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COMMENTARY

# Rad protein: An essential player in L-type $Ca^{2+}$ channel localization and modulation in cardiomyocytes

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## Introduction

L-type $Ca^{2+}$ channels (LTCCs, cardiac-specific subtype $Ca_{v1.2}$) are the primary gateway for $Ca^{2+}$ entry ($I_{Ca,L}$) in cardiomyocytes and are essential for excitation–contraction (EC) coupling. In adult ventricular cardiomyocytes, $I_{Ca,L}$ predominantly localizes to transverse (t-) tubules where they lie in close apposition to junctional sarcoplasmic reticulum (SR). During EC coupling, $I_{Ca,L}$ triggers SR $Ca^{2+}$ release, which initiates contraction.

$Ca_{v1.2}$ is a key point of (dys)regulation in health and disease, for example, changes in $Ca_{v1.2}$ activity, sarcolemmal density, or subcellular organization have been shown to result in cardiac arrhythmias, heart failure and sudden cardiac death (Best and Kamp, 2012). $Ca_{v1.2}$ is a multi-subunit protein comprised of a pore-forming $\alpha_{1C}$ and regulatory $\beta_{2B}$ and $\alpha_{2\delta}$ subunits. During the fight-or-flight response, β-adrenergic signaling stimulates $I_{Ca,L}$. A long-standing view of the underlying mechanism has involved protein kinase A (PKA)-dependent phosphorylation of $Ca_{v1.2}$ $\alpha_{1c}$ and/or $\beta_{2B}$ subunits, however, studies carried out over the past two decades have challenged this view (compare reviews Kamp and Hell, 2000; Benitah et al., 2010; Colecraft, 2020). Indeed, amino acid truncation and/or substitution studies have suggested that the proposed regulatory residues on $\alpha_{1c}$ and $\beta_{2B}$ are dispensable for β-adrenergic activation of $I_{Ca,L}$.

Ras associated with diabetes (Rad) (Reynet and Kahn, 1993) belongs to the highly conserved RGK subfamily of small GTP-binding proteins (Rad, Rem, Rem2, and Gem/Kir, see Fig. 1, inset) that regulate LTCCs (Colecraft, 2020). Within this subfamily, Rad (Reynet and Kahn, 1993) and Rem (Finlin and Andres, 1997) are predominantly expressed in the heart. Although the Ras superfamily is typically associated with cell growth, proliferation, and adaptation (Wennerberg et al., 2005), Rad has emerged as a major LTCC modulator. It is suggested that Rad inhibits $I_{Ca,L}$ via an association with the $\beta_2$ auxiliary subunit (e.g., Finlin et al., 2003) and that during β-adrenergic signaling, this inhibition is reduced (e.g., (Liu et al, 2020). Clarification of how Rad regulates LTCC and cardiomyocyte function may become more important in the disease state, where $Ca^{2+}$ dysregulation is a critical issue.

## Challenges in studying multimeric channel regulation

Investigating the effects of endogenous Rad on LTCC is challenging. Early studies carried out in heterologous expression systems showed that Rad and other RGK subfamily members can suppress LTCC activity (Béguin et al, 2001; Finlin et al, 2003, 2006; Ward et al, 2004). From this work, it was initially proposed (Béguin et al, 2001) that the underlying mechanism could involve channel trafficking, however, later studies showed that Rad can modulate channel activity directly and that this interaction requires the presence of $\beta_{2B}$, at least in these models (e.g., Finlin et al., 2003). To overcome challenges in recapitulating native organization and stoichiometry within the LTCC multimeric complex in these expression models, Wang et al. (2010) used silencing RNA in cultured adult rat cardiomyocytes to demonstrate an inhibitory effect of endogenous Rad. While this enabled the investigation of endogenous Rad function, the method involved short-term cell culture which is associated with cardiomyocyte dedifferentiation. Nevertheless, subsequent development of a global (Manning et al., 2013), then cardiac-specific (Ahern et al, 2019) Rad knock-out mouse models have further supported a role for Rad in regulating $I_{Ca,L}$. In an earlier issue of *JGP*, Elmore et al. (2024) investigated the role of the Rad C-terminus by generating full-length and C-terminally truncated Rad knock-in mice. By attaching an N-terminal 3xFlag affinity tag, they could also visualize wild-type and truncated Rad protein distribution in isolated adult ventricular cardiomyocytes.

## Effect of Rad on $I_{Ca,L}$ and subcellular distribution

Studies in expression systems, cultured, and freshly isolated cardiomyocytes show that reduced Rad expression is associated

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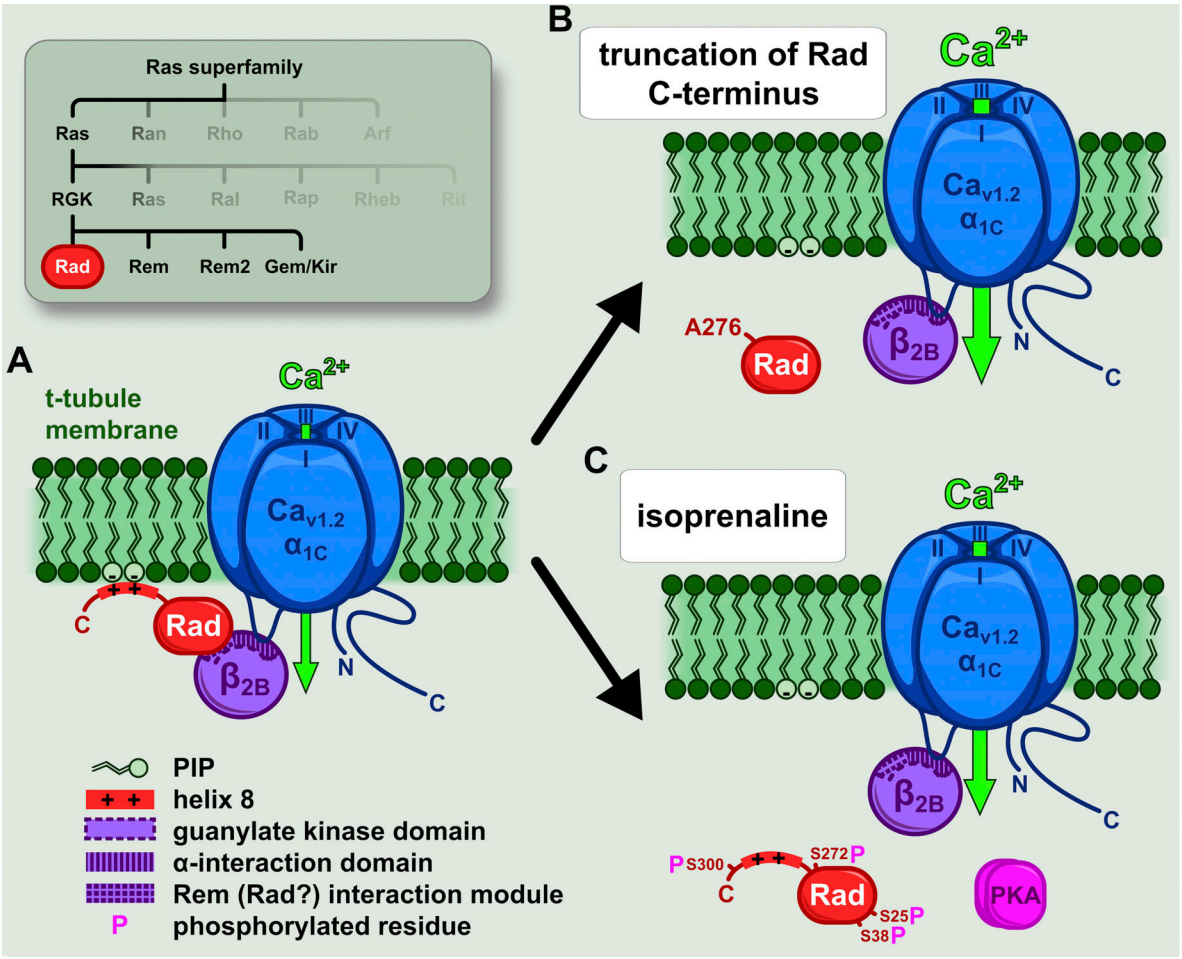


Figure 1. **Schematic of $Ca_{v1.2}$ $\alpha_{1C}$ and $\beta_{2B}$ subunits and proposed mechanism of Rad regulation. (A)** Basal $I_{Ca,L}$ is inhibited by Rad, an effect dependent on an interaction with $\beta_{2B}$ and association of Rad with the t-tubular membrane. The lipid interaction depends on helix 8, a polybasic region of the C-terminus. **(B)** Truncation of the Rad C-terminus by introducing a stop codon at Ala277 dissociates Rad from the t-tubule membrane and relieves $I_{Ca,L}$ inhibition. **(C)** Application of isoprenaline results in Rad phosphorylation by PKA, dissociation of a Rad fraction from the t-tubule membrane and relief of $I_{Ca,L}$ inhibition. The inset shows Rad in relation to other RGK subfamily, Ras family, and Ras superfamily members.

with increased $I_{Ca,L}$ density, leftward shift of the voltage-dependence of activation and little change to the steady-state voltage-dependent inactivation (Finlin et al, 2003; Wang et al, 2010; Manning et al, 2013; Ahern et al, 2019). A recent study by Liu et al. (2020) using proximity proteomics showed that Rad is located in close proximity to $\alpha_{1C}$ and $\beta_{2B}$ in ventricular cardiomyocytes. In the earlier issue, Elmore et al. (2024) used anti-FLAG immunocytochemistry to show a striated distribution of Rad that has a similar visual registration as that of $\alpha_{1C}$ labelling. Certainly, further investigation into Rad distribution and its role in $I_{Ca,L}$ regulation in cardiomyocytes from atria (such as in rat) and from larger mammals that have lower t-tubular density remains to be explored.

### Importance of C-terminus in Rad and other proteins in RGK subfamily

Though it has been shown that inhibition of $I_{Ca,L}$ by Rad requires $\beta_{2B}$ (e.g., Finlin et al., 2003), the details of this mechanism remain unclear. Studies with Rem have shown that its inhibition of $I_{Ca,L}$ depends on its C-terminus, which is (also) responsible for its localization to the plasma membrane and interaction with phosphatidylinositol (PIP) lipids (Correll et al., 2007; Xu et al., 2010). Elmore et al. (2024) tested this idea for Rad by introducing a stop codon at Ala277. They showed that truncated Rad abolished the inhibitory effect of full-length Rad on $I_{Ca,L}$ (Fig. 1), as well as its striated distribution. Elmore et al. (2024) go on to carry out further analysis using AlphaFold, which predicted a membrane interacting domain at helix 8 of Rad, which was deleted in truncated Rad. Elmore et al. (2024) observed that labelling of truncated Rad did not show a striated pattern and this may indicate that the deletion may have also disrupted the Rad–$\beta_{2B}$ interaction. If Rad links $Ca_{v1.2}$, PKA, and PIP, it might also interact with other scaffolding proteins including A kinase anchoring proteins (AKAPs), caveolin-3, and/or lipid rafts. Although previous studies have shown that PKA-dependent stimulation of $I_{Ca,L}$ is partly dependent on caveolin-3 (e.g., Bryant et al., 2018), fusion of a caveolin-3 binding domain to Rem did not alter basal $I_{Ca,L}$ or its response to PKA in adult feline cardiomyocytes (Correll et al., 2017). Nevertheless, this possible connection, alongside a possible involvement of AKAPs and the

cytoskeleton (e.g., Bilan et al., 1998; Ward et al., 2002) may also suggest a role for Rad in the maintenance of microdomains, t-tubules, or other structures.

### Involvement of other proteins and regulatory pathways

A key aspect to the interaction between Rad and $Ca_{v1.2}$ is that it provides the basis for the effects of PKA on $I_{Ca,L}$. Previous studies have shown that a reduction in Rad expression was associated with a reduction (Wang et al., 2010) or loss (Manning et al, 2013; Ahern et al, 2019) of isoprenaline's stimulatory effect on $I_{Ca,L}$. On the other hand, while overexpression of Rad reduced $I_{Ca,L}$, this effect was not relieved with isoprenaline (Wang et al., 2010; see also Xu et al., 2010 for Rem). More recently, Liu et al. (2020) showed that the close association of Rad with $\alpha_{1C}$ and $\beta_{2B}$ is reduced with isoprenaline treatment. They also showed that mutation of 35 proposed PKA phosphorylation sites on $\alpha_{1C}$ and 28 on $\beta_{2B}$ did not impair the isoprenaline effect, supporting previous studies that found phosphorylation of $Ca_{v1.2}$ to be unnecessary for PKA stimulation. Instead, the mechanism appears to require 4 serine residues on Rad (Papa et al, 2022). The data obtained by Elmore et al. (2024) lends further support to a role for endogenous Rad in PKA modulation of $I_{Ca,L}$. In the earlier issue, they showed that isoprenaline treatment was associated with reduced t-tubular 3xFLAG-Rad labelling (Fig. 1). Moreover, isoprenaline was not effective in modulating $I_{Ca,L}$ in cells from the truncated Rad mice. Thus, these studies suggest that phosphorylation sites on $Ca_{v1.2}$ may at best modulate the inhibitory effect of Rad though any role remains to be clarified.

Alterations in Rad expression have also been associated with other changes in cellular $Ca^{2+}$ handling. Several studies have reported that reduced Rad expression is associated with larger $Ca^{2+}$ transients (Wang et al, 2010; Manning et al, 2013; Ahern et al, 2019), without an effect on the $Na^+$–$Ca^{2+}$ exchanger (Wang et al., 2010). An effect on SR $Ca^{2+}$ content (Wang et al., 2010; Manning et al., 2013 versus Ahern et al, 2019), or diastolic $Ca^{2+}$ movements (Wang et al., 2010 versus Manning et al., 2013) remain to be clarified. While it is unclear whether Rad has other targets, heart tissue from the global Rad knock-out (KO) mouse showed increased phosphorylation of both calmodulin-dependent kinase 2 and phospholamban (Manning et al., 2013). Moreover, the loss of the frequency-dependence of diastolic and systolic $Ca^{2+}$ levels associated with Rad KO was recovered with KN-93 treatment (Manning et al., 2013). While these changes have not been reported in cardiac-specific KO or Rad truncation mouse models, they highlight the possibility that Rad could be a point of regulatory crosstalk and local organization. Indeed, RGK proteins contain interaction domains for calmodulin and 14-3-3 proteins (Kelly, 2005).

### Therapeutic perspectives of Rad–$Ca_{v1.2}$ interaction

As Elmore et al. (2024) discuss, the Rad–$\beta_{2B}$ interaction is a potential therapeutic target. Chang et al. (2007) reported a reduction in Rad expression in human heart failure, while a single nucleotide polymorphism has been identified in the Rad gene in patients with congestive heart failure (Lynch et al., 2002). Elmore et al. (2024) showed that truncation of the Rad C-terminus increased left ventricular ejection fraction and abolished a β-adrenergic response. Meanwhile, Manning et al. (2013) observed increased spontaneous $Ca^{2+}$ and electrical activity in cardiomyocytes from the global Rad KO mouse. Thus, fine tuning of $I_{Ca,L}$ remains an important challenge to maintain contractile capacity whilst avoiding arrhythmogenic behavior and $Ca^{2+}$-dependent cytotoxicity.

Elmore et al. (2024) suggest that a possible route to targeting the Rad–$\beta_{2B}$ interaction could be PIP. Although therapeutic PIP has some precedence (Burg et al., 2022; Murata et al., 2004), PIP is involved in many cellular processes including t-tubule formation, membrane trafficking and maintaining electrostatic effects. Further work clarifying how PIP interacts with Rad or RGK proteins will be useful in guiding these strategies, as well as exploring novel approaches to tune Rad proteins (Xie et al., 2023). Similarly, LTCC subpopulations (Best and Kamp, 2012) should be considered in this aspect.

### Conclusion

In summary, the intricate interplay between Rad and $Ca_{v1.2}$ underscores the complexity of cellular signaling mechanisms and the need for meticulous experimental design to gain more insights into the underlying molecular mechanisms. With the elegant use of transgenic mouse models, Elmore et al. (2024) provide new insights into Rad-dependent LTCC modulation. The importance of Rad structure in LTCC modulation adds novelty in the growing field of designing new therapeutic targets.

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