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Peptidomimetic Targeting of Cav β 2 Overcomes Dysregulation of the L-Type Calcium Channel Density and Recovers Cardiac Function

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Peptidomimetic Targeting of $Ca_v\beta_2$ Overcomes Dysregulation of the L-Type Calcium Channel Density and Recovers Cardiac Function

BACKGROUND: L-type calcium channels (LTCCs) play important roles in regulating cardiomyocyte physiology, which is governed by appropriate LTCC trafficking to and density at the cell surface. Factors influencing the expression, half-life, subcellular trafficking, and gating of LTCCs are therefore critically involved in conditions of cardiac physiology and disease.

METHODS: Yeast 2-hybrid screenings, biochemical and molecular evaluations, protein interaction assays, fluorescence microscopy, structural molecular modeling, and functional studies were used to investigate the molecular mechanisms through which the LTCC $Ca_v\beta_2$ chaperone regulates channel density at the plasma membrane.

RESULTS: On the basis of our previous results, we found a direct linear correlation between the total amount of the LTCC pore-forming $Ca_v\alpha_1.2$ and the Akt-dependent phosphorylation status of $Ca_v\beta_2$ both in a mouse model of diabetic cardiac disease and in 6 diabetic and 7 nondiabetic cardiomyopathy patients with aortic stenosis undergoing aortic valve replacement. Mechanistically, we demonstrate that a conformational change in $Ca_v\beta_2$ triggered by Akt phosphorylation increases LTCC density at the cardiac plasma membrane, and thus the inward calcium current, through a complex pathway involving reduction of $Ca_v\alpha_1.2$ retrograde trafficking and protein degradation through the prevention of dynamin-mediated LTCC endocytosis; promotion of $Ca_v\alpha_1.2$ anterograde trafficking by blocking Kir/Gem-dependent sequestration of $Ca_v\beta_2$, thus facilitating the chaperoning of $Ca_v\alpha_1.2$; and promotion of $Ca_v\alpha_1.2$ transcription by the prevention of Kir/Gem-mediated shuttling of $Ca_v\beta_2$ to the nucleus, where it limits the transcription of $Ca_v\alpha_1.2$ through recruitment of the heterochromatin protein 1 γ epigenetic repressor to the *Cacna1c* promoter. On the basis of this mechanism, we developed a novel mimetic peptide that, through targeting of $Ca_v\beta_2$, corrects LTCC life-cycle alterations, facilitating the proper function of cardiac cells. Delivery of mimetic peptide into a mouse model of diabetic cardiac disease associated with LTCC abnormalities restored impaired calcium balance and recovered cardiac function.

CONCLUSIONS: We have uncovered novel mechanisms modulating LTCC trafficking and life cycle and provide proof of concept for the use of $Ca_v\beta_2$ mimetic peptide as a novel therapeutic tool for the improvement of cardiac conditions correlated with alterations in LTCC levels and function.

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Clinical Perspective

What Is New?

- A tight control of the Ca²⁺ cardiac machinery is essential for normal cardiac physiology. Alterations in the voltage-dependent L-type calcium channel (LTCC), the key mediator of intracellular Ca²⁺ entry, are associated with various life-threatening cardiovascular conditions such as pathologic hypertrophy, atrial fibrillation, hypertension, and diabetic cardiomyopathy. The potential use of positive Ca²⁺ modulators for the treatment of cardiovascular pathologies has received considerable attention during the past decades. However, hitherto, pharmacologic approaches aimed at enhancing Ca²⁺ current and inotropism in systolic heart failure have frequently been found to favor arrhythmogenesis and diastolic dysfunction, thereby limiting their use in the clinic.
- Here, we identified a novel peptidomimetic therapeutic tool for the treatment of cardiovascular diseases that, via an unconventional mechanism, bypasses the arrhythmogenic limitations of current channel-activator inotropes. In particular, by dissecting new regulatory pathways modulating the LTCC life cycle, we generated a mimetic peptide that, through multiple levels of regulation, specifically targets the LTCC Ca_vβ2 chaperone, thereby controlling LTCC assembly and density at the plasma membrane while preserving its physiologic channel function.

What Are the Clinical Implications?

- We provide proof of concept for the exploitation of a novel therapy based on mimetic peptide technology and demonstrate that cardiac dysfunctions (eg, diabetic cardiomyopathy) associated with LTCC abnormalities can be effectively treated by in vivo administration of the generated mimetic peptide. Thus, mimetic peptide technology associated with nanocarriers for safe and cardiac-specific delivery may provide novel innovative therapeutic tools for the treatment of cardiac diseases.

Maintenance of calcium homeostasis is critical for preserving the physiology of the cell. A variety of complex mechanisms intervene in the regulation of intracellular levels of Ca²⁺ and its compartmentalization between subcellular compartments. In the heart, Ca²⁺ release from the sarcoplasmic reticulum (SR), the major intracellular Ca²⁺ store, to the cytosol is regulated through a process called Ca²⁺-induced Ca²⁺ release.^{1,2} Ca²⁺-induced Ca²⁺ release is initiated by the entry of Ca²⁺ into cardiac cells through sarcolemmal voltage-dependent L-type Ca²⁺ channels (LTCCs), which triggers the release of Ca²⁺ from the SR to the cytoplasm through ryanodine receptor 2. A close association between LTCC and ryanodine receptor 2 is required for efficient Ca²⁺-in-

duced Ca²⁺ release and is dependent on the density and organization of LTCCs within the T-tubular invagination of the plasma membrane.² The increase in free intracellular Ca²⁺ allows Ca²⁺ to bind to troponin C, initiating muscle contraction. This is terminated subsequently by the removal of cytosolic Ca²⁺ through its reuptake into the SR via the cardiac SR Ca²⁺-ATPase and, to a lesser extent, by other Ca²⁺ transport systems. This process is a key regulator of cardiac excitation-contraction coupling and a major determinant for intrinsic physiologic properties of the beating heart.

Modifications in the appropriate trafficking of LTCC to and density at the cell surface and changes in its subcellular localization and gating properties can cause functional alterations in the overall inward Ca²⁺ current (*I_{Ca,L}*)³ and consequently the cellular Ca²⁺ machinery. In line with this, acquired and genetically determined LTCC dysfunction has been found to be associated with various human diseases,⁴ including life-threatening cardiovascular pathologies.⁴⁻¹⁴

LTCCs are expressed in all excitable cells (ie, striated, smooth muscle, and neuronal cells).¹⁵ The cardiac LTCC is composed of the pore-forming Ca_vα1.2 and the accessory Ca_vβ2 and Ca_vα2δ subunits.¹⁵⁻¹⁷ Ca_vβ2, the cytoplasmic chaperone of Ca_vα1.2, is composed of a globular domain (including an Src homology 3 [SH3] and a guanylate kinase-like [GK] domain) and a C-terminal coiled-coil tail (C-tail) and belongs to the membrane-associated GK family, which is involved in intracellular signaling and the establishment/maintenance of cell polarity.¹⁶ Ca_vβ2 binds to the pore unit at the α-interaction domain,¹⁸ affecting its trafficking to the plasma-membrane,¹⁹⁻²¹ its internalization,²² and its gating properties.^{17,23} However, although LTCC channel physiology has been intensively studied during the last decades, more in-depth insights into the mechanisms regulating the trafficking and function of LTCCs are still required. Here, we unraveled new regulatory pathways modulating the LTCC life cycle and generated a mimetic peptide (MP) that, through multiple levels of regulation, specifically targets Ca_vβ2, thereby controlling LTCC levels at the plasma membrane. Additionally, we provide proof of concept for in vivo administration of MP as an effective therapeutic strategy for the treatment of cardiac disorders associated with LTCC abnormalities.

METHODS

Detailed procedures are provided in the [online-only Data Supplement](#).

Plasmids

Yeast 2-hybrid bait constructs were generated in the pGBKT7 vector (Clontech), and the mouse adult heart cDNA library was generated with the Make Your Own Mate & Plate Library System (Clontech). For the nanoluciferase and bioluminescence

resonance energy transfer (BRET) assays, cDNAs were cloned into the pNLF1-N and pNLF1-N and the HaloTag- pFN21A vector (Promega), respectively.

Western and Dot Blot Analyses

Ca_vα1.2 Ca_vβ2 (S8b-1), HA, and transferrin receptor were from Abcam; c-myc 9E10 was from Santa Cruz; HP-1γ 42S2 came from Millipore; GAPDH 14C10 was purchased from Cell Signaling Technology; Polyarginine 9R was from Cell Applications; and goat anti-mouse and anti-rabbit horseradish peroxidase was purchased from Thermo Fisher Scientific. A rabbit polyclonal antibody against the phosphorylated Akt consensus site of Ca_vβ2 was generated by GenScript.

Nanoluciferase Assay

In NanoLuc Luciferase (Promega), HEK293 cells pretreated for 30 minutes with 20 μmol/L cycloheximide were transfected as described in the Results section. Time-course analysis was performed with a Synergy 4 instrument (BioTek).

NanoBRET Assay

In the NanoBRET Assay (Promega), HEK293-transfected cells were treated with 100 nmol/L NanoBRET 618 ligand, and signals were detected 5 hours after treatment. For the peptide-protein binding assay, tetramethylrhodamine peptides were used. Signals were detected with a Synergy 4 instrument (BioTek).

Human Patients

Patients with aortic stenosis who underwent aortic valve replacement were recruited retrospectively from the biobank available at the University Hospital of Verona, Division of Cardiac Surgery, Verona, Italy. Approval for studies on human tissue samples was obtained from the ethics committee of the University Hospital of Verona. All patients or their relatives gave informed consent before surgery.

Animals

AQ4 All procedures on mice were performed according to institutional guidelines in compliance with national (D.L. N.26, 04/03/2014) and international law and policies (new directive 2010/63/EU). The protocol was approved by the Italian Ministry of Health. Special attention was paid to animal welfare to minimize the number of animals used and their suffering. All experiments were performed on 12-week-old male mice with at least 8 animals per group. Diabetes mellitus was induced in adult C57B6/J male mice by intraperitoneal injection of streptozotocin (50 mg·kg⁻¹·d⁻¹) for 5 days. At 2 weeks after the last injection of streptozotocin, whole blood was obtained and glucose levels were measured with the Accu-Chek active blood glucose monitoring system (Roche). Mice with hyperglycemia (>300 mg/dL) were used for the study. Citrate buffer-treated mice were used as nondiabetic controls. After streptozotocin treatment, mice were treated with peptides (2.5 mg·kg⁻¹·d⁻¹ IP) as described in the Results section.

Statistical Analysis

Data are presented as mean±SD. The normality of the data was assessed with the Kolmogorov-Smirnov test. Statistical

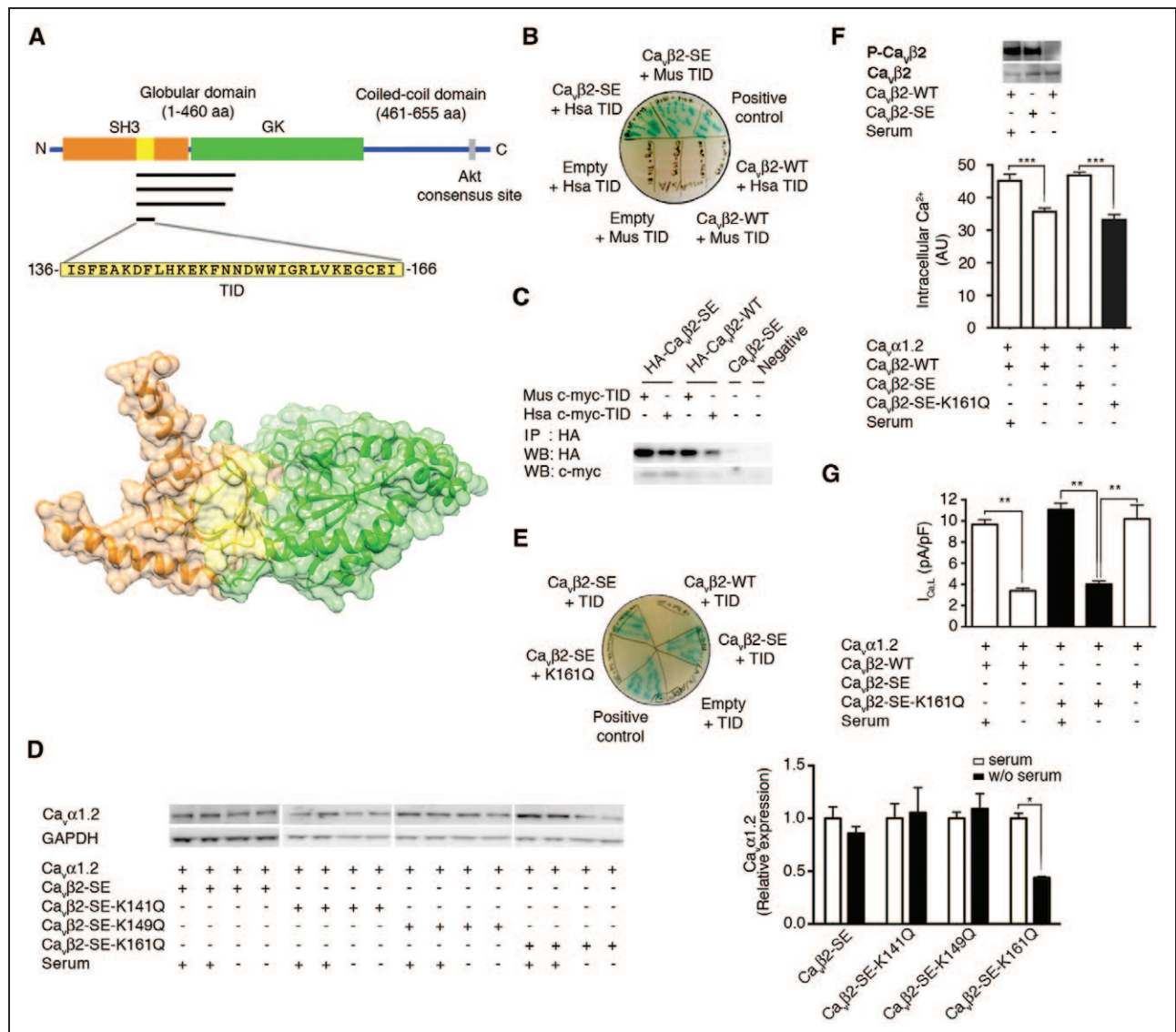
comparison was performed in at least 3 independent experiments with the Mann-Whitney test, and comparisons between groups were analyzed by repeated-measures ANOVA combined with the Tukey multicomparison. The Pearson correlation coefficient test was used for the analysis of human data. Prism 6.0 software (GraphPad Software) was used to assess the normality of the data and for statistical calculation. A value of *P*<0.05 was considered significant.

RESULTS

Ca_vβ2 Molecular Reorganization Stabilizes Ca_vα1

We previously demonstrated that Ca_vα1.2 total protein levels are affected by the phosphorylation status of Ca_vβ2 at the Akt consensus site in its C-tail.²⁴ To determine the mechanism by which phosphorylated Ca_vβ2 controls the Ca_vα1.2 level, we performed yeast 2-hybrid screenings of human and mouse heart cDNA libraries using phosphomimetic Ca_vβ2 (Ca_vβ2-SE²⁴) as bait. Unexpectedly, several positive clones corresponded to the SH3 region in the globular domain of Ca_vβ2 (Figure 1A), and cotransformation assays confirmed the binding of Ca_vβ2-SE, but not wild-type Ca_vβ2, to the globular region of Ca_vβ2 (Figure 1B). The specificity of the interaction was further confirmed by coimmunoprecipitation assays (Figure 1C). Analysis of the Ca_vβ2 structure revealed a solvent accessible sequence within the minimal common region of the identified Ca_vβ2 clones (Figure 1A), which we predicted to be responsible for the binding to the Ca_vβ2 C-tail on Akt phosphorylation. Taken together, these results suggest that the Akt-dependent protective effect on Ca_vα1.2 stability is not mediated through interaction with other proteins but relies entirely on Ca_vβ2 structural rearrangements, triggered by Akt-mediated phosphorylation of its C-terminal coiled-coil region, allowing interaction with a minimal region within the Ca_vβ2-SH3 globular domain that we called the tail-interacting domain (TID).

To determine whether the TID directly affects Ca_vα1.2 protein stability, we used site-specific mutagenesis to replace positively charged lysines (K) at positions 141, 149, and 161 in the TID sequence with glutamines (Q) to destroy any potential ionic interaction between the TID and the C-tail. In cells cotransfected with Ca_vα1.2 and Ca_vβ2-SE, similar protein levels of Ca_vα1.2 were found in the presence and absence of Akt activation (ie, with and without serum, corresponding to phosphorylated and nonphosphorylated Ca_vβ2, respectively; Figure 1F, inset), whereas significantly reduced Ca_vα1.2 levels were found in cells cotransfected with K161Q-mutant Ca_vβ2-SE under serum free conditions (Figure 1D). Consistently, the K161Q mutation prevented the interaction with Ca_vβ2-SE in the yeast 2-hybrid



system (Figure 1E), suggesting a direct role of the TID in the protective effect of Ca_vβ2-SE on Ca_vα1.2 stability. Similarly, the intracellular Ca²⁺ concentration was significantly higher in cells cotransfected with Ca_vα1.2 and Ca_vβ2-SE compared with wild-type Ca_vβ2 after serum starvation, whereas cotransfection with K161Q-mutant Ca_vβ2-SE ablated this effect (Figure 1F). In addition, I_{CaL} was significantly reduced in cells transfected with K161Q-mutant Ca_vβ2-SE compared with Ca_vβ2-SE (Figure 1G). Taken together, these results provide evidence that the TID within the Ca_vβ2-SH3 domain plays a direct role in stabilizing Ca_vα1.2.

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MP Affects LTCC Protein Stability and Function In Vitro

We next designed an array of partially overlapping peptides covering the C-tail and tested their effect on $Ca_v\alpha1.2$ stability through binding to the $Ca_v\beta2$ -TID. Cotransfection of cells with $Ca_v\alpha1.2$, $Ca_v\beta2$, and individual peptides, followed by Western blot analyses (Figure 2A) and intracellular Ca^{2+} concentration measurements (Figure 2B), identified several peptides that efficiently regulated $Ca_v\alpha1.2$ protein amounts and increased intracellular Ca^{2+} concentration without inducing apoptosis (Figure 2A). For our subsequent studies, we selected peptide 11 designated as MP, the effect of which was most similar to that of $Ca_v\beta2$ -SE. Computational docking simulation of the interaction between the MP and the $Ca_v\beta2$ -TID (Figure 2C) and additional site-specific mutagenesis (Figure I in the online-only Data Supplement) further defined the TID-MP binding pocket.

Next, to facilitate intracellular uptake, MP was fused to an oligoarginine (R7W) cell-penetrating peptide.²⁵ The R7W was fused to the N-terminus of the MP, which, on the basis of the $Ca_v\beta2$ -MP 3D model (Figure 2C), was not predicted to create steric hindrance or to affect MP function. To monitor the extent and specificity by which R7W-MP binds to $Ca_v\beta2$, we performed a BRET protein interaction assay between a $Ca_v\beta2$ -NanoLuc donor and increasing doses of R7W-MP or R7W-scramble peptide conjugated to the tetramethylrhodamine fluorophore (acceptor). This revealed binding of R7W-MP to $Ca_v\beta2$ in a dose-dependent manner, a finding specific for the $Ca_v\beta2$ -SH3 domain and not the GK domain (Figure 2D). Dot blot analyses after $Ca_v\beta2$ immunoprecipitation on lysates from cells transfected with $Ca_v\beta2$ constructs and treated with R7W-peptides supported the above results (Figure IIA in the online-only Data Supplement). Furthermore,

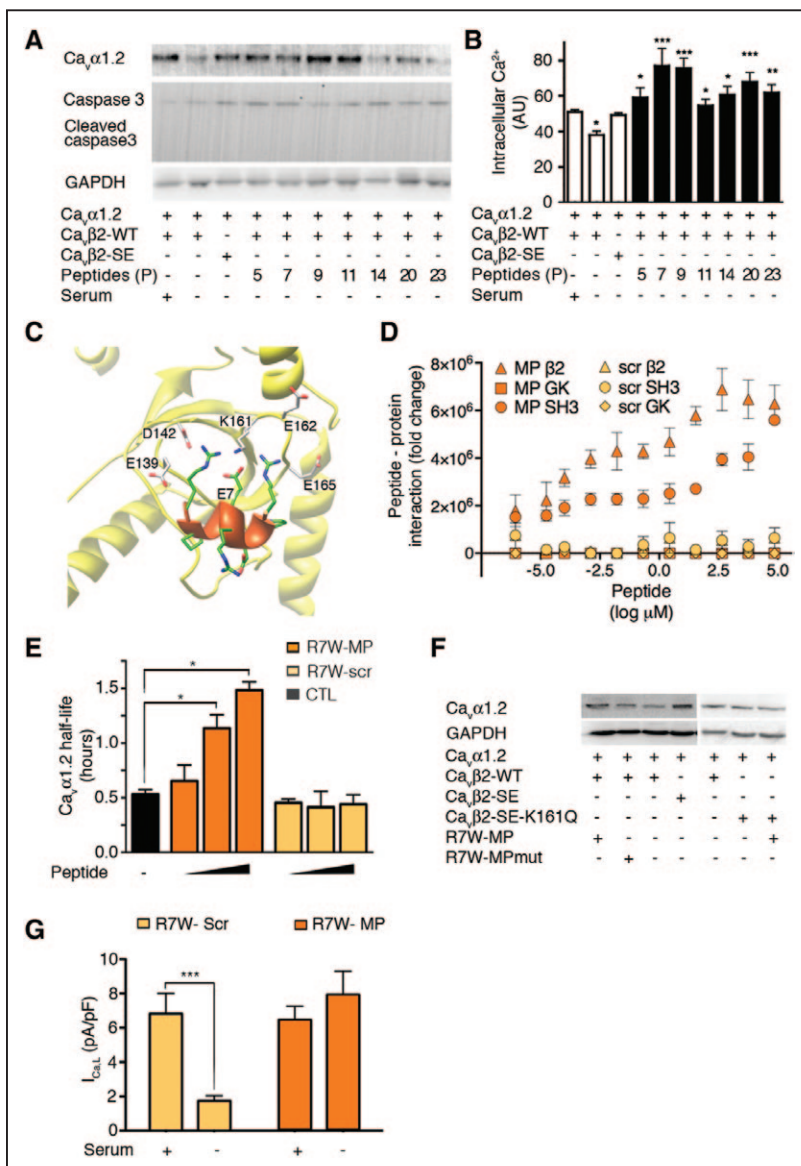


Figure 2. The mimetic peptide (MP) R7W-MP binds to $Ca_v\beta2$ and affects $Ca_v\alpha1.2$ protein stability and function.

A, Western blot analysis of $Ca_v\alpha1.2$ and Caspase 3 in total protein lysates from HEK293 cells transfected as indicated (n=3). **B**, Intracellular Ca^{2+} levels in HEK293 cells transfected as indicated (n=8; 1-way ANOVA). **C**, Molecular model of the MP (orange) docked onto the tail-interacting domain (TID) region of $Ca_v\beta2$ (yellow), revealing the formation of electrostatic interactions. **D**, Dose-dependent binding of R7W-MP-tetramethylrhodamine (TMR) and R7W-scramble (R7W-scr)-TMR to $Ca_v\beta2$ -NanoLuc, Src homology 3 (SH3)-NanoLuc, and guanylate kinase-like (GK)-NanoLuc as measured by bioluminescence resonance energy transfer assay in transfected HEK293 cells (n=6). **E**, $Ca_v\alpha1.2$ half-life measured by a NanoLuc luciferase assay. HEK293 cells were transfected with $Ca_v\alpha1.2$ -NanoLuc and treated with incremental doses (0.12, 1.3, and 10.2 μ mol/L) of R7W-MP and R7W-scr as indicated (n=6; 1-way ANOVA). **F**, Western blot analysis for $Ca_v\alpha1.2$ in total protein lysates from serum-starved HEK293 cells transfected and treated as indicated (n=4; Mann-Whitney test). **G**, Ca^{2+} current measurements in tSA201 cells transfected and treated as indicated (n=15; 1-way ANOVA). All data are shown as mean \pm SD. * P <0.05; ** P <0.01; *** P <0.001.

R7W-MP was found to colocalize with $Ca_v\beta 2$ in adult cardiomyocytes (Figure IIB in the online-only Data Supplement). As expected, R7W-MP was shown to increase the half-life (Figure 2E) and total protein levels (Figure IIC in the online-only Data Supplement) of $Ca_v\alpha 1.2$ in a dose-dependent manner. Replacement of either K161 in the TID sequence or its direct interacting residue in MP (E7) with glutamines (Q) ablated the protective effect of R7W-MP on $Ca_v\alpha 1.2$ protein levels (Figure 2F) and half-life (Figure IID in the online-only Data Supplement), confirming the specificity of the TID-MP interaction. R7W-MP, but not R7W-scramble, was found to efficiently preserve $I_{Ca,L}$ (Figure 2G) without affecting the steady-state voltage dependency of the channel under serum free conditions (data not shown).

R7W-MP Prevents Dynamin From Binding to $Ca_v\beta 2$ and Protects $Ca_v\alpha 1.2$ From Protein Degradation

The $Ca_v\beta 2$ -SH3 domain was previously shown to down-regulate LTCC density at the plasma membrane through interaction with dynamin, a GTPase involved in endocytosis and vesiculation.²² Furthermore, a dynamin-specific inhibitor was found to protect $Ca_v\alpha 1.2$ from protein degradation²² and to extend its half-life (data not shown). This led us to question whether R7W-MP might compete with dynamin for binding to the $Ca_v\beta 2$ -SH3 domain, thereby enhancing the $Ca_v\alpha 1.2$ half-life. To test this hypothesis, we performed a BRET assay that revealed that R7W-MP indeed prevents dynamin from binding to $Ca_v\beta 2$ (Figure 3A). In addition, we demonstrated that $Ca_v\beta 2$ dissociates from $Ca_v\alpha 1.2$ when bound to dynamin, whereas R7W-MP recovered the interaction of $Ca_v\alpha 1.2$ with its chaperone (Figure 3B). Finally, in a cell surface protein biotinylation assay, a significant reduction in biotinylated $Ca_v\alpha 1.2$ (membrane fraction) was found in cells cotransfected with dynamin, an effect that was fully counteracted by R7W-MP (Figure 3C).

R7W-MP Facilitates $Ca_v\alpha 1.2$ Chaperoning to the Plasma Membrane by Preventing Kir/Gem Binding to $Ca_v\beta 2$

Kir/Gem, together with Rad, Rem, and Rem2, is a member of the RGK small GTP-binding protein family²⁶ known to negatively affect the amount of LTCCs at the cell surface.^{27,28} In particular, overexpression of Kir/Gem in *Xenopus* oocytes was reported to compromise the association of $Ca_v\beta 3$ with $Ca_v\alpha 1.2$ and to prevent the trafficking of the channel to the plasma membrane by sequestering $Ca_v\beta 3$ in intracellular compartments.²⁷ From this finding and evidence that Kir/Gem binds to $Ca_v\beta 3$ through a region within its SH3 domain,²⁹ we

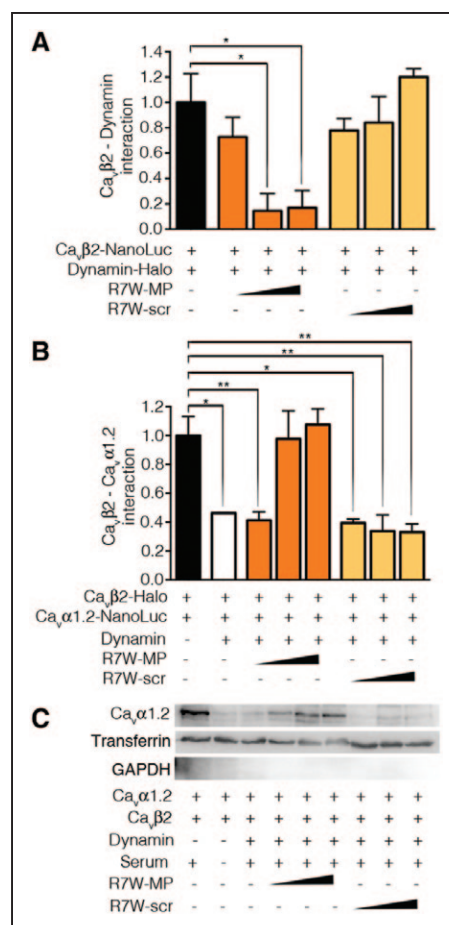


Figure 3. R7W-mimetic peptide (MP) prevents dynamin from binding to $Ca_v\beta 2$ and enhances $Ca_v\alpha 1.2$ density at the plasma membrane.

A, $Ca_v\beta 2$ -dynamin protein affinity as measured by bioluminescence resonance energy transfer (BRET) assay in HEK293 cells transfected with $Ca_v\beta 2$ -NanoLuc and Dynamin-Halo and treated as indicated (n=6). **B**, $Ca_v\beta 2$ - $Ca_v\alpha 1.2$ protein affinity as measured by BRET assay in HEK293 cells transfected with $Ca_v\beta 2$ -Halo and $Ca_v\alpha 1.2$ -NanoLuc and treated as indicated (n=6). **C**, Cell surface biotinylation assay performed in HEK293 cells transfected and treated as indicated (n=4). All data are shown as mean±SD. Peptide doses were 0.12, 1.3, and 10.2 μmol/L. R7W-scr indicates R7W-scramble. *P<0.05; **P<0.01 (Mann-Whitney test).

hypothesized that R7W-MP promotes anterograde trafficking of $Ca_v\alpha 1.2$ by preventing Kir/Gem from complexing with $Ca_v\beta 2$. A BRET interaction assay confirmed the binding of Kir/Gem to $Ca_v\beta 2$, which was compromised by R7W-MP (Figure 4A). In addition, the association of $Ca_v\beta 2$ with $Ca_v\alpha 1.2$, which was drastically affected by the presence of Kir/Gem, was gradually recovered by R7W-MP (Figure 4B). The above results were supported by a surface protein biotinylation assay showing that R7W-MP treatment is sufficient to prevent the Kir/Gem-mediated reduction of $Ca_v\alpha 1.2$ at the plasma membrane (Figure 4C).

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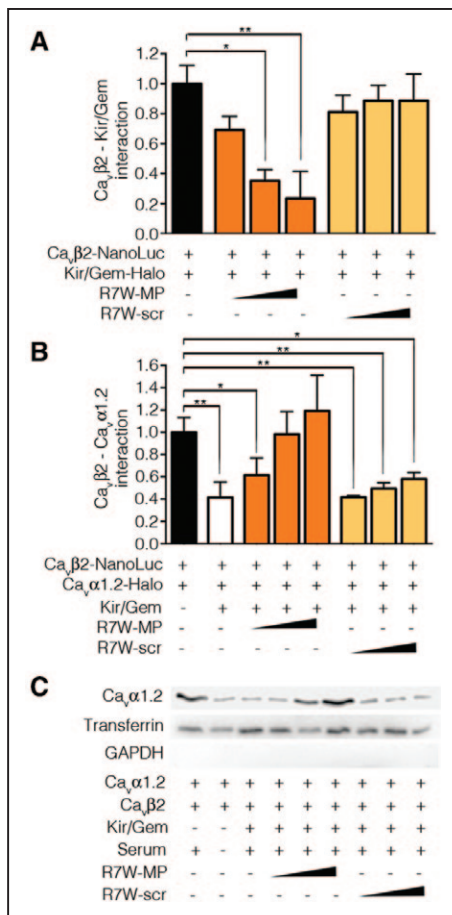


Figure 4. R7W-mimetic peptide (MP) prevents Kir/Gem from binding to Ca_vβ2 and enhances Ca_vα1.2 density at the plasma membrane.

A, Ca_vβ2-Kir/Gem protein affinity as measured by bioluminescence resonance energy transfer (BRET) assay in HEK293 cells treated as indicated (n=6). **B**, Ca_vβ2-Ca_vα1.2 protein affinity as measured by BRET assay in HEK293 cells treated as indicated (n=6). **C**, Cell surface biotinylation assay performed in HEK293 cells transfected and treated as indicated (n=6). All data are shown as mean±SD. Peptide doses were 0.12, 1.3, and 10.2 μmol/L. R7W-scr indicates R7W-scramble. *P<0.05; **P<0.01 (Mann-Whitney test).

R7W-MP Prevents Kir/Gem-Mediated Relocation of Ca_vβ2 to the Nucleus and Promotes *cacna1* Expression

Members of the RGK family show several nuclear import signals and have been reported to sequester and shuttle Ca_vβ3 and Ca_vβ4 from the cytoplasm to the nucleus.^{30,31} In addition, Ca_vβ1b, Ca_vβ4, and Ca_vβ3 were shown to interact with nuclear proteins and to modulate gene expression. In particular, the Ca_vβ4 short splice variant in brain was found to interact with the nuclear heterochromatin protein 1γ (HP1γ),³⁰ a member of the chromatin organization modifier superfamily, negatively regulating gene expression.³² Thus, we hypothesized

that, when bound to Kir/Gem, cytoplasmic Ca_vβ2 relocates to the nucleus, where it affects the transcription of a specific set of genes through recruitment of HP1γ. As predicted, overexpression of Kir/Gem in HL-1 cardiac cells resulted in the translocation of Ca_vβ2 from the cytosol/plasma membrane to the nucleus, where it was found to colocalize with HP1γ (Figure 5A). The specific interaction between Ca_vβ2 and HP1γ was confirmed by BRET (Figure 5B) and coimmunoprecipitation (Figure 5C) assays, whereas R7W-MP prevented the interaction (Figure 5B and 5C). In contrast, Ca_vβ2 did not interact with bromodomain 4,³³ an epigenetic factor that facilitates transcriptional activation (Figure III in the online-only Data Supplement). Taken together, these data point to a specific interaction between the nucleus-relocalized Ca_vβ2 and HP1γ that is potentially required for the regulation of determined genes.

In muscle progenitor cells, Ca_vβ1 was recently shown to translocate to the nucleus and to regulate gene expression by recognizing noncanonical heptameric sites at the promoter region of a number of genes.³⁴ To determine whether nuclear Ca_vβ2 might play a similar role and be part of a negative feedback loop in cardiac cells by regulating genes encoding LTCC subunits, we performed promoter analysis that identified 3 and 1 noncanonical sequences in the promoter region of *Cacna1c* (encoding Ca_vα1.2) and *Cacnb2* (encoding Ca_vβ2), respectively (Figure 5F and data not shown). Consistently, Ca_vα1.2 RNA levels were found to be reduced in Kir/Gem-transfected HL-1 cells (causing translocation of Ca_vβ2 to the nucleus), whereas Ca_vα1.2 expression was recovered by R7W-MP treatment (Figure 5D). In contrast, Ca_vβ2 expression was unaltered under all analyzed conditions (data not shown). Cotransfection with HP1γ-specific RNAi (Figure IV in the online-only Data Supplement) prevented the reduction in Ca_vα1.2 RNA levels (Figure 5E), demonstrating that the ability of nuclear Ca_vβ2 to repress LTCC pore unit expression is dependent on its interaction with HP1γ. Consistent with a direct role of nuclear Ca_vβ2 in regulating *Cacna1c* gene expression, chromatin immunoprecipitation with Ca_vβ2 antibody, followed by quantitative polymerase chain reaction analysis for the above promoter sequences revealed enrichment for the NC1 site at the *Cacna1c* promoter, which was prevented by R7W-MP treatment (Figure 5F). Furthermore, a luciferase-based promoter assay confirmed the negative regulatory effect of nuclear Ca_vβ2 on *Cacna1* gene expression (Figure 5G), supporting the idea that relocalized Ca_vβ2 contributes to adjusting channel density in cells via a negative feedback loop.

R7W-MP Restores Cardiac Function In Vivo Through Modulation of LTCC

Our next objective was to explore the use of R7W-MP for potential cardiac treatments in vivo. After dot blot

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ORIGINAL RESEARCH
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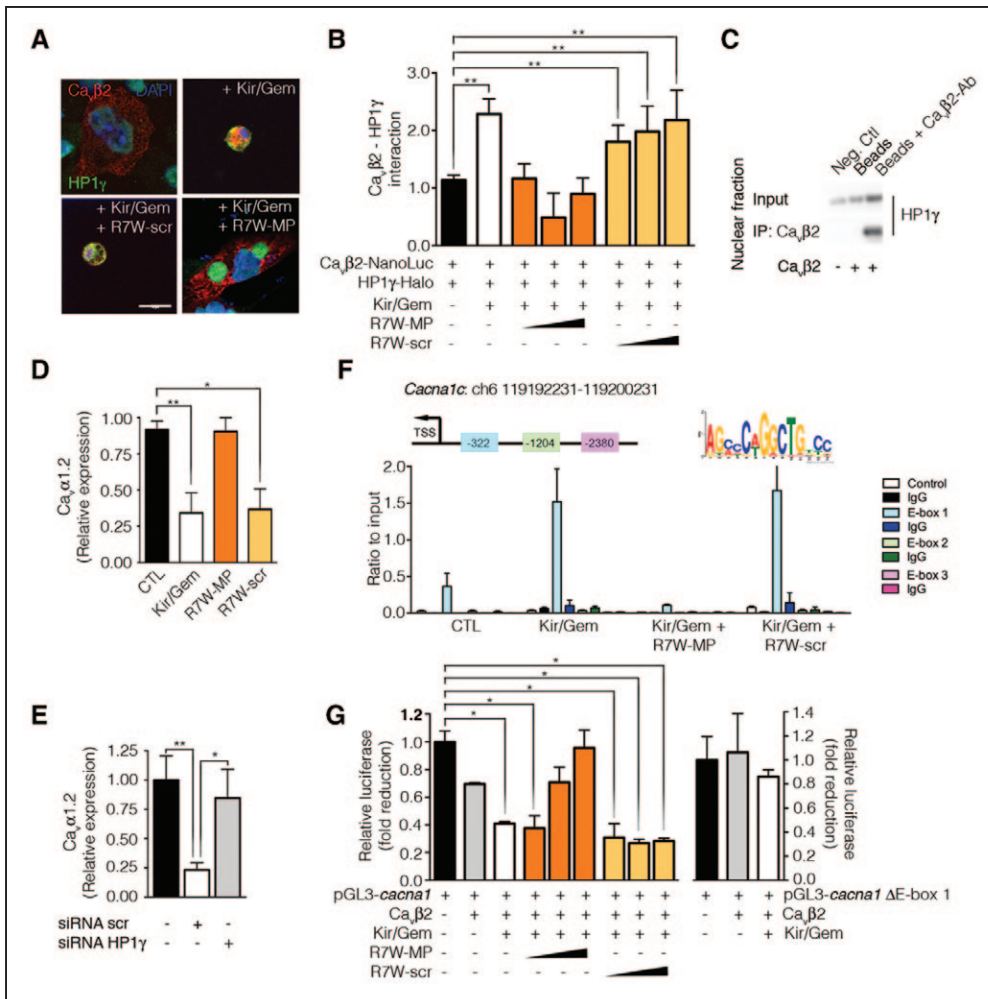


Figure 5. R7W-mimetic peptide (MP) prevents relocation of $Ca_v\beta 2$ to the nucleus and promotes *cacna1* expression. **A**, Immunofluorescence of $Ca_v\beta 2$ (red) and heterochromatin protein 1 γ (HP1 γ ; green) in HL-1 cells transiently transfected with $Ca_v\beta 2$ -DsRed and Kir/Gem constructs and treated as indicated. Bar=10 μ m (n=15). **B**, $Ca_v\beta 2$ -Hp1 γ protein interaction as measured by bioluminescence resonance energy transfer (BRET) assay in HEK293 cells transfected with $Ca_v\beta 2$ -NanoLuc and Hp1 γ -Halo and treated as indicated (n=6). **C**, Coimmunoprecipitation assay from the nuclear fraction of HEK293 cells, confirming the interaction of Hp1 γ with $Ca_v\beta 2$. Representative experiments are shown (n=4). Cells were transfected as indicated. **D**, Quantitative reverse transcription-polymerase chain reaction analysis for $Ca_v\alpha 1.2$ expression in HL1 cells transfected as indicated (n=4). **E**, **Top**, Consensus $Ca_v\beta 2$ DNA-binding motifs in the promoter of the *cacna1c* gene. **Bottom**, Chromatin immunoprecipitation with $Ca_v\beta 2$ antibody in HL-1 cells treated as indicated (n=3). **F**, Promoter luciferase assay in HEK293 cells transfected as indicated. All data are shown as mean \pm SD. Peptide doses were 0.12, 1.3, and 10.2 μ mol/L. R7W-scr indicates R7W-scramble. * P <0.05; ** P <0.01 (Mann-Whitney test).

analysis confirming the delivery of administered R7W-MP to the heart, the effect of R7W-MP on cardiac function was monitored (Figure VA in the online-only Data Supplement). In vivo echocardiographic analyses revealed no differences between groups (Table I in the online-only Data Supplement), and $Ca_v\alpha 1.2$ protein levels were unchanged (Figure VB in the online-only Data Supplement). Furthermore, electrophysiologic properties of isolated adult cardiomyocytes were unaffected (Figure VC-VF in the online-only Data Supplement), and in vivo epicardial multiple lead recording showed no significant changes in cardiac excitability and refractoriness, pointing toward

a low index of proarrhythmogenicity (Figure VG in the online-only Data Supplement).

To investigate the potential therapeutic application of R7W-MP, we assessed its effect in a mouse model of streptozotocin-induced diabetes mellitus in which alteration of cardiac contractility and reduced LTCC density are secondary consequences of the disease^{10,35-39} (Figure 6A and 6B and Tables II and III in the online-only Data Supplement). Notably, R7W-MP treatment normalized ventricular systolic function and restored $Ca_v\alpha 1.2$ protein levels in diabetic mice (Figure 6A and 6B and Table III in the online-only Data Supplement). In addition, functional analyses

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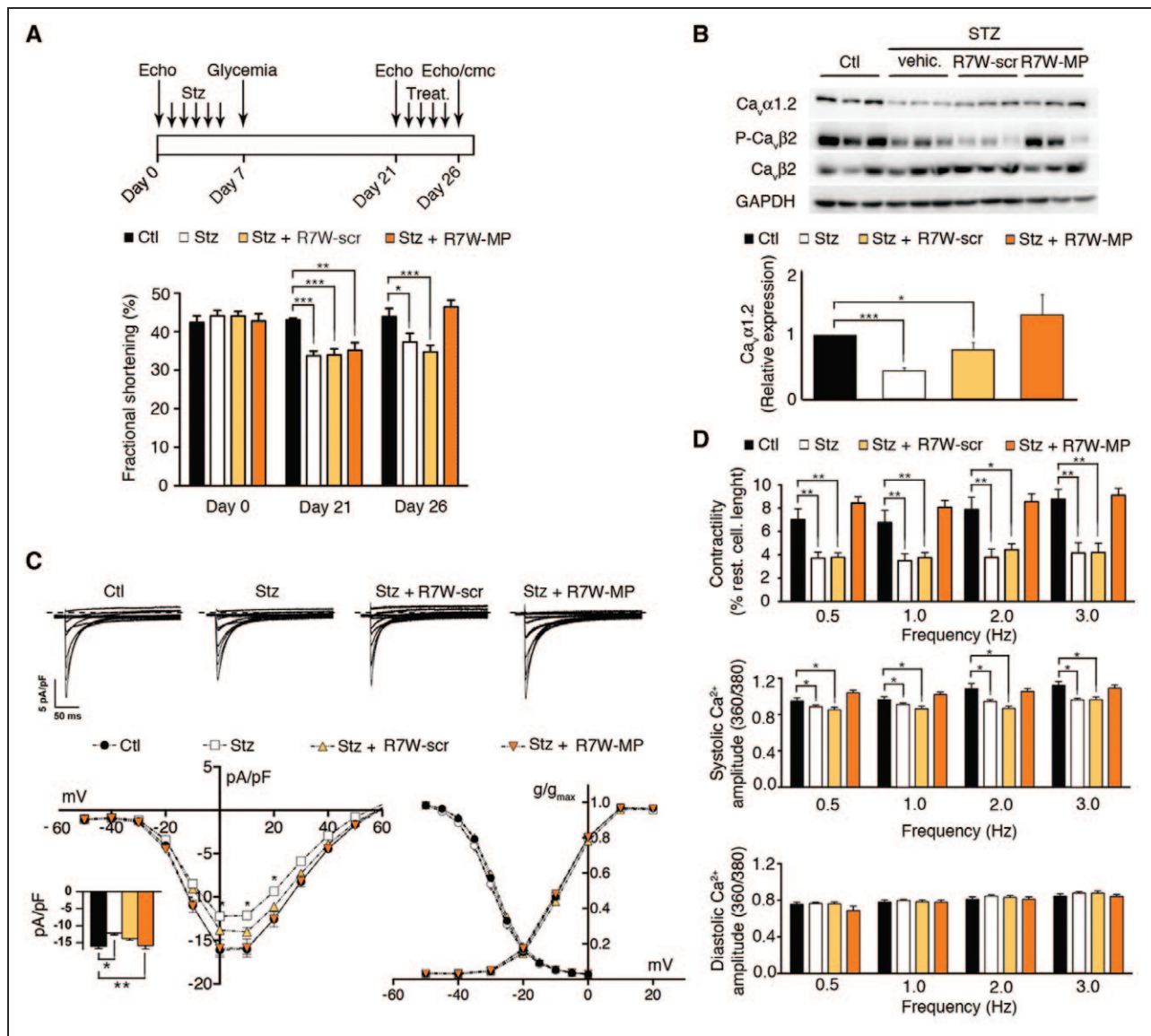


Figure 6. R7W-mimetic peptide (R7W-MP) restores cardiac function in a mouse model of diabetic cardiomyopathy.

A, Design of the study (top) and fractional shortening (%; index of heart contraction; bottom) as determined by echocardiographic analysis on streptozotocin (Stz)-treated mice injected as indicated (n=10). **B**, Western blot analysis and densitometry for $Ca_v\alpha1.2$, $Ca_v\beta2$, and P- $Ca_v\beta2$ in left ventricular homogenates. **C**, Ca^{2+} current measurements in adult cardiomyocytes isolated from treated mice. Single traces (top), I/V relationships (left bottom), and steady-state activation/inactivation curves (right bottom) are shown (n=15). **D**, Contractility (top) and Ca^{2+} transients (middle, systolic; bottom, diastolic) in adult cardiomyocytes isolated from treated mice at different pacing frequencies (Hz; n=15). Stz and peptides were administered daily by intraperitoneal injection as indicated. All data are shown as mean \pm SD. R7W-scr indicates R7W-scramble. * P <0.05; ** P <0.01; *** P <0.001 vs control.

of cardiomyocytes from the same mice revealed restoration of cell contractility, systolic Ca^{2+} amplitude, and I_{CaL} (Figure 6C and 6D), as well as normalization of I_{CaL} inactivation kinetics (Figure VI in the online-only Data Supplement), in R7W-MP treated mice. These results show the ability of R7W-MP to reduce LTCC turnover and to correct myocardial dysfunction associated with diabetes mellitus, a secondary complication of diabetes mellitus.

On the basis of these outcomes, we explored the potential translatability of our results and retrospectively analyzed

left ventricular biopsies from diabetic and nondiabetic cardiomyopathy patients with aortic stenosis undergoing aortic valve replacement. Interestingly, we found a direct linear correlation between the total amount of $Ca_v\alpha1.2$ and the Akt-dependent phosphorylation status of $Ca_v\beta2$ (Figure 7). F7

DISCUSSION

The $Ca_v\beta2$ LTCC subunit is known to dictate the appropriate chaperoning of $Ca_v\alpha1.2$ to and density at the

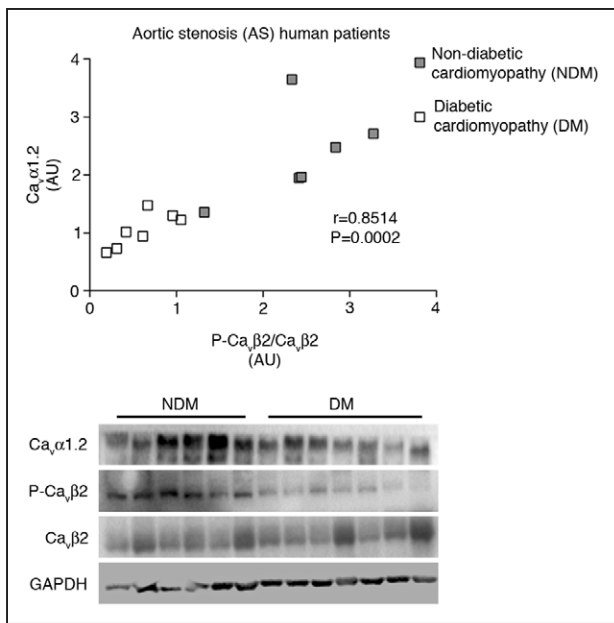


Figure 7. Phosphorylation status of $Ca_v\beta_2$ at the Akt consensus site correlates with $Ca_v\alpha_{1.2}$ levels in the myocardium of patients with diabetic and nondiabetic cardiomyopathy associated with aortic stenosis.

Densitometry (top) and Western blot analysis (bottom) on total protein lysates from left ventricular biopsies. A significant correlation was observed (Pearson correlation coefficient). Protein levels were normalized to GAPDH. AU indicates arbitrary units.

cell surface, which is necessary for initiating Ca^{2+} influx and thus proper excitation-contraction coupling in the heart. Under stress conditions, the SH3-dependent

scaffolding properties of $Ca_v\beta_2$ are altered, thus affecting the assembly, intracellular mobility, and function of the LTCC multiprotein complex. Here, we provide new insights into Ca^{2+} channel physiology and demonstrate that the density of the pore unit is regulated by molecular reorganization of $Ca_v\beta_2$, which is initiated by Akt-mediated phosphorylation of its C-terminal tail, allowing it to bind to the central SH3 domain. From this finding, we generated an MP that, by targeting the binding region (TID) within the SH3 domain of $Ca_v\beta_2$, increases $Ca_v\alpha_{1.2}$ density at the plasma membrane. Mechanistically, our results point toward a complex regulatory system that involves multiple levels of regulation (Figure 8): reduces $Ca_v\alpha_{1.2}$ retrograde trafficking and protein degradation by preventing dynamin-mediated LTCC endocytosis; promotes $Ca_v\alpha_{1.2}$ anterograde trafficking by blocking Kir/Gem-dependent sequestration of $Ca_v\beta_2$, thus facilitating the chaperoning of $Ca_v\alpha_{1.2}$; and promotes $Ca_v\alpha_{1.2}$ transcription by preventing Kir/Gem-mediated shuttling of $Ca_v\beta_2$ to the nucleus, where it limits the transcription of $Ca_v\alpha_{1.2}$ through recruitment of the HP1 γ epigenetic repressor to the *Cacna1c* promoter. This last mechanism shows that $Ca_v\beta_2$ plays a critical role in a previously unknown negative feedback pathway controlling the highly regulated expression of LTCC. Future work should address whether nuclear $Ca_v\beta_2$ might be involved in the modulation of a family of genes encoding proteins associated with the regulation of Ca^{2+} handling, sarcomeric assembly, and Ca^{2+} sensitivity of myofilaments, thereby affecting contractility.^{34,40-42}

Our results provide the basis for the development of a potential novel therapy for the treatment of cardiac

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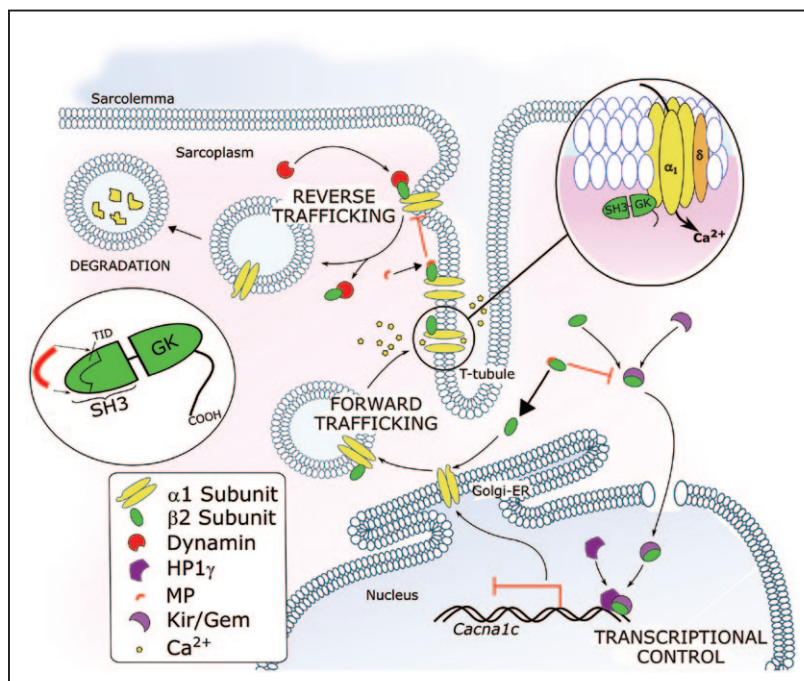


Figure 8. Working model.

Mimetic peptide binds to the Src homology 3 (SH3) domain of $Ca_v\beta_2$ subunit and facilitates L-type Ca^{2+} channel density at the plasma membrane by promoting forward trafficking, reducing reverse trafficking, and inducing gene expression of $Ca_v\alpha_{1.2}$ subunit. GK indicates guanylate kinase-like; and HP1 γ , heterochromatin protein 1 γ .

disorders associated with reduced LTCC density. The MP generated in this study falls in the class of positive Ca^{2+} modulators, which has received considerable attention in the past decades. However, although remarkably efficient at enhancing Ca^{2+} current and inotropism in systolic heart failure, this class of compounds was also found to favor arrhythmogenesis, thereby limiting their clinical use. For instance, the deleterious effects of the LTCC-activator BAYK8644 was shown to be due at least partly to unwanted modification of gating kinetics of the LTCC pore and Ca^{2+} -independent effects on ryanodine receptor gating, increasing resting SR Ca^{2+} release.⁴³ In contrast, the MP described here acts unconventionally by direct chaperone-mediated modulation of LTCC density and, via its direct action on the life cycle of the channel, restores physiologic levels of LTCC only when its density at the plasma membrane is reduced. Additionally, we found that $\text{P-Ca}_v\beta_2$ levels, which, as expected, were reduced in diabetic mice, were normalized to control levels after MP treatment. This leads to the intriguing hypothesis that, by binding to $\text{Ca}_v\beta_2$, MP might facilitate the proximity or availability of the LTCC chaperone for the active Akt at the plasma membrane. If this is the case, in addition to its effect on $\text{Ca}_v\alpha_1.2$ density at the plasma membrane, MP stimulates the “physiologic” Akt-dependent stabilizing effect on LTCC density and function,²⁴ thereby initiating a possible forward mechanism. More dedicated and in-depth studies are required to address this possibility. Although an MP-based therapy might be envisioned, as also supported by the striking linear correlation between phosphorylated $\text{Ca}_v\beta_2$ and $\text{Ca}_v\alpha_1.2$ density in our preliminary retrospective studies of human patient biopsies, further compound optimization, toxicity studies, and larger-scale prospective analyses are required. For instance, the detrimental effects previously observed with supraphysiologic levels of $\text{Ca}_v\beta_2$ and $\text{Ca}_v\alpha_1.2$ ^{44–48} raise the question of whether MP administration may cause similar issues. However, although further and more exhaustive studies are required (ie, short- to long-term pharmacokinetic analysis), it is promising that MP administration did not result in any abnormal increase in $\text{Ca}_v\alpha_1.2$ density or I_{CaL} . Additionally, MP did not affect $\text{Ca}_v\beta_2$ protein levels in either stressed or unstressed conditions. Another issue is the broad tissue distribution of $\text{Ca}_v\beta_2$, necessitating careful re-evaluation of the nature and type of carrier to use because the current R7W carrier is not specific to the heart and thus may also have potential effects at other locations such as the vasculature or the sympathetic nervous system. In this regard, the recent development of novel drug carriers (ie, cell-specific aptamers⁴⁹ or functionalized nanoparticles⁵⁰) might allow specific delivery to the heart and thus minimization of short- or long-term secondary side effects.

CONCLUSION

This study identifies new regulatory mechanisms controlling the LTCC life cycle and provides proof of concept for a novel therapy for the treatment of cardiac conditions associated with reduced LTCC density.

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DISCLOSURES

None.

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FOOTNOTES

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- AQ4—Should “D.L. N.26, 04/03/2014” be “D.L. No. 26, March 4, 2014”?
- AQ5—Replace “C-terminal coiled-coil region” with “C-tail”?
- AQ6—If you have color in your proof, please indicate whether you approve the color charge by returning the color agreement with your corrections. The color agreement can be found at <http://links.lww.com/circ/A10>. The rate is \$653 per 1 printed color page. If you prefer black and white figures, please indicate so on your proof corrections. Please note: There is no online-only color option for Circulation articles. If your article is black and white in print, the online version will also be black and white. If you have any questions, please contact circulationjournal@lww.com.
- AQ7—Please confirm “short- to long-term pharmacokinetic analysis.”

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