Dominant Negative Suppression of Rad Leads to QT Prolongation and Causes Ventricular Arrhythmias via Modulation of L-type Ca\textsuperscript{2+} Channels in the Heart

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Abstract—Disorders of L-type Ca\textsuperscript{2+} channels can cause severe cardiac arrhythmias. A subclass of small GTP-binding proteins, the RGK family, regulates L-type Ca\textsuperscript{2+} current (I\textsubscript{Ca,L}) in heterologous expression systems. Among these proteins, Rad (Ras associated with diabetes) is highly expressed in the heart, although its role in the heart remains unknown. Here we show that overexpression of dominant negative mutant Rad (S105N) led to an increase in I\textsubscript{Ca,L} and action potential prolongation via upregulation of L-type Ca\textsuperscript{2+} channel expression in the plasma membrane of guinea pig ventricular cardiomyocytes. To verify the in vivo physiological role of Rad in the heart, a mouse model of cardiac-specific Rad suppression was created by overexpressing S105N Rad, using the \(\alpha\)-myosin heavy chain promoter. Microelectrode studies revealed that action potential duration was significantly prolonged with visible identification of a small plateau phase in S105N Rad transgenic mice, when compared with wild-type littermate mice. Telemetric electrocardiograms on unrestrained mice revealed that S105N Rad transgenic mice had significant QT prolongation and diverse arrhythmias such as sinus node dysfunction, atrioventricular block, and ventricular extrasystoles, whereas no arrhythmias were observed in wild-type mice. Furthermore, administration of epinephrine induced frequent ventricular extrasystoles and ventricular tachycardia in S105N Rad transgenic mice. This study provides novel evidence that the suppression of Rad activity in the heart can induce ventricular tachycardia, suggesting that the Rad-associated signaling pathway may play a role in arrhythmogenesis in diverse cardiac diseases. (Circ Res. 2007;101:69-77.)

Key Words: G protein \(\square\) L-type Ca\textsuperscript{2+} channels \(\square\) arrhythmia

Rad (Ras associated with diabetes) is the prototypic member of the newly emerging RGK family of proteins, a group of Ras-related GTPases that includes Rad, Gem, and Rem.\textsuperscript{1} Rad was initially identified by subtraction cloning as an mRNA overexpressed in skeletal muscle in a subset of patients with type 2 diabetes mellitus.\textsuperscript{2} Among the RGK proteins, Rad is abundantly expressed in skeletal and cardiac muscle.\textsuperscript{2} It interacts with various signal transduction molecules such as Rho kinase, calmodulin, and calmodulin-dependent protein kinase II, leading to inhibition of their downstream signals.\textsuperscript{3-5} In epithelial or fibroblastic cells, overexpression of Rad results in stress fiber and focal adhesion disassembly, implicating an involvement in cytoskeletal regulation through the Rho kinase pathway.\textsuperscript{3} In vascular smooth muscle cells, focal gene transduction of Rad attenuates neointimal formation after balloon injury by inhibiting smooth muscle proliferation and migration activated through the Rho kinase pathway.\textsuperscript{6} Furthermore, overexpression of Rad in skeletal muscle worsens diet-induced insulin resistance and glucose intolerance, which is consistent with the observed upregulation of Rad in diabetic patients.\textsuperscript{7} Despite the critical roles of Rad in diverse biological processes, its function in the heart is still unknown.

Recently, RGK proteins were found to suppress voltage-gated L-type Ca\textsuperscript{2+} currents (I\textsubscript{Ca,L}) in heterologous expression systems and insulin-secreting \(\beta\) cells of the pancreas.\textsuperscript{8-10} This was shown to occur via an interaction with Ca\textsuperscript{2+} channel \(\beta\) subunits, and the finding suggests that Rad may play an important role in cellular Ca\textsuperscript{2+} homeostasis. Indeed, overexpression of Gem in PC12 cells and MIN6 cells prevents Ca\textsuperscript{2+}-triggered exocytosis via inhibition of L-type Ca\textsuperscript{2+} channels.\textsuperscript{11} In the heart, Ca\textsuperscript{2+} is essential for electrical activity and is a direct activator of the myofilaments in contraction. Among the many Ca\textsuperscript{2+} handling proteins, cardiac L-type Ca\textsuperscript{2+} channels play central roles in initiation of excitation–contraction coupling and in cardiac electrophysiological properties. Abnormal function of Rad in the heart might therefore lead to various cardiac disorders such as arrhythmias or contractile dysfunction.
We show here that dominant negative suppression of Rad led to the enhancement of $I_{\text{Ca,L}}$ by facilitating channel expression in the plasma membrane of cardiomyocytes, resulting in prolongation of the action potential. Furthermore, transgenic mice with heart-specific overexpression of dominant negative mutant Rad (S105N) displayed ventricular arrhythmias as a consequence of QT prolongation. Our results constitute the first evidence that Rad plays an important role in regulating cardiac electrophysiological properties and that Rad could be a key molecule for understanding the mechanism of arrhythmogenesis in cardiovascular diseases.

**Materials and Methods**

All experimental procedures and protocols were approved by the Animal Care and Use Committee of Keio University and conformed to the NIH Guide for the Care and Use of Laboratory Animals. An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

**Myocyte Isolation and Cultures**

Myocytes were isolated from the left ventricles of adult guinea pigs and mice using enzymatic digestions as previously described, with slight modifications. After isolation, ventricular myocytes were cultured in DMEM containing 10% FBS and 1% penicillin–streptomycin (all from Invitrogen, Carlsbad, Calif) for 24 hours.

**Production of S105N Rad Transgenic Mouse**

The complete mutant mouse S105N Rad (with the serine at the 105-aa position substituted to arginine, to inhibit GTP binding) cDNA construct was subcloned into the region downstream of the $\alpha$-myosin heavy chain promoter previously subcloned into the PBS2 SK+ plasmid. The complete transgene was isolated using Not I digestion of the PBS2 SK+ plasmid. Transgenic mice were generated by the Animal Laboratory Center of Keio University by cDNA microinjection of fertilized C57BL/6 SJL oocytes using standard techniques.

**Statistical Analysis**

All data are shown as means±SEM. Statistical differences were determined using repeated-measures ANOVA, and $P<0.05$ was considered significant.

**Results**

**Rad Interacts With L-Type Ca$^{2+}$ Channel $\beta$ Subunits**

Previous reports have shown that Rad interacts with Ca$^{2+}$ channel $\beta$ subunits in heterologous expression systems, resulting in the inhibition of $I_{\text{Ca,L}}$. To examine whether Rad physically interacts with the $\beta$ subunits, glutathione S-transferase (GST) pull-down assays were performed. As shown in Figure 1A, recombinant GST wild-type (WT) Rad bound to the $\beta_{2a}$ subunit and WT Rad or S105N Rad. HEK293 cells were cotransfected with hemagglutinin (HA)-tagged Rad and Flag-tagged $\beta_{2a}$ subunits. The cell lysate was subjected to immunoprecipitation and visualized by immunoblotting with antibodies as indicated.

![Figure 1](https://example.com/figure1.png)

**Figure 1. Interaction between Rad and the L-type Ca$^{2+}$ channel $\beta$ subunit. A, In vitro binding between $\beta_{2a}$ subunit and WT Rad or S105N Rad. Lysates from Cos7 cells transiently transfected with purified recombinant GST-WT Rad or GST-S105N Rad were incubated with glutathione–Sepharose beads. B, In vivo binding between $\beta_{2a}$ subunit and WT Rad or S105N Rad. HEK293 cells were cotransfected with hemagglutinin (HA)-tagged Rad and Flag-tagged $\beta_{2a}$ subunits. The cell lysate was subjected to immunoprecipitation and visualized by immunoblotting with antibodies as indicated. C, Dose-dependent inhibition of the interaction between WT Rad and $\beta$ subunit by overexpression of S105N Rad. The table denotes the molar ratio of transfected plasmid.**
Figure 2. Rad regulates L-type Ca\(^{2+}\) channel function via \(\alpha\) subunit trafficking to plasma membrane. A. Localization of WT Rad and S105N Rad in HEK293 cells. B. Localization of Ca\(^{2+}\) channel \(\alpha_{1c}\) subunits in HEK293 cells, when expressed with or without \(\beta_{2a}\) subunit. C and D, Subcellular localization of GFP-\(\alpha_{1c}\) subunit (green) and Flag-tagged \(\beta_{2a}\) subunit (blue) in a cell coexpressing hemagglutinin-tagged WT Rad or S105N Rad (red). The localizations of each subunit are depicted diagrammatically on the left. E, Representative Ba\(^{2+}\) current traces in HEK293 cells transiently cotransfected with GFP-\(\alpha_{1c}\) subunit, \(\beta_{2a}\) subunit, and blank (n=7), GFP-\(\alpha_{1c}\) subunit, \(\beta_{2a}\) subunit, and WT Rad (n=4), or GFP-\(\alpha_{1c}\) subunit, \(\beta_{2a}\) subunit, and S105N Rad (n=6). ns indicates not significant vs control, *P<0.01 vs control. 

Subunits showed an interaction with WT Rad (Figure 1B), but no interaction with S105N Rad was detected. Because S105N Rad, which could bind GDP but not GTP, is known to function as a dominant negative mutant in the regulation of neointimal formation after vascular injury,\(^6\) we examined whether S105N Rad showed dominant negative inhibition of the interaction between WT Rad and \(\beta\) subunits. A plasmid encoding S105N Rad fused with Myc tag at its N terminus was cotransfected with WT Rad and \(\beta_{2a}\)-subunit plasmids into HEK293 cells. As shown in Figure 1C, Myc-tagged S105N Rad suppressed the interaction between WT Rad and \(\beta_{2a}\) subunits in a dose-dependent manner, indicating that S105N Rad did function as a dominant negative mutant for the interaction between WT Rad and \(\beta_{2a}\) subunits.

Based on these results, we next studied whether the interaction between Rad and the \(\beta\) subunit affected the trafficking of Ca\(^{2+}\) channel \(\alpha\) subunit to the plasma membrane. To do this, green fluorescent protein (GFP)-fused Ca,1,2 (cardiac \(\alpha_{1c}\) subunit) and \(\beta_{2a}\) subunit were coimmunostained with WT Rad or S105N Rad in HEK293 cells and visualized by confocal microscopy. Both WT Rad and S105N Rad were localized at the plasma membrane, when expressed alone (Figure 2A). Although individually expressed \(\alpha_{1c}\) subunit was localized mainly in the cytoplasm (Figure 2B), cotransfection with \(\beta_{2a}\) subunits resulted in the translocation of \(\alpha_{1c}\) subunits from the cytoplasm to plasma membrane, confirming that the \(\beta\) subunit plays a chaperon-like role with the \(\alpha_{1c}\) subunit (Figure 2B). When WT Rad was expressed together with both the \(\alpha_{1c}\) and \(\beta_{2a}\) subunits, the \(\alpha_{1c}\) subunit showed a cytoplasmic distribution, whereas WT Rad and the \(\beta_{2a}\) subunit were still colocalized at the plasma membrane, indicating that WT Rad disrupted the binding of \(\alpha_{1c}\) subunit to \(\beta_{2a}\) subunit (Figure 2C). In contrast, S105N Rad did not affect the localization of the \(\alpha_{1c}\) and \(\beta_{2a}\) subunits (Figure 2D). These results indicated that the interaction between Rad and the \(\beta\) subunit regulated the trafficking of the \(\alpha_{1c}\) subunit to the plasma membrane.

To examine whether Rad regulates the function of L-type Ca\(^{2+}\) channels, we recorded Ba\(^{2+}\) currents in HEK293 cells using the whole-cell patch clamp technique. Currents conducted by L-type Ca\(^{2+}\) channels were recorded with 4 mmol/L Ba\(^{2+}\) in the external solution as a charge carrier. The currents in cells in which S105N Rad was coexpressed with GFP-\(\alpha_{1c}\) subunit and \(\beta_{2a}\) subunit were similar to those in control cells (average current densities for GFP-\(\alpha_{1c}\), subunit, \(\beta_{2a}\) subunit, and S105N Rad: 9.4±0.9 pA/pF [n=6]; for GFP-\(\alpha_{1c}\), subunit, \(\beta_{2a}\) subunit, and blank: 8.8±0.9 pA/pF [n=7]; not significant), whereas in cells expressing the GFP-\(\alpha_{1c}\), subunit, \(\beta_{2a}\) subunit, and S105N Rad: 9.4±0.9 pA/pF [n=6]; for GFP-\(\alpha_{1c}\), subunit, \(\beta_{2a}\) subunit, and blank: 8.8±0.9 pA/pF [n=7]; not significant), whereas in cells expressing the GFP-\(\alpha_{1c}\), subunit, \(\beta_{2a}\) subunit, and blank: 8.8±0.9 pA/pF [n=7]; not significant).
Rad Regulates $I_{\text{Ca,L}}$ Via Inhibition of $\alpha$ Subunit Trafficking to Plasma Membrane in Guinea Pig Ventricular Cardiomyocytes

To investigate the physiological role of Rad in the heart, we transduced adenovirus (Ad) encoding WT Rad or S105N Rad into guinea pig ventricular cardiomyocytes and recorded $I_{\text{Ca,L}}$ using the whole-cell patch clamp technique. Interestingly, the peak $I_{\text{Ca,L}}$ was larger in the cells overexpressing S105N Rad than in controls (12.0±1.5 pA/pF at 0 mV [n=7] in Ad-S105N Rad–transduced cells versus 5.4±1.0 pA/pF at 0 mV [n=8] in Ad-GFP–transduced controls; P<0.05), whereas $I_{\text{Ca,L}}$ was dramatically smaller in cells overexpressing WT Rad (1.4±0.1 pA/pF at 0 mV [n=6] in Ad-WT Rad–transduced cells versus control; P<0.01) (Figure 3A and 3B). Because S105N Rad itself does not affect the $I_{\text{Ca,L}}$, as shown in Figure 2F, enhancement of $I_{\text{Ca,L}}$ by S105N Rad in cardiomyocytes might be attributable to the dominant negative suppression of endogenous Rad activity. Because the L-type Ca$^{2+}$ channel contributes to the ion influx and plateau phase of the cardiac action potential, we investigated whether Rad-mediated regulation of $I_{\text{Ca,L}}$ might affect the action potential in heart cells. Action potentials were recorded in guinea pig ventricular cells transduced with Ad-GFP (control), Ad-WT Rad, and Ad-S105N Rad. As expected, overexpression of WT Rad resulted in a dramatic shortening of the action potential duration (APD) and abolished the robust action potential plateau (APD$_{90}$ 128±7.76 ms [n=5] in Ad-WT Rad–transduced cells versus 267±26.3 ms [n=8] in Ad-GFP–transduced control cells; P<0.01), whereas S105N Rad significantly prolonged the APD without changing the action potential configuration (538±7.0 ms [n=6] in Ad-S105N Rad–transduced cells versus control; P<0.01) (Figure 3C and 3D).

In Vivo Phenotype of Dominant Negative Suppression of Rad in Mouse Heart

To investigate the physiological role of endogenous Rad in vivo, we took advantage of the dominant negative mutant Rad. Because the expression of $\alpha_1$ subunit alone (0.19±0.04 pA/pF [n=5]), indicating the association of WT Rad-mediated suppression with the $\beta$ subunit (Figure 2E and 2F). These results confirmed that WT Rad dramatically suppressed the function of the L-type Ca$^{2+}$ channel, whereas S105N Rad did not.

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In Vivo Phenotype of Dominant Negative Suppression of Rad in Mouse Heart

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to engineer an in vivo model of Rad disruption, obtained by overexpressing the S105N Rad under the control of the α-myosin heavy chain promoter. In transgenic (TG) mice aged 12 weeks, the total Rad protein expression in whole heart was 6 times greater than in their WT littermates (Figure 5A), indicating that the expression level of S105N Rad was approximately 5 times that of endogenous Rad. This level of exogenous S105N Rad expression in TG mice should have been sufficient to suppress the endogenous Rad/β subunit interaction, as shown in Figure 1B. Thus, we performed coimmunoprecipitation assays to confirm the effect of S105N Rad overexpression on the endogenous Rad/β subunit interaction in whole hearts. As shown in Figure 5B, the total amount of Rad that bound to the β subunits was much less in S105N TG mouse heart than that in WT mouse heart. Furthermore, we used Western blot analysis to compare αs1c protein expression in the plasma membranes of S105N Rad TG and WT mice. The αs1c protein expression was significantly greater in S105N Rad TG mice than WT mice, supporting the proposition that dominant negative suppression...

Figure 4. Rad modulates L-type Ca2+ channel membrane expression in guinea pig ventricular cardiomyocytes. Quantitative Western blot analysis comparing αs1c subunit protein extracted from plasma membranes of cardiomyocytes transduced with Ad-GFP (control), Ad-WT Rad, or Ad-S105N Rad after 24 hours in culture. N-Cadherin was used as an internal control for the membrane protein fraction. M indicates molecular size markers. Data are from 5 independent experiments. *P<0.05 vs control.

Figure 5. S105N Rad suppresses the in vivo interaction between Rad and β subunit in the heart. A, Western blot analysis comparing total Rad protein level in cardiac muscle from WT and S105N Rad TG mice. Data are from 5 WT and 5 S105N Rad TG mice, *P<0.05 vs WT. B, In vivo interaction between Rad and β subunit in the heart. Lysates from whole heart were coimmunoprecipitated with anti-β2 subunit antibody, and associated Rad proteins were detected by Western blotting. C, Western blot of cardiac αs1c subunit protein in the membrane fraction from WT and S105N Rad TG mouse hearts. N-Cadherin was used as an internal control for the membrane fraction. M indicates molecular size markers. Data are from 5 WT and 5 S105N Rad TG mice. *P<0.05 vs WT. D, Immunohistochemical analysis of αs1c subunits in WT and S105N Rad TG ventricular cardiomyocytes. The white squares in the upper panels indicate the regions shown at higher magnification below. Vinculin was used as a T-tubule marker. Scale bar=10 μm. Pooled data for relative αs1c mean fluorescence in WT (n=10) and S105N Rad TG mouse cardiomyocytes (n=10).
sion of Rad facilitates α_{tc} subunit expression at the plasma membrane (Figure 5C). Accordingly, immunohistological analysis revealed that the T tubules of ventricular cardiomyocytes isolated from S105N Rad TG mouse hearts were more intensely immunoreactive for α_{tc} subunits than those of WT mice (Figure 5D). The relative mean fluorescence of α_{tc} subunits in the T-tubule areas to that in non–T-tubule area (between T tubules) were significantly greater in the S105N subunits in the T-tubule areas to that in non–T-tubule area (between T tubules) were significantly greater in the S105N Rad TG mouse cells than in the WT mouse cells, indicating that S105N Rad facilitated the α_{tc} subunit trafficking to the T tubules in cardiomyocytes (Figure 5D).

Next, action potentials were recorded from left ventricular papillary muscles using conventional microelectrode techniques. The APD was measured at 3 different pacing cycle lengths (100 ms, 150 ms, and 200 ms). As shown in Figure 6A, the APD was prolonged, and a subtle plateau phase was observed in S105N Rad TG mice. Consistent with the in vitro data in guinea pig cells, the APD_{50} and APD_{90} were both longer in S105N Rad TG mice than in WT mice at each pacing cycle length (Figure 6B; APD_{90} at 100 ms pacing cycle length, 33.9±2.5 ms [n=8] in S105N TG mice, versus 20.9±2.6 ms [n=8] in WT mice; P<0.05). There were no significant differences in resting membrane potential or V_{max} between WT and TG mice (Figure 6C and 6D), suggesting that dominant negative suppression of Rad did not affect the inward rectifying K^+ currents and Na^+ currents. We also examined the transient outward potassium current (I_{to}), which mainly affects repolarization in the mouse action potential. There were no significant differences between the WT and S105N Rad TG mice in either peak or sustained I_{to} current densities, implying that Rad did not affect I_{to} function (Figure 6E).

As expected from the APD data, surface electrocardiograms showed QT prolongation in S105N Rad TG mice compared with WT mice (Figure 7A). Both QT and QTc intervals in S105N Rad TG mice were significantly longer than those in WT mice (QTc, 60.1±3.0 ms [n=8] in S105N Rad TG mice, versus 47.3±3.3 ms [n=8] in WT mice; P<0.05; Figure 7B). No significant differences were detected in other ECG parameters, such as RR, PR, and QRS intervals, as described in Table I in the online data supplement. Given the importance of QT prolongation as a cause of lethal ventricular arrhythmias, we investigated whether the dominant negative suppression of endogenous Rad activity produced arrhythmias in S105N Rad TG mice. In vivo ECGs were recorded from freely moving mice for 24 hours. No arrhythmias were observed in the recordings from WT mice (n=8). In contrast, among the TG mice (n=8), we recorded...
transient sinus arrest and second-degree atrioventricular block in 6 mice each, and ventricular extrasystoles in 5 mice (Figure 7C). In some patients with long QT syndrome, fatal ventricular arrhythmias occur under physical exertion and emotional stress.17,18 To mimic these circumstances, we injected epinephrine (2 mg/kg) into the peritoneum of S105N Rad TG mice (n=8) and WT mice (n=8). Under the epinephrine loading, nonsustained ventricular tachycardia was induced in 3 of the TG mice (Figure 7D), and consecutive ventricular extrasystoles were more frequently observed (7 TG mice), although no ventricular arrhythmias were observed in WT mice (see supplemental Table II).

Because the increase in \( I_{\text{Ca,L}} \) should facilitate nodal conduction, the abnormal nodal function observed in TG mice seemed anomalous. Rad is also known to suppress the Rho signaling pathway by direct interaction with its substrate, ROCK.3 Furthermore, disruption of Rho signaling results in progressive atrioventricular conduction defects, probably attributable to a dramatic decrease of connexin40.19,20 We examined the atrial expression of connexin40 in mouse hearts by Western blot analysis. As expected, connexin40 expression was significantly lower in S105N Rad TG mice than WT mice (Figure 7E).

**Discussion**

The major finding of this study was that dominant negative suppression of endogenous Rad in the heart resulted in an increase in \( I_{\text{Ca,L}} \), via upregulation of L-type Ca\(^{2+}\) channel expression at the plasma membrane. Using a transgenic approach for cardiac-specific inhibition of Rad by expressing the dominant negative form of Rad (S105N), the present study provides important new insights into the physiological function of Rad in regulating cardiac electrophysiology. Although mouse echocardiography showed no significant changes in cardiac size or function in transgenic animals at 12 weeks of age (supplemental Figure I), the phenotype of the transgenic animals comprised a prolonged QT interval, nodal dysfunction, and extrasystoles. These ECG phenotypes were
accompanied by prolonged APDs and enhanced \( I_{\text{Ca,L}} \), which were attributable to the upregulation of L-type \( \text{Ca}^{2+} \) channels in T tubules. Under baseline conditions, TG mice displayed ventricular extrasystoles but no ventricular tachycardia. Furthermore, epinephrine administration exacerbated the ventricular arrhythmias in TG mice but did not induce arrhythmias in WT mice. These data indicate that the Rad signaling pathway plays an important role in cardiac antiarrhythmia via the strong suppression of \( I_{\text{Ca,L}} \).

We did not predict any nodal dysfunction in TG mice because an increase of \( I_{\text{Ca,L}} \) would be expected to facilitate nodal conduction. The induction of nodal dysfunction was probably attributable, at least in part, to the reduction of connexin40 expression in TG mice. Previous studies have shown that Rad binds directly to ROCK, resulting in the inhibition of Rho/ROCK signaling pathways,\(^{1,6} \) which regulates the connexin40 expression in mouse hearts.\(^{10} \) The GST pull-down assays revealed that S105N Rad, as well as WT Rad, could physically interact with ROCK (data not shown). This implies that overexpression of S105N Rad in TG mice may have led to a decrease of connexin40 expression via suppression of the Rho signaling pathways. Furthermore, this inhibitory effect of Rad on Rho pathways was likely to have been extremely strong in the heart because Rad binds to ROCK2, which is highly expressed in the heart, whereas the other isoform (ROCK1) is preferentially expressed in non-cardiac tissues such as lung, liver, spleen, kidney, and testis.\(^{21} \) This distinct tissue-specific expression of ROCK isoforms might explain the opposite effects of Gem and Rad on nodal conduction because Gem predominantly binds to ROCK1.\(^{3} \) However, further studies are needed to test this hypothesis.

One major cause of mortality in patients with diabetes mellitus is diabetic cardiomyopathy, which occurs independently from diabetes-mediated vascular complications. Pereira et al\(^{22} \) reported that the systolic dysfunction of type 2 diabetic mice is partly attributable to a reduction of \( I_{\text{Ca,L}} \), implicating Rad-mediated \( \text{Ca}^{2+} \) channel regulation as a possible factor in diabetic cardiomyopathy. Furthermore, diabetic cardiomyopathy is characterized by electrical remodeling, metabolic remodeling with malignant biochemical processes, and anatomical remodeling with progressive loss of cardiomyocytes.\(^{23} \) The abnormal prolongation of QT interval is the most prominent electrical remodeling that occurs in diabetic hearts. QT prolongation is a significant predictor of mortality in diabetes patients because it is associated with an increased risk of sudden cardiac death caused by lethal ventricular arrhythmias.\(^{24} \) One of the mechanisms for QT prolongation in diabetes mellitus is depression of multiple ion currents including the transient outward current, \( I_{\text{K,trans}} \), and the delayed rectifier K\(^+\) current.\(^{22,25} \) Given that Rad mRNA is upregulated in type 2 diabetes patients\(^{2} \) and Rad protein expression in diabetic mouse heart is upregulated relative to WT mice (data not shown), Rad-mediated regulation of \( I_{\text{Ca,L}} \) might be involved in the electrophysiological remodeling in diabetic cardiomyopathy. Dominant negative suppression of Rad led to QT prolongation and induction of arrhythmias even in the nondiabetic mice used in this study. Considering these experimental and clinical data, it is plausible that upregulation of Rad in diabetic patients might function as a negative regulator to counteract QT prolongation by compensating for the decreased outward K\(^+\) currents with downregulation of \( I_{\text{Ca,L}} \). If so, the preservation of Rad function might be a potential strategy for the prevention of lethal ventricular arrhythmias in diabetic cardiomyopathy.

Our data clearly demonstrated that Rad regulated the trafficking of \( \text{Ca}^{2+} \) channel \( \alpha \) subunit in both heterologous systems and cardiomyocytes. However, the precise mechanisms of RGK protein-mediated modulation of L-type \( \text{Ca}^{2+} \) channels remain to be clarified. Consistent with our data, Beguin et al\(^{9} \) showed that inhibition of \( I_{\text{Ca,L}} \) by another RGK protein, Gem, is attributable to the decreased expression of \( \alpha \) subunits in the plasma membrane. However, in our study, the complete inhibition of \( I_{\text{Ca,L}} \) 24 hours after transduction of WT Rad in guinea pig cells could not be explained solely by the suppression of \( \alpha \) subunit trafficking to the plasma membrane because our Western blot data still detected a small amount of \( \text{Ca}^{2+} \) channel expression in the T tubules. These channels may have remained because the turnover of L-type \( \text{Ca}^{2+} \) channels in the plasma membrane is 36 to 48 hours\(^{26} \); thus a 24 hour culture period might not be sufficient for the complete degradation of preexisting channels. Therefore, other mechanisms for suppression of \( I_{\text{Ca,L}} \) by Rad, unrelated to trafficking, are also likely to be involved. One possibility is the direct inhibition of L-type \( \text{Ca}^{2+} \) channels by association of Rad with channel subunits. Rem2 has recently been shown to almost completely suppress \( I_{\text{Ca,L}} \) without altering channel expression at the plasma membrane,\(^{27,28} \) which supports this hypothesis. The possibility remains that Rad may regulate the expression of other RGK proteins, which in turn alter L-type \( \text{Ca}^{2+} \) channel function. However, the expression level of Rem protein did not change with overexpression of Rad (data not shown), which leads us to conclude that Rem is not associated with the Rad-mediated regulation of the L-type \( \text{Ca}^{2+} \) channel. Further studies are required to identify the multiple mechanisms involved in regulation of the L-type \( \text{Ca}^{2+} \) channel by Rad.

In summary, dominant negative suppression of Rad in the heart induced QT prolongation and ventricular arrhythmias, caused by the augmentation of \( I_{\text{Ca,L}} \). The finding that Rad regulates L-type \( \text{Ca}^{2+} \) channel function in the heart suggests that the Rad-associated signaling pathway may play a role in arrhythmogenesis in diverse cardiac diseases.

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Disclosures

None.
References


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Expanded Materials and Methods

Plasmid constructs and recombinant adenoviruses

The full-length coding sequence of mouse Rad cDNA (GenBank accession No. BC057138) was obtained by RT-PCR from mouse cDNA. Mouse Rad was subcloned in the pCMV expression vector as hemagglutinin (HA)-tagged protein (V79020; Invitrogen), Myc-tagged protein (K6003-1; Clontech Laboratories), and GST gene fused pGEX-6P vector (27-4598-01; GE Healthcare). Human Ca_{\beta2a} (kindly provided by Dr Roger Hullin, Cologne, Germany) was subcloned into pCMV expression vector as FLAG-tagged protein. The point mutation, S105N, was introduced into Rad by site-directed mutagenesis. The HA-tagged mouse wild-type (WT) and S105N mutant Rad were then cloned into the multiple cloning sites of adenovirus shuttle vectors (kindly provided by Dr Eduardo Marbán, Baltimore, USA). These constructs are bicistronic (through an internal ribosome entry site), are driven by a cytomegalovirus promoter, and carry green fluorescent protein (GFP) as a reporter. Detailed methods for the adenovirus vector construction have been described.\textsuperscript{1} Aliquots of adenoviruses (equivalent to \( \sim 1 \times 10^7 \) plaque forming units) were added to the culture medium in the 35-mm dishes 2 hours after cell isolation.

Plasmid transfection

A total of 10 µg of pCMV blank vector, HA- or Myc-tagged Rad, Flag-tagged human Ca_{\beta2a} subunit, and GFP-fused rabbit Ca_{\alpha1c} subunit plasmid cDNA (kindly provided by Dr Manfred Grabner, Innsbruck, Austria) per 10-cm dish was transfected into HEK293 cells using Lipofectamine Plus as per the manufacturer’s instructions (11514015; Invitrogen). OptiMEM I medium (31985-070; GIBCO) was used to dilute the reagent and DNA, and the cells
were incubated for 3 hours. After transfection, the HEK293 cells were cultured in fresh DMEM for 24 to 48 hours before use in experiments. For the experiments examining the dominant negative effect of S105N Rad on the interaction between wild-type (WT) Rad and the β subunit (Figure 1B), 10 µg of plasmid was transfected into HEK293 cells with the molar ratios as indicated.

**Electrophysiology**

The L-type calcium current (I\textsubscript{Ca,L}) and action potential were recorded using the whole-cell patch clamp technique with an Axopatch 200B amplifier (Axon Instruments, Foster City, Calif). All recordings from myocytes were performed at 37°C. Cells were superfused in solution containing (in mmol/L) 140 NaCl, 5 KCl, 1 MgCl\textsubscript{2}, 10 HEPES, 2 CaCl\textsubscript{2}, and 10 glucose (pH 7.4, adjusted with NaOH). For I\textsubscript{Ca,L} recordings, the external solution was replaced with a Na\textsuperscript{+}, K\textsuperscript{+}-free solution containing (in mmol/L) 140 choline chloride, 5 CsCl, 1 MgCl\textsubscript{2}, 10 HEPES, 1.8 CaCl\textsubscript{2}, and 10 glucose (pH 7.4, adjusted with CsOH), after establishing whole-cell clamp mode. The micropipette electrode solution for measuring I\textsubscript{Ca,L} was composed of (in mmol/L) 120 CsCl, 10 TEACl, 1 MgCl\textsubscript{2}, 10 HEPES, 10 EGTA, and 5 MgATP. L-type calcium currents were elicited by 300-ms depolarizing steps from -40 to 50 mV, in 10-mV increments.

Transient outward current (I\textsubscript{o}) was recorded in an external solution containing (in mmol/L) 140 choline chloride, 5 CsCl, 1 MgCl\textsubscript{2}, 10 HEPES, 1.8 CaCl\textsubscript{2}, and 10 glucose (pH 7.4, adjusted with CsOH). The micropipette electrode solution for I\textsubscript{o} was composed of (in mmol/L) 130 KCl, 10 TEACl, 1 MgCl\textsubscript{2}, 10 HEPES, 10 EGTA, and 5 MgATP. I\textsubscript{o} was elicited by 4000-ms depolarizing steps from -40 to 60 mV, in 10-mV increments. The sustained I\textsubscript{o} was measured at the end of the step pulses.
Action potentials were recorded in an external solution containing (in mmol/L) 140 NaCl, 5 KCl, 1 MgCl₂, 10 HEPES, 1.8 CaCl₂, and 10 glucose (pH 7.4, adjusted with NaOH). The micropipette electrode solution for recording action potentials was composed of (in mmol/L) 130 K-glutamate, 9 KCl, 10 HEPES, 2 EGTA, and 5 MgATP (pH 7.2, adjusted with KOH). Action potentials were initiated by short depolarizing current pulses (2-3 ms, 500-800 pA) at 2 Hz. Ba²⁺ currents were recorded at 25°C in an external solution containing (in mmol/L) NaCl 140, CsCl 5, BaCl₂ 4, MgCl₂ 1.0, glucose 10, and HEPES 10 (pH 7.4, adjusted with NaOH). Borosilicate glass pipettes were pulled and fire-polished to final tip resistances of 1 to 3 MΩ when filled with internal recording solution. Uncompensated capacitance currents in response to small hyperpolarizing voltage steps were recorded for off-line integration to measure cell capacitance. Cells were allowed to equilibrate for at least 5 minutes after whole-cell access was obtained. A xenon arc lamp was used to view GFP fluorescence at 488/530 nm (excitation/emission). Transduced cells were recognized by their obvious green fluorescence.

The transmembrane action potential was recorded with glass microelectrodes filled with 2.7 mol/L KCl (pipette resistance, 10-20 MΩ) connected to a high-input impedance amplifier (MEZ-8300; Nihon Kohden).² The amplified signal was filtered with a 4-pole Bessel filter (NF-3625; NF Electronic Instruments, Tokyo, Japan) set at 2 kHz, then digitized with an A/D converter (PCI-MIO-16E4; National Instruments Japan, Tokyo, Japan) at a sampling frequency of 10 kHz and saved to a computer (Macintosh G3, Apple) using Igor Pro 4 and NIDAQ Tools software (Wavemetrics Inc, Lake Oswego, Oregon). The maximum diastolic potential, maximum positive deflection of the phase 0 upstroke, amplitude of the action potential, and action potential duration at 50% and 90% repolarization (APD₅₀ and APD₉₀) were measured.
Immunoprecipitation and immunoblotting

Forty-eight hours after transfection, HEK293 cells were washed with PBS and lysed with lysis buffer containing 10 mmol/L HEPES, 20 mmol/L Tris, 250 mmol/L NaCl, 2% Triton X-100, 0.5 mmol/L DTT, 10 mmol/L MgCl₂, 10 μmol/L GTPγS, and 1 μL/mL protease inhibitor mixture (pH 7.4). Cell lysates were centrifuged at 15 000 rpm for 30 minutes and 1 mg of supernatant protein was used for immunoprecipitation. Cell lysates were incubated with 20 μL protein A-agarose (719408; Roche) and 10 μg anti-Flag polyclonal antibody (F7425; Sigma-Aldrich), and rotated for 4 hours at 4°C. The cells were then pelleted and washed 3 times with 1 mL of lysis buffer. The pellet and 20 μg of input proteins were detected on a 4% to 12% Bis-Tris Gel (NP0321; Invitrogen), and then transferred to nitrocellulose membrane. Monoclonal anti-HA antibody (1:1000; H9658; Sigma-Aldrich) was used as the primary antibody and detected with anti-mouse IgG HRP-linked antibody (1:1000; #7076; Cell Signaling Technology). For the reverse immunoprecipitation, protein A-agarose and 10 μL of monoclonal anti-HA antibody were used for the immunoprecipitation, and rabbit anti-Flag antibody (1:500) and anti-Rabbit IgG HRP-linked antibody (1:2000; #7074; Cell Signaling Technology) were used for immunoblotting. Anti-mouse Myc monoclonal antibody (1:500; 631206; Clontech Laboratories) was used for Myc-Rad immunoblotting.

For native myocardial immunoprecipitation, mouse heart was homogenized with lysis buffer containing 10 mmol/L HEPES, 20 mmol/L Tris, 250 mmol/L NaCl, 0.5% Triton X-100, 0.5 mmol/L DTT, 10 mmol/L MgCl₂, 10 μmol/L GTPγS, and 1 μL/mL protease inhibitor mixture (pH 7.4). Proteins were centrifuged at 15 000 rpm for 30 minutes. Supernatant proteins (1 mg) were incubated with 20 μL protein A-agarose beads and 10 μg anti-L-type Ca²⁺ channel β₂ subunit polyclonal antibody (ARP34955; Aviva Systems Biology), and rotated for 8 hours at 4°C. The cells were then pelleted and washed 3 times with 1 mL of lysis buffer. The pellet and 20 μg of input proteins were detected by immunoblotting. Polyclonal
anti-Rad polyclonal antibody (1:1000; kindly provided by Dr. C. Ronald Kahn) was used as the primary antibody and detected with HRP-linked secondary antibody.

**GST pulldown assay**

A total of 500 mg of lysates from Cos7 cells expressing Cavβ2a was incubated with Glutathione Sepharose 4B (17-0756-01; GE Healthcare) for 2 hours at 4°C, for binding to GST, GST-WT Rad or GST-S105N Rad. The proteins were pelleted and washed 3 times with 1 mL of lysis buffer. The pellets and supernatants from these incubations were subjected to immunoblotting with the primary antibody (anti-Rad polyclonal antibody, anti-L-type Ca^{2+} channel beta-2 subunit polyclonal antibody, or 1:200 anti-GST monoclonal antibody [SCB138; Santa Cruz Biotechnology]) and detected with HRP-linked secondary antibody.

**Immunoblotting for the Cav1.2 Ca^{2+} channel protein**

The membrane protein fraction was collected by ultracentrifugation. Twenty micrograms of membrane protein fraction was incubated with NuPAGE LDS sample buffer (NP0007; Invitrogen) at room temperature for 2 hours. Protein samples were detected on a 3% to 8% Tris Acetate Gel (EA0378; Invitrogen) and transferred to nitrocellulose membrane. After blocking with 5% BSA for an hour, the nitrocellulose membrane was incubated at 4°C overnight with primary antibody (1:200 rabbit anti-cardiac Cav1.2 polyclonal antibody [ACC-013; Alamone Labs] or for mouse cadherin, 1:500 mouse anti-N-cadherin monoclonal antibody [333900; Zymed Laboratories]) and detected with HRP-linked secondary antibody.

**Immunofluorescence**

To assess protein expression and localization, HEK293 cells were fixed with 4% paraformaldehyde at 24 to 36 hours after transfection, then washed 3 times with PBS and
incubated for 30 minutes with PBS containing 10% BSA and 0.2% Triton X-100. The cells were then incubated with 1:500 monoclonal anti-HA antibody or 1:200 anti-Flag polyclonal antibody (as primary antibodies) overnight at 4°C, washed 3 times with PBS, then incubated with 1:500 Alexa594 goat anti-mouse IgG (A11012; Molecular Probes) and 1:500 Alexa488 or Alexa647 goat anti-rabbit IgG (A11029, A21244; Molecular Probes) as secondary antibodies for 1 hour at 37°C. For isolated guinea pig cardiomyocytes, cells were fixed with 4% paraformaldehyde, then washed 3 times with PBS and incubated for 1 hour with PBS containing 10% BSA and 0.5% Triton X-100. Cardiomyocytes were incubated with 1:20 anti-rabbit Ca\textsubscript{1.2} polyclonal antibody (ACC-013; Alamone Labs) and 1:40 monoclonal anti-vinculin antibody (V9131; Sigma-Aldrich) as the primary antibodies overnight at 4°C, washed 3 times with PBS, then incubated with secondary antibody overnight at 4°C. Specimens were visualized on a confocal microscope (LSM510 META; Carl Zeiss). Relative mean $\alpha_{1c}$ subunit fluorescence was analyzed using LSM 510 Version 3.0 software. Ten adjacent areas containing T-tubules were chosen, as were areas without T-tubules, and fluorescence intensities from those areas were averaged and used to calculate the ratio of mean fluorescence of areas with T-tubules to mean fluorescence of areas without T-tubules ($n = 10$).

**Telemetric electrocardiogram recordings**

Three-month-old mice were anesthetized (intraperitoneal pentobarbital, 40-50 mg/kg) and fitted with telemetric electrocardiogram (ECG) transmitters (Data Science, Minneapolis, Minn). The transmitter was inserted into the peritoneal sac, and the 2 ECG electrodes were placed subcutaneously near the right shoulder and left leg to approximate lead II of the Einthoven surface ECG. All analyses were performed on recordings that were obtained starting at least 48 hours after the implantation procedure. Full-disclosure 24 hour recordings
were analyzed off-line. The QT interval was corrected for heart rate using the following formula established for mice,\textsuperscript{5} with QT and RR expressed in ms: $\text{QTc} = \text{QT}/(\text{RR/100})^{1/2}$. 
References


## Supplemental Table 1

### ECG parameters in mice

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<tr>
<th></th>
<th>WT</th>
<th>S105N Rad TG</th>
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<tr>
<td>RR (ms)</td>
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<td>107.2 ± 5.4</td>
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<td>PR (ms)</td>
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<tr>
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<tr>
<td>QT (ms)</td>
<td>47.2 ± 4.6</td>
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<tr>
<td>QTc (ms)</td>
<td>47.3 ± 3.3</td>
<td>60.1 ± 2.7*</td>
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Data are expressed as mean ± SEM of 8 WT and 8 TG mice.

* $P < .05$ versus WT
Supplemental Table 2

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<th>WT (n = 8)</th>
<th>S105N Rad TG (n = 8)</th>
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<tr>
<td>SN dysfunction</td>
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<tr>
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<table>
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<tr>
<td>NSVT</td>
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SN, sinus node; AV, atrioventricular; NSVT, nonsustained ventricular tachycardia
Supplemental Figure 1

Figure 1. Echocardiography in mice.
Transthoracic echocardiography was carried out using a Sonos 1000 echocardiogram (Hewlett-Packard) equipped with a 10-MHz linear-array transducer. The heart was imaged in two-dimensional mode. M-mode scanning along the long axis view was examined, and the thickness of the interventricular septum and left ventricular free wall, left ventricular end-diastolic dimension, left ventricular end-systolic dimension, and fractional shortening were measured. There were no significant differences in echo parameters between WT and S105N Rad TG mice.

<table>
<thead>
<tr>
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<th>S105N Rad TG (n = 5)</th>
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<td>IVS (mm)</td>
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<td>PW (mm)</td>
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<tr>
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<td>%FS</td>
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<td>heart rate (bpm)</td>
<td>553 ± 7.6</td>
<td>538 ± 7.2</td>
<td>ns</td>
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IVS, interventricular septum; PW, posterior wall; LVDd, left ventricular end-diastolic dimension; LVDs, left ventricular end-systolic dimension; FS, fractional shortening; ns, not significant versus WT.