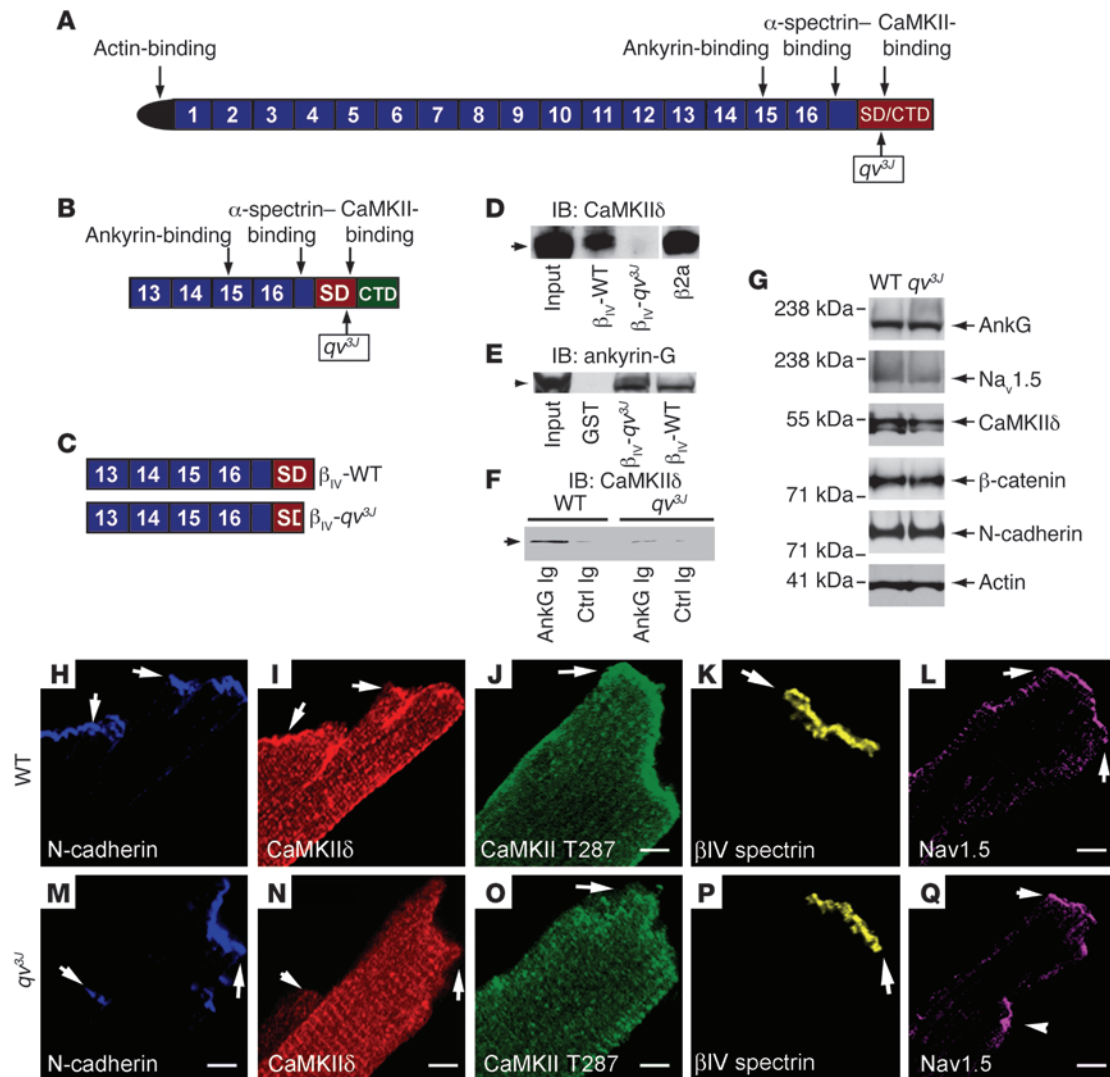
Figure 3
β_{IV}-spectrin is required for CaMKIIδ targeting. (**A**) β_{IV}-spectrin organization in WT and qv^{3J} animals. qv^{3J} animals lacked a CaMKII-binding domain, but retained actin-, ankyrin-, and α-spectrin–binding domains. (**B** and **C**) Schematic of control GST–β_{IV}-spectrin fusion protein encompassing spectrin repeats 13–17 and the CaMKII-binding domain (βIV-WT) and truncated mutant lacking CaMKII-binding domain (βIV-qv^{3J}). (**D**) β_{IV}-spectrin WT and qv^{3J} GST fusion proteins were incubated with detergent-soluble rat heart lysate and analyzed by immunoblot (CaMKII). L-type Ca^{2+} channel β2a subunit was also expressed as a GST fusion protein and used as positive control. CaMKIIδ bound to the β2a and WT GST fusion proteins, but not to the qv^{3J} GST fusion protein. Lanes were run on the same gel but were noncontiguous (white line). (**E**) β_{IV}-spectrin WT and qv^{3J} GST fusion proteins retained binding activity for ankyrin-G. (**F**) Coimmunoprecipitation studies showing association of ankyrin-G and CaMKIIδ in WT, but not qv^{3J}, hearts. (**G**) Expression of ankyrin-G, Na$_v$1.5, CaMKIIδ, β-catenin, and N-cadherin in heart lysates from WT and qv^{3J} animals. Actin is shown as loading control. (**H–Q**) Permeabilized adult rat cardiomyocytes from (**H–L**) WT and (**M–Q**) qv^{3J} hearts were immunostained for (**H** and **M**) N-cadherin, (**I** and **N**) total CaMKIIδ, (**J** and **O**) CaMKII-phospho-T287, (**K** and **P**) β_{IV}-spectrin, and (**L** and **Q**) Na$_v$1.5. Localization of CaMKIIδ (total and phospho-T287) to the intercalated disc (white arrows) was disrupted in qv^{3J} cardiomyocytes. Scale bars: 10 μm (**H–Q**).

vated CaMKIIδ (identified by CaMKII T287 phosphorylation) at the intercalated disc of qv^{3J} ventricular cardiomyocytes (Figure 3, J and O). Finally, consistent with the noted lack of effect of the qv^{3J} allele on ankyrin-G binding, we observed no difference in Na$_v$1.5 (Figure 3, L and Q) or ankyrin-G (data not shown) intercalated disc targeting. We believe our findings identify β_{IV}-spectrin as a novel CaMKII targeting protein in excitable cardiac cells.

β_{IV}-spectrin is required for local CaMKII signaling in cardiomyocytes. We tested the requirement of the β_{IV}-spectrin–dependent CaMKII targeting pathway for normal cardiomyocyte function by assess-

ing the activity of the resident intercalated disc voltage-gated Na$^+$ channel in primary qv^{3J} ventricular cardiomyocytes. qv^{3J} cardiomyocytes displayed aberrant CaMKII-dependent Na$^+$ channel regulation at baseline and in response to the β-adrenergic receptor agonist isoproterenol, which activates CaMKII (5). Specifically, qv^{3J} myocytes showed a significant increase in peak Na$^+$ current (I_{Na}; Figure 4, A–C) and a depolarizing shift in steady-state inactivation (Figure 4, D and E) compared with WT mouse ventricular cardiomyocytes. Additionally, persistent I_{Na} ($I_{Na,p}$), measured as current amplitude 50 ms after the peak value, was significantly
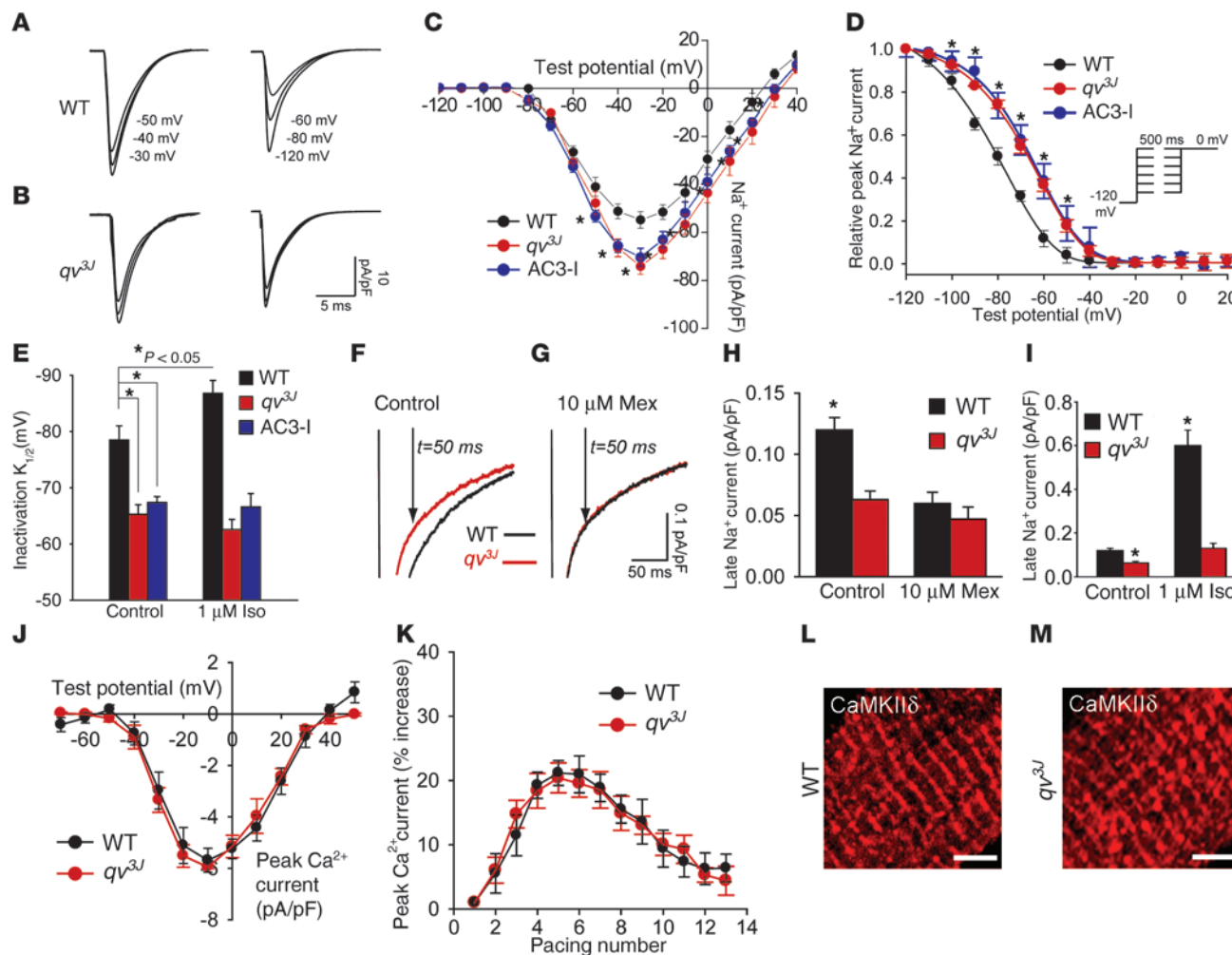
Figure 4
β_{IV}-spectrin/CaMKII complex is required for myocyte Na$^+$ channel function. (**A** and **B**) Whole-cell patch clamp I_{Na} traces from WT and qv^{3J} cardiomyocytes. Test pulse potential is listed next to each trace. (**C**) Current-voltage relationship for cardiomyocytes from WT, qv^{3J}, and AC3-I mice ($n = 8$ per group). *$P < 0.05$ versus WT. (**D** and **E**) Voltage-gated Na$^+$ channel steady-state inactivation measured from WT, qv^{3J}, and AC3-I cardiomyocytes ($n = 8$ per group). *$P < 0.05$ versus WT. Pulse protocol is shown in the inset of **D**. (**F** and **G**) $I_{Na,p}$ from WT and qv^{3J} mice with or without 10 μM mexiletine. Persistent current was determined as amplitude 50 ms after time of peak. (**H** and **I**) $I_{Na,p}$ in WT ($n = 10$) and qv^{3J} ($n = 17$) cardiomyocytes at baseline and in the presence of 10 μm mexiletine or isoproterenol. *$P < 0.05$. (**J**) Whole-cell patch clamp Ca^{2+} current-voltage relationship for WT ($n = 9$) and qv^{3J} ($n = 8$) cardiomyocytes. (**K**) Ca^{2+} current facilitation (peak current in response to a train of depolarizing voltage pulses to 0 mV) in WT ($n = 9$) and qv^{3J} ($n = 7$) cardiomyocytes. Peak current is expressed as percent increase from first pulse. (**L** and **M**) Transverse-tubule CaMKII labeling was preserved in qv^{3J} mouse myocytes. Scale bars: 5 μm (**L** and **M**).

reduced in qv^{3J} myocytes compared with littermate controls (Figure 4, F–I). Differences in $I_{Na,p}$ between genotypes were eliminated by mexiletine (Figure 4, F–H), a cardiac Na$_v$1.5 antagonist used to treat patients with type 3 long QT syndrome, a genetic syndrome that predisposes affected hearts to increased $I_{Na,p}$ and arrhythmias. Isoproterenol (1 μM) produced a depolarizing shift in steady-state inactivation and increased $I_{Na,p}$ in ventricular cardiomyocytes derived from WT mice, but not qv^{3J} mice or AC3-I transgenic mice (Figure 4, E and I), which overexpress a CaMKII-inhibitory peptide (5). In fact, we verified that differences in intercalated disc Na$^+$ channel properties were due to specific elimination of CaMKII by testing the identical parameters in ventricular cardiomyocytes derived from AC3-I mice. Notably, all tested I_{Na} parameters were identical between AC3-I and qv^{3J} mice (Figure 4,

C–E). These findings demonstrate that the CaMKII-binding function of β_{IV}-spectrin is required for CaMKII actions on I_{Na} in excitable cardiac myocytes.

Finally, past work has demonstrated a clear role of CaMKII in regulation of L-type Ca^{2+} channel (Ca$_v$1.2) Ca^{2+} current ($I_{Ca,L}$) facilitation (35). As this functional property is specific to the cardiomyocyte transverse-tubule membrane network (35), we tested whether loss of CaMKII targeting by β_{IV}-spectrin in qv^{3J} mice would display defects in Ca^{2+} current facilitation. Consistent with a highly selective role of β_{IV}-spectrin in the organization of CaMKII at the intercalated disc, we observed no differences in peak Ca^{2+} current or Ca^{2+} current facilitation between qv^{3J} and WT cardiomyocytes (Figure 4, J–M). Moreover, WT and qv^{3J} cardiomyocytes showed no difference in either total myocyte K$^+$ current or the transient
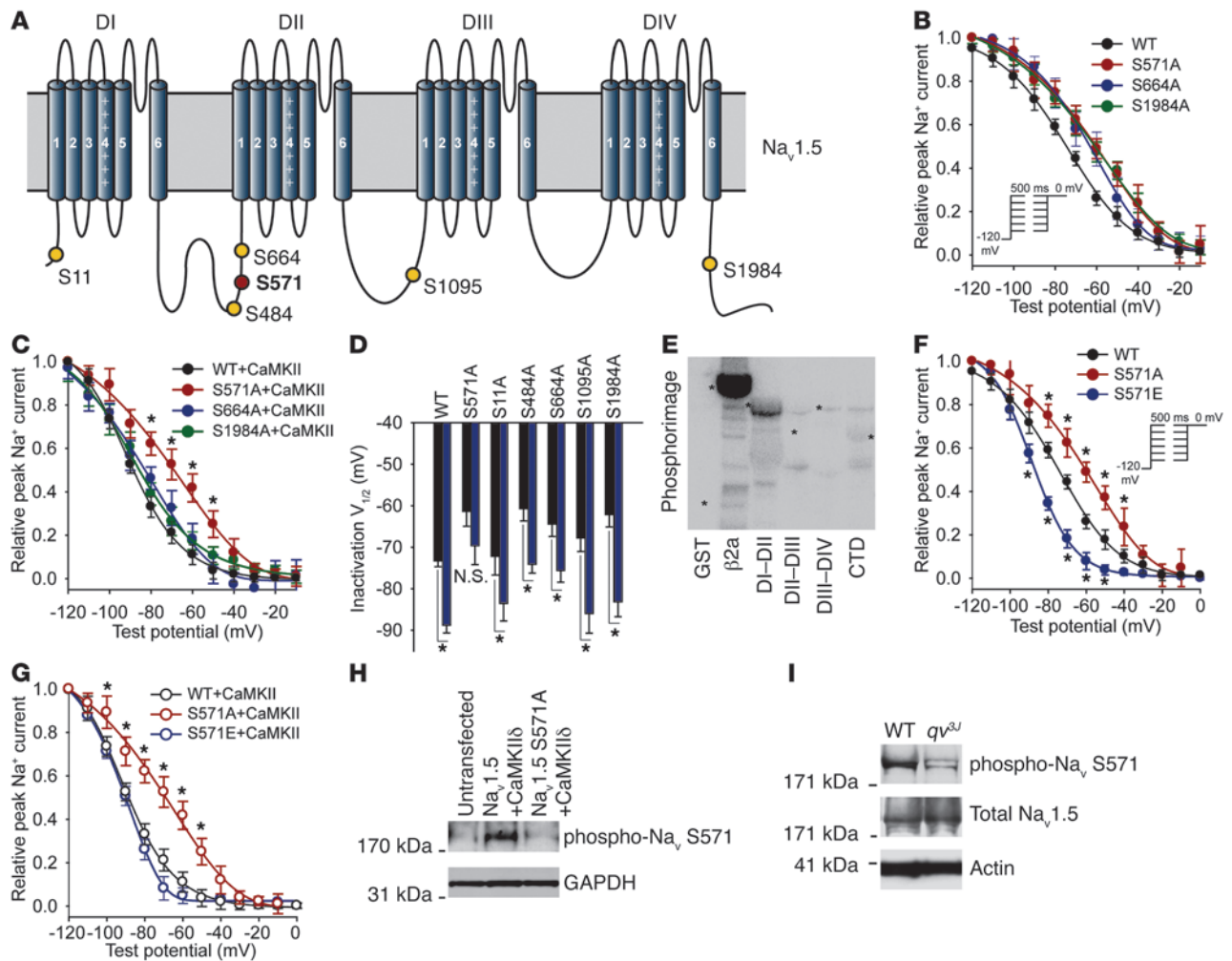
Figure 5
β_{IV}-spectrin/CaMKII regulates Na$_v$1.5 phosphorylation. (**A**) CaMKII consensus phosphorylation sites in intracellular domains of Na$_v$1.5. (**B** and **C**) Na$^+$ channel steady-state inactivation measured from WT and Na$_v$1.5 mutant channels expressed in HEK cells. *$P < 0.05$ versus WT. (**D**) Summary data showing depolarizing shift in steady-state inactivation $V_{1/2}$ following CaMKII activation for WT and all mutants except S571A. Black, before CaMKII activation; blue, after CaMKII activation. (**E**) CaMKII phosphorylation assay on intracellular domains of Na$_v$1.5. β2a was used as positive control. Asterisks denote location of purified proteins. (**F** and **G**) Na$^+$ channel steady-state inactivation measured from WT, S571A, and S571E (phosphomimetic) channels expressed in HEK cells. *$P < 0.05$ versus WT. (**H**) Na$_v$1.5 S571 antibody recognized WT, but not Na$_v$1.5 S571A mutant, channels. WT and mutant channels were expressed at equivalent levels in HEK293 cells overexpressing active CaMKIIδ. (**I**) Immunoblots showing reduced levels of phospho–Na$_v$1.5 S571, but unchanged total Na$_v$1.5 levels, in qv^{3J} versus WT heart lysates. Actin is shown as loading control.

outward K$^+$ current (I_{to}; Supplemental Figure 6; $n = 12$ myocytes/genotype; $P = $ NS), the primary murine repolarizing current (46) and a target for CaMKII in heart (47, 48). Collectively, these data provide the first description to our knowledge of the molecular mechanism underlying CaMKII targeting to the cardiomyocyte intercalated disc, and establish β_{IV}-spectrin as a bona fide CaMKII targeting protein in vivo. Notably, although our data link the β_{IV}-spectrin/CaMKII complex with Na$_v$1.5 regulation (and not $I_{Ca,L}$ or I_{to} regulation), they do not exclude other intercalated disc proteins (e.g., channels, receptors, and signaling molecules) as potential targets of the regulatory complex.

CaMKII regulates Na$_v$1.5 via direct phosphorylation of Na$_v$1.5 S571. While CaMKII has previously been shown to regulate cardiac I_{Na} (16, 18), the molecular mechanism underlying this modulation

has remained elusive. Based on our findings, we hypothesized that CaMKII directly regulates I_{Na} by direct phosphorylation of Na$_v$1.5. To test this hypothesis in cells, we measured I_{Na} properties from a library of full-length human Na$_v$1.5 (*SCN5A*) mutants engineered to harbor single serine-to-alanine mutations at all putative consensus CaMKII phosphorylation sites (RXX[S/T]; Figure 5A). Notably, with the exception of S571A (localized in the cytoplasmic domain loop DI–DII), WT Na$_v$1.5 and all mutant Na$_v$1.5 channels showed a significant depolarizing shift in Na$^+$ channel steady-state inactivation in the presence of CaMKII T287D (Figure 5, B–D). Based on these findings, we tested an additional S571E (phosphomimetic) mutant and observed a significant leftward shift in steady-state inactivation at baseline, with no CaMKII response in I_{Na} (Figure 5, F and G). These biophysical data strongly suggest that Na$_v$1.5 is
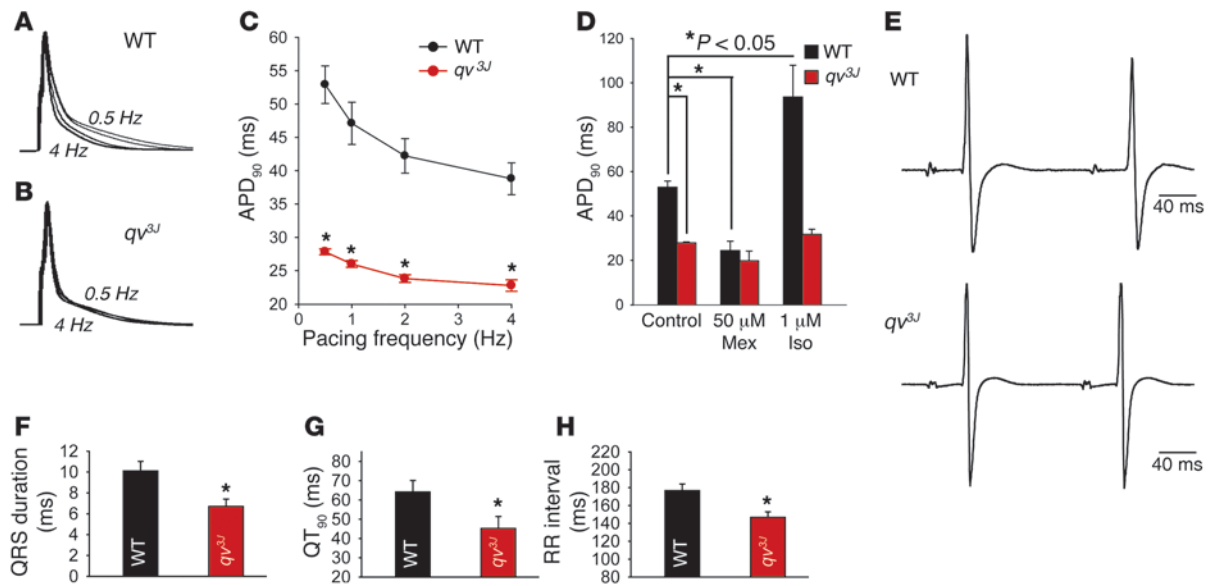
Figure 6
β_{IV} spectrin/CaMKII signaling complex is critical for normal cardiac cell membrane excitability. (**A** and **B**) Representative APs and (**C**) APD_{90} in WT ($n = 14$) and qv^{3J} ($n = 16$) cardiomyocytes paced at 4, 2, 1, or 0.5 Hz. *$P < 0.05$. (**D**) Differences in APD_{90} were eliminated by application of 50 μM mexiletine, but exaggerated by 1 μM isoproterenol (pacing frequency, 0.5 Hz). $n = 14$ (WT); 16 (qv^{3J}). *$P < 0.05$. (**E**) Representative electrocardiograms recorded from Langendorff-perfused WT and qv^{3J} hearts and summary data for (**F**) QRS duration (**G**) QT interval at 90% repolarization (QT_{90}), and (**H**) RR interval for qv^{3J} versus WT. $n = 10$ (WT); 10 (qv^{3J}). *$P < 0.05$.

directly phosphorylated by CaMKII, and that the CaMKII regulation is dependent on $Na_v1.5$ S571. In vitro phosphorylation assays (using purified $Na_v1.5$ cytoplasmic domains and activated CaMKII) validated our functional findings, showing that $Na_v1.5$ was directly phosphorylated on DI–DII (Figure 5E) and that S571 was a primary site for this regulation (Supplemental Figure 7). Together, these data identify S571 in the DI–DII loop as a critical site for regulation of $Na_v1.5$ activity by CaMKII.

β_{IV}-spectrin regulates direct phosphorylation of $Na_v1.5$ by CaMKII. We created a custom phosphospecific antibody against $Na_v1.5$ S571 to test the role of the β_{IV}-spectrin/CaMKII pathway for $Na_v1.5$ regulation in heart (Figure 5, H and I). Consistent with (a) a role of β_{IV}-spectrin for CaMKII regulation in vivo and (b) a role of CaMKII for direct regulation of $Na_v1.5$ in vivo, we observed decreased levels of phospho-$Na_v1.5$ S571 in qv^{3J} compared with WT hearts (Figure 5I and Supplemental Figure 8). In contrast, total $Na_v1.5$ levels were unchanged between WT and qv^{3J} mice (Figure 5I). These data support the hypothesis that β_{IV}-spectrin creates a platform for regulation of cardiac Na^+ channel activity through direct phosphorylation by CaMKII.

β_{IV}-spectrin is critical for cardiac membrane excitability. We next tested the role of β_{IV}-spectrin for CaMKII-dependent regulation of cardiac excitability. Voltage-gated Na^+ channel current is required for the rapid upstroke of the cardiac AP and normal electrical conduction in myocardium (49). Furthermore, alterations in I_{Na} and AP prolongation contribute to congenital and acquired forms of heart disease and arrhythmia (50, 51). Based on our observed dysfunction of I_{Na} in qv^{3J} mice, we measured APs from isolated WT and qv^{3J} cardiomyocytes. As observed in other vertebrates, AP duration (APD) in WT mouse cardiomyocytes decreased with increased pacing frequency (APD adaptation, Figure 6, A and C). Notably, WT cardiomyocytes displayed a parallel reduction in $I_{Na,p}$ with increased pacing frequency (Supplemental Figure 9). Consistent with the measured decrease

in $I_{Na,p}$ in qv^{3J} myocytes, qv^{3J} myocyte APs (90% and 70% repolarization; APD_{90} and APD_{75}, respectively) were significantly shorter at all pacing frequencies compared with WT myocytes ($P < 0.05$; APD_{50}, $P = NS$; Figure 6, A–C and Supplemental Figure 10). Furthermore, qv^{3J} myocytes displayed diminished capacity for APD adaptation (Figure 6C). These differences, as well as differences in $I_{Na,p}$, were eliminated by mexiletine but increased by isoproterenol (Figure 4, F–I, Figure 6D, and Supplemental Figure 10). These data indicate that disruption of CaMKII signaling through the β_{IV}-spectrin pathway results in aberrant cardiomyocyte electrical activity.

We used whole-animal and isolated heart electrocardiograms to test the effect of the β_{IV}-spectrin/CaMKII pathway on in vivo cardiac function. qv^{3J} mice showed decreased QRS duration (a marker of more rapid intraventricular conduction; refs. 50, 52), QT interval, and RR interval (increased heart rate) on both surface and isolated heart electrocardiograms (Figure 6, E–I). These findings were consistent with our observations of increased I_{Na} availability, decreased $I_{Na,p}$, and shorter APs in qv^{3J} cardiomyocytes (Figure 4 and Figure 6, A–D).

We next investigated the effect of targeted disruption in CaMKII signaling on isoproterenol-induced cellular afterdepolarizations, which serve as important triggers for life-threatening cardiac electrical disturbances (53). At baseline, afterdepolarizations were infrequent in both WT and qv^{3J} ventricular cardiomyocytes (20% and 10% of cells, respectively; $n = 10$; $P = NS$; Figure 7, A, B, and G). However, 1 μM isoproterenol significantly increased afterdepolarizations in WT but not qv^{3J} cardiomyocytes (WT, 70%, $P < 0.05$ versus control; qv^{3J}, 10%, $P = NS$ versus control; $n = 10$ per group; Figure 7, B, E, and G). Mexiletine completely eliminated isoproterenol-induced afterdepolarizations (0% in WT and qv^{3J}; $P = NS$; $n = 5$ per group; Figure 7, C, F, and G). Collectively, these data provide compelling evidence for the role of the β_{IV}-spectrin/

Figure 7

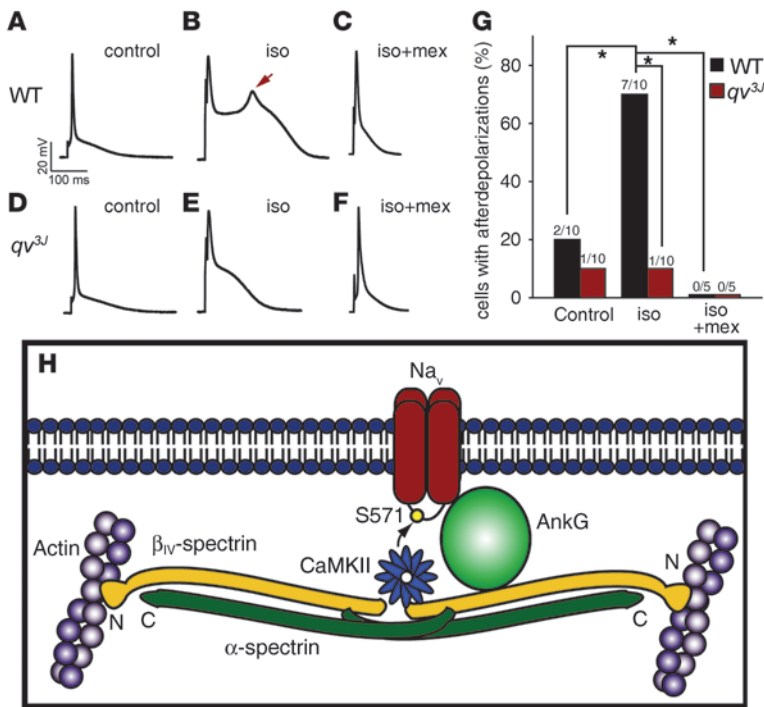β_{IV}-spectrin/CaMKII complex regulates afterdepolarization formation in response to isoproterenol treatment. Representative APs recorded from WT and qv^{3J} cardiomyocytes at baseline (**A** and **D**) and following application of 1 μM isoproterenol (**B** and **E**) or isoproterenol applied with 10 μM mexiletine (**C** and **F**). (**G**) Summary data showing percent cells displaying afterdepolarizations in WT and qv^{3J} at baseline ($n = 10$ per genotype) and following application of isoproterenol ($n = 10$ per genotype) or isoproterenol applied with mexiletine ($n = 5$ per genotype). *$P < 0.05$. (**H**) β_{IV}-spectrin–based complex targets CaMKII to effector proteins in excitable cells.

CaMKII signaling complex in normal myocyte physiology and suggest that modulation of this complex may represent a novel target to suppress persistent component of I_{Na} for arrhythmia treatment (Figure 7H).

The β_{IV}-spectrin–dependent targeting pathway is conserved in excitable neurons. While implicated in membrane biogenesis and maintenance, as well as ion channel clustering in the nervous system, we believe a role for β_{IV}-spectrin in neuronal signaling is unprecedented. Based on our findings from heart, we tested the requirement of β_{IV}-spectrin for local CaMKII organization in brain. In cerebellar Purkinje neurons, β_{IV}-spectrin was localized to AISs with ankyrin-G (39, 40, 54) and associated with ankyrin-G in detergent-soluble cerebellar lysates (Figure 8A). In agreement with our findings from heart, CaMKII associated with β_{IV}-spectrin in brain lysates (Figure 8B). Moreover, CaMKII was coexpressed with β_{IV}-spectrin in Purkinje neuron AISs, but also localized to the neuronal cell body (Figure 8C). Consistent with our findings from heart, qv^{3J} mice displayed aberrant targeting of CaMKII to the AIS (Figure 8C). Notably, CaMKII expression in the neuronal cell body was unaffected in qv^{3J} cerebellum. In contrast, both ankyrin-G and β_{IV}-spectrin were normally localized at qv^{3J} AIS, although β_{IV}-spectrin staining levels at the AIS were modestly decreased compared with WT cerebellum (Figure 8, D and E). Importantly, these findings indicate that the β_{IV}-spectrin pathway has evolved to target CaMKII gene products to specific membrane domains in functionally diverse excitable cells.

Discussion

Little is known about the cellular pathways that determine membrane excitability. Using a computational approach, we identified more than 30 putative CaMKII-binding proteins, including mitochondrial, nuclear, cytoskeletal, and membrane proteins. We performed comprehensive analyses that authenticated 1 candidate, β_{IV}-spectrin, as a CaMKII-anchoring protein at select excitable membrane domains, including the Purkinje cell AIS and the cardiomyocyte intercalated disc. Notably, mice harboring a mutant β_{IV}-spectrin lacking CaMKII binding activity showed defective membrane-specific CaMKII targeting, resulting in aberrant substrate activity, dysfunctional cellular excitability, and abnormal cardiac electrophysiology. Interestingly, our data also support the concept that modulating this complex may serve as an unexpected molecular target for arrhythmia therapy through suppression of late I_{Na} (i.e., $I_{Na,p}$; refs. 55, 56).

From bacteria to humans, regulated membrane excitability is central to life. Membrane ion flux drives salt and water balance, neurotransmitter release, myocyte contraction, and hormone secretion. Importantly, as recognized by Walter Cannon nearly a century ago (57), membrane excitability is remarkably fluid, constantly evolving to balance the relationship between an organism and its environment. Loss of ion homeostasis leads to disordered membrane excitability, which is a major factor in cardiac and neurological diseases. However, despite remarkable inroads into animal, tissue, and cellular physiology over the past century, arguably, the last major therapeutic breakthroughs in membrane excitability for human health and disease were the beta-blocker (discovered in the late 1950s; ref. 58), the ACE inhibitor (in the 1970s; ref. 59), and selective serotonin reuptake inhibitors (in the 1970s; ref. 60). Identification of new therapeutic agents to treat disorders of membrane excitability depends upon a more complete understanding of the cellular pathways regulating cell membrane excitability and organ function.

Previously identified CaMKII-binding partners have also served as direct substrates. For example, CaMKII associates with and phosphorylates the voltage-gated Ca^{2+} channel β_{2a} subunit to modulate transverse-tubule L-type Ca^{2+} channel facilitation in cardiomyocytes (35). Similarly, direct binding of CaMKII with NR2B regulates NMDA receptor activity in vitro (61). In contrast, we postulate that β_{IV}-spectrin, the CaMKII-targeting protein identified herein, will behave more analogous to AKAP proteins to organize the local signaling environment for multiple target substrates (Figure 8 and ref. 62). In fact, we predict that the β_{IV}-spectrin qv^{3J} mice will serve as an excellent in vivo model to identify and/or validate other potential CaMKII substrates at the cardiac intercalated disc or neuronal AIS.

Initial mRNA studies did not identify significant levels of β_{IV}-spectrin in multiple tissues including heart and pancreas (39, 45, 63). Thus, our discovery of β_{IV}-spectrin in the heart was initially surprising. However, β_{IV}-spectrin has subsequently been identified at select membranes of pancreatic β cells, where β_{IV}-spectrin was also not identified by whole-tissue mRNA analysis (39). Thus, our present findings of a strategic concentration of β_{IV}-spectrin at the ventricular cardiomyocyte intercalated disc are consistent with the notion that this protein pathway has evolved as a master
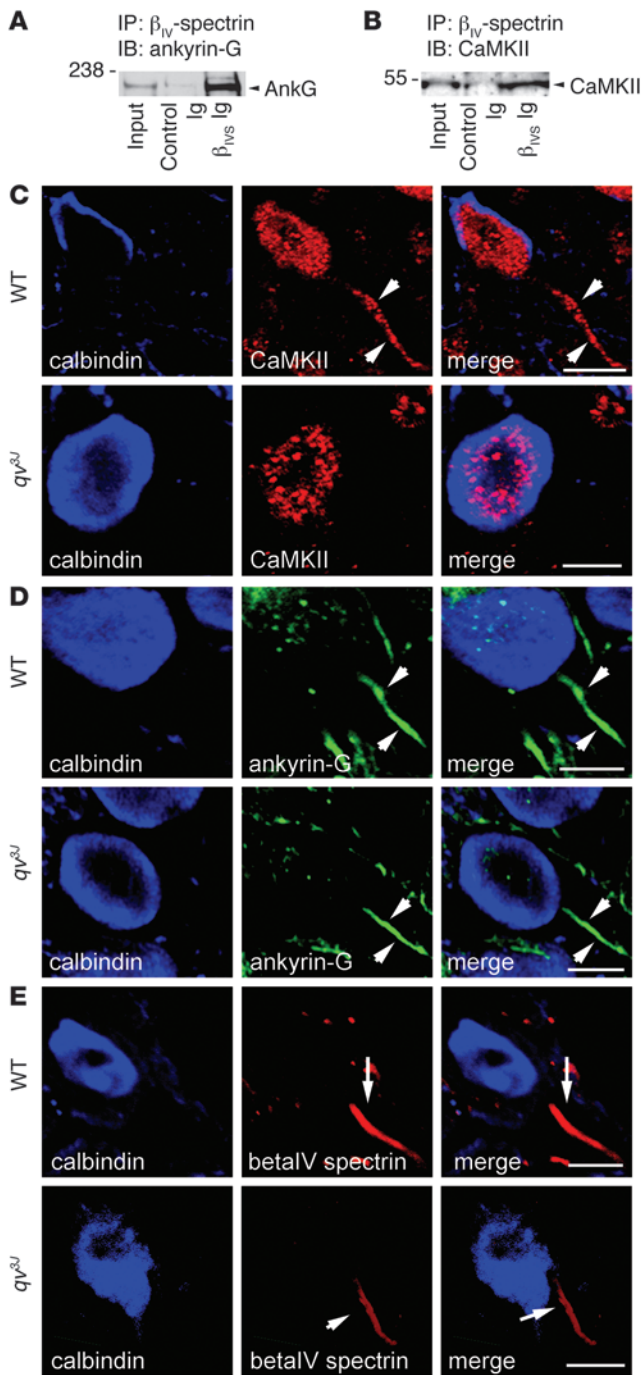
Figure 8
β_{IV}-spectrin targets CaMKII in neurons. (**A** and **B**) β_{IV}-spectrin associated with both ankyrin-G and CaMKII from mouse cerebellar lysates. (**C**) CaMKII (red) was observed at AISs (white arrows) in WT, but not qv^{3J}, cerebellum. (**D**) AIS integrity (shown by positive ankyrin-G staining) was not markedly affected in qv^{3J} mice. (**E**) β_{IV}-spectrin (red) was present at qv^{3J} mouse AISs, but at reduced levels compared with WT cerebellum. Calbindin (blue) was used to label the Purkinje cell body in **C**–**E**. Scale bars: 10 μm (**C**–**E**).

actions with ankyrin-G) and cell excitability. Independent studies from several groups demonstrate that cardiac Na$_v$1.5 is highly concentrated at the vertebrate myocyte intercalated disc (between 80% and 95%; refs. 43, 64–66). An important future goal will be to determine the mechanisms underlying regulation of this minor nonintercalated disc (i.e., transverse-tubule and peripheral sarcolemmal) population of Na$_v$1.5 channels.

Our findings identify the β_{IV}-spectrin/CaMKII complex as an essential component for I_{Na} regulation in the cardiomyocyte. In fact, qv^{3J} myocytes with targeted disruption of this complex show abnormal I_{Na} inactivation properties, decreased $I_{Na,p}$, and altered cell excitability. Additionally, our findings illustrate that loss of this complex in myocytes does not affect other major cardiac ion currents, including total K$^+$ current, $I_{Ca,L}$, or I_{to}. Nevertheless, our findings do not rule out the possibility that other targets of the β_{IV}-spectrin/CaMKII complex are important in cardiomyocytes. In fact, we predict that other resident intercalated disc proteins (including ion channels, transporters, receptors, cytoskeletal proteins, and signaling molecules) may also be modulated by the β_{IV}-spectrin/CaMKII complex and contribute to observed differences in APD$_{75}$ and APD$_{90}$ in qv^{3J} myocytes. An important immediate future goal will be to identify additional targets for the β_{IV}-spectrin/CaMKII complex, in both small and large animal models, that may yield unique insights into the relationship between CaMKII signaling and pathophysiology. For example, although both large and small animals show APD adaptation in response to changes in pacing frequency (Supplemental Figure 9 and ref. 67), $I_{Na,p}$ is an important determinant of this behavior in mice, but the potential role of $I_{Na,p}$ is less clear in larger animals, where APD adaptation is primarily attributed to K$^+$ and/or Ca^{2+} channel activity (reviewed in ref. 67). Thus, we anticipate that studies in different animal models will be useful for studying all aspects of the β_{IV}-spectrin/ CaMKII signaling complex.

While our findings identify β_{IV}-spectrin as a component of vertebrate heart, they also raise many exciting questions regarding the regulation of this complex gene and resulting gene products in heart. For example, while we have identified 2 isoforms in heart ($\Sigma 1$ and $\Sigma 6$), 6 β_{IV}-spectrin splice forms are expressed in brain (29, 39, 63). Future experiments must address how these gene products are differentially expressed throughout development and across specific cardiac excitable cells, and whether other β_{IV}-spectrin isoforms are found in heart. Moreover, while it is likely that each isoform has a unique role in heart (e.g., $\beta_{IV\Sigma6}$-spectrin localizes Na$_v$ channels to AISs in neurons, ref. 28), the specific functions of β_{IV}-spectrin splice variants in heart remain unknown. Based on conservation of the CaMKII- and ankyrin-G–binding motifs in $\Sigma 1$ and $\Sigma 6$ isoforms, we anticipate that both isoforms will be important for regulation of Na$_v$ channel activity and cell excitability. Finally, it is notable that we observed a small shift in the mobility of cardiac β_{IV}-spectrin isoforms compared with their cerebellar β_{IV}-spectrin

regulator of local excitable cell signaling in select cells of the brain, heart, and pancreas (albeit at significantly lower expression levels in heart compared with brain; Figure 2E). Furthermore, we predict that β_{IV}-spectrin may also be localized to key membrane domains in other tissues to regulate local signaling pathways. For example, β_{IV}-spectrin is localized to the neuromuscular junction, where it interacts with skeletal muscle Na$^+$ channels (Na$_v$1.4) and ankyrin-G (M.N. Rasband, unpublished observations).

Our present study revealed that β_{IV}-spectrin exerted spatial control over CaMKII-dependent regulation of intercalated disc voltage-gated Na$^+$ channels (targeted by β_{IV}-spectrin–dependent inter-

counterparts (Figure 2E). While we hypothesize that this small shift may represent tissue-specific modification of residues found in both isoforms, additional experiments will be necessary to identify whether β_{IV}-spectrin polypeptides display differential posttranslational modifications as well as to define how these potential modifications may differentially regulate β_{IV}-spectrin function.

In addition to heart, β_{IV}-spectrin also targets CaMKII to AISs in cerebellar Purkinje neurons (Figure 8). In both central and peripheral nervous systems, β_{IV}-spectrin is critical for the biogenesis and maintenance of local membrane domains (32, 40, 68). Moreover, ankyrin-G recruits voltage-gated Na$^+$ and K$^+$ channels to neuronal membrane domains (69). Therefore, we speculate that β_{IV}-spectrin and ankyrin-G coordinate coupling between CaMKII and brain ion channels to regulate neuronal function and coordinate the cell's response to external stimuli (e.g., transmitted by increased catecholamine levels; Figure 7H). It is notable that, similar to β_{IV}-spectrin qv^{3J} mice and mice lacking ankyrin-G in the cerebellum, CaMKIIβ-deficient mice display defects in Purkinje cell excitability and pronounced ataxia (14). Moreover, human mutations in spectrin have been identified in patients with spinocerebellar ataxia, while genome-wide association studies have linked variability in ankyrin-G expression with bipolar disorder (70). Thus, compelling data from humans and animal models strongly support the β_{IV}-spectrin–based signaling platform as a nodal point for regulating excitable cell function in health and disease.

Methods

Computational analysis of putative CaMKII binding partners and in vitro binding. Novel CaMKII binding partners were identified by screening the human genome for proteins containing an 8–amino acid stretch ([LMI]-x-[RHK]-Q-[ED]-[ST]-x-[ED]) found in the CaMKII autoregulatory domain (35) using ScanProsite. Candidates were cloned from human tissue, and CaMKII-binding activity was assessed by in vitro binding assays using radiolabeled target proteins and activated CaMKII expressed in HEK cells and immunoprecipitated using monoclonal CaMKIIδ antibody (Santa Cruz) and TrueBlot anti-mouse Ig IP beads (eBioscience). Protein complexes were washed 5 times (50 mM Tris, pH 7.4; 1 mM EDTA; 1 mM EGTA; 500 mM NaCl; and 0.1% Triton X-100), resuspended in SDS sample buffer, and boiled for 5 minutes. Products were separated by SDS-PAGE. To ensure equal loading, gels were stained with Coomassie Blue. [^{35}S]-labeled in vitro translated target proteins bound to CaMKII were detected by phosphorimaging.

Animals. qv^{3J} mice and WT littermates were obtained from Jackson Laboratories. All experiments were performed in mice 8 weeks of age. The present studies were reviewed and approved by the IACUC of the University of Iowa.

Molecular biology. To generate cDNA to use for engineering fusion proteins, PCR primers were designed to TOPO-clone the C-terminal region of β_{IV}-spectrin from the human heart cDNA library (BD Biosciences) and the reverse transcribed cDNA from human heart tissue, based on a sequence obtained from GeneBank. To generate constructs for in vitro transcription/translation, fusion protein expression, and binding assay, cDNAs were engineered in-frame into pcDNA3.1$^+$ (Invitrogen) and pGEX6P1 (GE Healthcare). Vectors were completely sequenced. RNA was isolated and processed for RT-PCR as described previously, using oligo-dT to prime for cDNA production (71). Primers to identify β_{IV}-spectrin transcripts in heart and brain include primers directed against the N terminus to recognize full-length $\beta_{IV\Sigma1}$-spectrin in rat (forward, 5′-GGGATGGCTTT-GTCCTCACCC-3′; reverse, 5′-GCCAAGAGTTCCAACTTCTCC-3′; nucleotides 271–1,226 in rat [β_{IV}-spectrin-*Spnb4*; XM_218364], product shown in Figure 2) as well as C-terminal primers that recognize $\beta_{IV\Sigma1}$-spectrin

and $\beta_{IV\Sigma6}$-spectrin in mouse (β_{IV}-spectrin-*Spnb4*; NM_032610) and rat (forward, 5′-CCACGATCGAGAAACTCAAGG-3′; reverse, 5′-GGTGTCCGTC-GTGTCCAACG-3′; not shown).

Biochemistry and imaging. Adult heart immunoprecipitations were performed as described previously (72). The following antibodies were used for immunoblotting, immunoprecipitation, or immunostaining: antibodies against ankyrin-G (43), β_{IV}-spectrin (N-terminal domain, specific domain, and C-terminal domain; provided by M. Komada, Tokyo Institute of Technology, Yokohama, Japan; or obtained from Santa Cruz or LSBio; refs. 29, 40), Na$_v$1.5, and CaMKIIδ, CaMKIIδ (Santa Cruz), CaMKII-phospho-T287 (ABR), connexin43 (Invitrogen), N-cadherin (Abcam), β-catenin (BD Biosciences), actin (Sigma-Aldrich). Affinity-purified polyclonal antibody against phospho–Na$_v$1.5 S571 was developed and validated against WT and S571A mutant Na$_v$1.5 channels expressed in HEK cells. Adult cardiomyocytes and heart sections were isolated, immunostained, and imaged as described previously (43, 73).

In vitro binding. CaMKIIδ was in vitro translated using the TNT Coupled Reticulocyte Lysate Systems (Promega) and labeled with [^{35}S]-methionine. [^{35}S]-labeled CaMKIIδ was incubated with CTP-P, a biotinylated peptide mimicking the putative kinase-binding domain in human β_{IV}-spectrin, or CTP-C, a scrambled peptide, in 500 μl binding buffer (50 mM Tris, pH 7.4; 1 mM EDTA; 1 mM EGTA; 150 mM NaCl; 0.1% Triton X-100; and 1:1,000 protease inhibitor [Sigma-Aldrich]) overnight at 4°C. Protein complexes were washed 5 times (50 mM Tris, pH 7.4; 1 mM EDTA; 1 mM EGTA; 500 mM NaCl; and 0.1% Triton X-100), resuspended in SDS sample buffer, and boiled for 5 minutes. Products were separated by SDS-PAGE. To ensure equal loading, gels were stained with Coomassie Blue. [^{35}S]-labeled in vitro translated CaMKIIδ that bound to the peptide was detected by phosphorimaging.

APs. APs were recorded using the perforated (amphotericin B) patch-clamp technique at 36°C ± 1°C in Tyrode solution (bath). The pipette contained 130 mM potassium aspartate, 10 mM NaCl, 10 mM HEPES, 0.04 mM CaCl$_2$, 2.0 mM MgATP, 7.0 mM phosphocreatine, 0.1 mM NaGTP, and 240 μg/ml amphotericin B, with the pH adjusted to 7.2 with KOH. APs were evoked by brief current pulses 1.5–4 pA, 0.5–1 ms. APD was assessed as the time from the AP upstroke to 50%, 75%, and 90% repolarization to baseline (APD$_{50}$, APD$_{75}$, and APD$_{90}$, respectively).

Electrophysiology and electrocardiograms. See Supplemental Methods and Supplemental Figure 11.

Statistics. Data are presented as mean ± SEM. P values were assessed with a paired Student's t test (2-tailed) or ANOVA, as appropriate, for continuous data. The Bonferroni test was used for post-hoc testing. The null hypothesis was rejected for $P < 0.05$.

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