Rem GTPase interacts with the proximal Ca\textsubscript{v}1.2 C-terminus and modulates calcium-dependent channel inactivation

Chunyan Pang, Shawn M. Crump, Ling Jin, Robert N. Correll, Brian S. Finlin, Jonathan Satin and Douglas A. Andres\textsuperscript{*}

Department of Molecular and Cellular Biochemistry and Physiology; University of Kentucky College of Medicine; Lexington, KY USA

Key words: RGK GTPases, Ras, calcium channel, Ca\textsubscript{v}1.2, Rem, Rem2, Rad, Gem, calmodulin, calcium-dependent inactivation

Abbreviations: RGK, Rem, Rem2, Rad and Gem/Kir GTPase; CCT, Ca\textsubscript{v}1.2 C-terminus; VDCC, voltage-dependent calcium channel; Ca\textsubscript{v}\alpha, VDCC alpha subunit; Ca\textsubscript{v}\beta, VDCC beta subunit; HVA, high voltage-activated; AID, Ca\textsubscript{v}\alpha, loop I-II interaction domain; CDI, calcium-dependent inactivation; VDI, voltage-dependent inactivation; PIP, phosphorylated phosphatidylinositide lipids; GST, glutathione-S-transferase; GFP, green fluorescent protein; HA, hemagglutinin; PKA, protein kinase A; PKC, protein kinase C; CaM, calmodulin; CaMKII, calmodulin kinase II; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A

The Rem, Rem2, Rad and Gem/Kir (RGK) GTPases, comprise a subfamily of small Ras-related GTP-binding proteins, and have been shown to potentiate high voltage-activated Ca\textsuperscript{2+} channel current following overexpression. Although the molecular mechanisms underlying RGK-mediated Ca\textsuperscript{2+} channel regulation remain controversial, recent studies suggest that RGK proteins inhibit Ca\textsuperscript{2+} channel currents at the plasma membrane in part by interactions with accessory channel \beta subunits. In this paper, we extend our understanding of the molecular determinants required for RGK-mediated channel regulation by demonstrating a direct interaction between Rem and the proximal C-terminus of Ca\textsubscript{v}1.2 (PCT), including the CB/IQ domain known to contribute to Ca\textsuperscript{2+}/calmodulin (CaM)-mediated channel regulation. The Rem2 and Rad GTPases display similar patterns of PCT binding, suggesting that the Ca\textsubscript{v}1.2 C-terminus represents a common binding partner for all RGK proteins. In vitro Rem:PCT binding is disrupted by Ca\textsuperscript{2+}/CaM, and this effect is not due to Ca\textsuperscript{2+}/CaM binding to the Rem C-terminus. In addition, co-overexpression of CaM partially relieves Rem-mediated L-type Ca\textsuperscript{2+} channel inhibition and slows the kinetics of Ca\textsuperscript{2+}-dependent channel inactivation. Taken together, these results suggest that the association of Rem with the PCT represents a crucial molecular determinant in RGK-mediated Ca\textsuperscript{2+} channel regulation and that the physiological function of the RGK GTPases must be re-evaluated. Rather than serving as endogenous inhibitors of Ca\textsuperscript{2+} channel activity, these studies indicate that RGK proteins may play a more nuanced role, regulating Ca\textsuperscript{2+} currents via modulation of Ca\textsuperscript{2+}/CaM-mediated channel inactivation kinetics.

Introduction

High voltage-activated (HVA) Ca\textsuperscript{2+} channels allow Ca\textsuperscript{2+} influx upon membrane depolarization to regulate a wide variety of cellular functions including excitation-contraction coupling in muscle cells, neurotransmitter release and Ca\textsuperscript{2+} transients in neurons, and hormone secretion in endocrine cells.\textsuperscript{1} L-type Ca\textsuperscript{2+} channels minimally consist of a pore-forming \alpha\textsubscript{1} subunit, an intracellular \beta subunit, and a transmembrane complex \alpha/\delta,\textsuperscript{1} with each of the subunits contributing to the modulation of the kinetics and voltage-dependence of channel gating properties.\textsuperscript{1,2} Intracellular domains of the pore-forming \alpha\textsubscript{1} subunit serve as scaffolds for the targeting and localization of a diverse array of regulatory molecules. A conserved \alpha\textsubscript{1} interaction domain (AID) located between the first and second repeats of the \alpha\textsubscript{1} subunit (loop I-II), serves as a high affinity binding site for all Ca\textsuperscript{2+} channel \beta subunits (Ca\textsubscript{v}\beta).\textsuperscript{3} AID:Ca\textsubscript{v}\beta association promotes trafficking of channel complex to the plasma membrane and results in increased current densities.\textsuperscript{4,6} The Ca\textsubscript{v}1.2 C-terminus (CCT) serves as another major site of modulation. The L-type channel CCT (residues 1507–2171 for rabbit Ca\textsubscript{v}1.2) is targeted by a variety of regulatory pathways, including PKA, PKC, CaM, CaMKII and protein phosphatases PP1 and PP2A.\textsuperscript{1,3,8} A conserved CB/IQ domain and regions proximal to the CB/IQ have been reported to contribute to channel auto-regulation, including Ca\textsuperscript{2+}-dependent inactivation (CDI) through Ca\textsuperscript{2+}-regulated interactions with calmodulin (CaM).\textsuperscript{9} Ca\textsuperscript{2+} free CaM (ApoCaM) appears to be constitutively tethered to the Ca\textsubscript{v}1.2 C-terminus,\textsuperscript{10,11} and a recent study by Pitt and colleagues suggests that Ca\textsuperscript{2+}-bound CaM elicits a conformational change within the Ca\textsubscript{v}1.2 C-terminus which promotes binding to the I-II intracellular linker, resulting in channel inactivation.\textsuperscript{13} In skeletal and cardiac muscle cells, the distal C-terminus of L-type calcium channels is subject to proteolytic cleavage,\textsuperscript{14,16} and the released distal C-terminal
RGK proteins associate with the CaV1.2 channel complex through Caβ subunit binding.28,41,43 Here, we expand upon the potential molecular mechanisms involved in RGK-mediated Ca2+ channel regulation by identifying a direct interaction between Rem and the proximal C-terminus of CaV1.2 (CCT) and provide evidence supporting a role for CCT association in Rem-dependent Ca2+ channel regulation.

Results

Rem interacts with the L-type, but not T-type CaVα,–CCT in vitro. Rem inhibits I\text{\textsubscript{Ca,L}} in part through association with auxiliary Caβ subunits,28,30,41,43 but the contribution of the channel α\textsubscript{1}-subunit to Rem regulation remains poorly characterized.26 To explore whether Rem interacts with the CaV1.2 C-terminus, tsA201 cells were transiently co-transfected with expression vectors encoding 3xFlag-tagged Rem and either pCDNA3.1 + 3xHA-α empty vector), HA-CCT-FL or the indicated HA-tagged CCT truncation mutants. 48 h post-transfection, cells were harvested, and cell lysate (0.5 mg) was subjected to immunoprecipitation with anti-HA antibody as described under “Materials and Methods”. The entire bound fraction or a portion of the unbound fraction (2.5 μl) was analyzed by immunoblotting with biotin-Flag to detect Rem. (C) Cell lysate (5 μg) was immunoblotted with anti-HA antibody to monitor expression of CCT-FL and the corresponding truncation mutants used in (B). (D) TsA201 cells were transiently co-transfected with vectors expressing Flag-Rem and either pCDNA3.1 + 3xHAα, HA-CaV1.2 CCT or HA-CaV3.2 CCT. Co-immunoprecipitation was performed using HA antibody as described in (B) and Rem binding examined by biotin-Flag immunoblotting. Results in each panel are representative of three independent experiments.

Figure 1. Rem interacts with the proximal and distal domains of CCT. (A) Schematic of the CCT truncation mutants with the Rem interaction status indicated on the right. (B) TsA201 cells were transiently co-transfected with expression vectors encoding 3xFlag-tagged Rem and either pCDNA3.1 + 3xHAα empty vector), HA-CCT-FL or the indicated HA-tagged CCT truncation mutants. 48 h post-transfection, cells were harvested, and cell lysate (0.5 mg) was subjected to immunoprecipitation with anti-HA antibody as described under "Materials and Methods". The entire bound fraction or a portion of the unbound fraction (2.5 μl) was analyzed by immunoblotting with biotin-Flag to detect Rem. (C) Cell lysate (5 μg) was immunoblotted with anti-HA antibody to monitor expression of CCT-FL and the corresponding truncation mutants used in (B). (D) TsA201 cells were transiently co-transfected with vectors expressing Flag-Rem and either pCDNA3.1 + 3xHAα, HA-CaV1.2 CCT or HA-CaV3.2 CCT. Co-immunoprecipitation was performed using HA antibody as described in (B) and Rem binding examined by biotin-Flag immunoblotting. Results in each panel are representative of three independent experiments.
indicate that Rem interacts with both the proximal and distal portions of the L-type CCT, but not with the T-type CCT.

**In vitro association of RGK proteins with the proximal CCT.** To further characterize the nature of the Rem-CCT association, we next used ^35^S-labeled, in vitro translated CCT fragments and recombinant glutathione-S-transferase (GST) fused Rem (GST-Rem) and GST-Rem-(1-265) to explore the Rem-CCT interaction. The generation of ^35^S-labeled CCT-FL or the other three CCT truncation mutants is shown in Figure 2A. GST-Rem directly bound ^35^S-labeled PCT (Fig. 2B, top), but failed to associate with MCT (Fig. 2B, middle). Interestingly, recombinant Rem also failed to interact with DCT (Fig. 2B and bottom) suggesting that Rem binding with the distal CCT may require additional cellular binding factors. Surprisingly, the conserved RGK C-terminus appears to contribute to CCT binding, since GST-Rem-(1-265) failed to associate with either CCT-FL or PCT (Fig. 2C). In addition, while previous work has suggested that RGK-mediated Ca\(^{2+}\) channel regulation may be GTP-dependent, both GDP-bound and GTPγS-bound Rem displaying equivalent in vitro PCT binding (Fig. 2B, top).

If CCT association is required for RGK-mediated channel regulation, we reasoned that CCT binding should be a common property for all RGK proteins. Importantly, when expressed in tsA201 cells, both Rem2 and Rad GTPases co-immunoprecipitate with the proximal and distal, but not the medial domain, of CCT (Fig. 3A and B). Thus, the ability to bind CCT is a conserved property for RGK family proteins and suggests that CCT association may contribute to RGK-mediated channel regulation.

Rem-mediated \(I_{\text{Ca,L}}\) inhibition does not require the distal CCT. To explore the contribution of the distal Ca\(_{1,2}\) C-terminus to Rem-dependent regulation of Ca\(^{2+}\) channel current, we examined the modulation of Ca\(_{1,2}\) (1-1905), a deletion mutant lacking the final 265 residues of the C-terminus. Co-expression of Ca\(_{1,2}\) (1-1905) with Ca\(_{1,2}\)β\(_{ih}\) + GFP in tsA201 cells resulted in appreciable whole cell currents (12.0 ± 4.4 pA/pF, filled squares, \(n = 4\)) (Fig. 3C). Importantly, expression of wild-type GFRP-Rem resulted in a potent reduction in ionic current expression (0.3 ± 0.3 pA/pF, open circles, \(n = 5\)), which is similar to the inhibition observed with full-length Ca\(_{1,2}\) + Ca\(_{1,2}\)β\(_{ih}\) (see Fig. 4D). Similar results were obtained using Ca\(_{1,2}\) (1-1905) + Ca\(_{1,2}\)β\(_{ih}\) (data not shown), suggesting that the C-terminus of Ca\(_{1,2}\) distal to position 1905 does not contribute to Rem-mediated regulation of Ca\(^{2+}\) channel current.

**Plasma membrane targeting contributes to Rem:CCT association.** The RGK C-terminus appears to play important roles in both plasma membrane targeting and Ca\(^{2+}\) channel regulation. To examine the importance of this domain in CCT binding, we analyzed the interaction of wild-type Rem and Rem-(1-265) with PCT using co-immunoprecipitation from tsA201 cell lysates. The Rem-(1-265) truncation mutant lacks the conserved RGK C-terminus, is not localized at the plasma membrane, and does not alter Ca\(^{2+}\) channel current expression. Consistent with the binding studies in Figure 2C, Rem-(1-265) failed to associate with HA-PCT, whereas full-length Rem displayed PCT binding (Fig. 4A). We next investigated whether the Rem C-terminus directly contributed to CCT binding. For these studies, we made use of two chimeric proteins in which the C-terminus of K-Ras4B and H-Ras were fused to Rem-(1-265). Addition of either plasma membrane trafficking motif has been found to restore both membrane localization and Ca\(^{2+}\) channel regulation, and co-immunoprecipitation analysis found that both chimeric proteins are capable of PCT binding (Fig. 4A). Therefore, Rem:CCT association is correlated with both Rem-mediated Ca\(^{2+}\) channel regulation and plasma membrane localization. As an added specificity control, Rem-(1-265)-HSAAX (mutation of the CAAX motif cysteine to serine) was generated. Mutation of the CAAX motif cysteine disrupts protein farnesylation and confocal microscopy confirms that Rem-(1-265)-HSAAX is not localized to the plasma membrane (Suppl. Fig. S1). Co-immunoprecipitation analysis found that Rem-(1-265)-HSAAX does not associate with CCT-FL (Fig. 4B) but retains Ca\(_{1,2}\)β\(_{ih}\) subunit binding (Fig. 4C). While GFP-Rem-(1-265)-HCAAX expression resulted in a potent reduction in ionic current expression (-1.1 ± 0.3 pA/pF, closed triangles, \(n = 8\)) when co-expressed with Ca\(_{1,2}\) + Ca\(_{1,2}\)β\(_{ih}\) in tsA201 cells (Fig. 4D), GFP-Rem-(1-265)-HSAAX did not significantly alter either current density or voltage-dependent channel gating properties (Fig. 4D). Whole cell currents elicited in the presence of Ca\(_{1,2}\) + Ca\(_{1,2}\)β\(_{ih}\) + GFP-Rem-(1-265)-HSAAX (-17.7 ± 3.9 pA/pF, filled squares, \(n = 9\)) were indistinguishable from control currents in cells expressing Ca\(_{1,2}\) + Ca\(_{1,2}\)β\(_{ih}\) + GFP (-18.5 ± 4.7 pA/pF, open circles, \(n = 10\)) (Fig. 4D). As a final specificity control, co-immunoprecipitation analysis found that the isolated Rem C-terminus (residues 266–297) does not directly associate with PCT (Fig. 4E). Taken together, these data suggest that plasma membrane localization, but not sequences...
within the C-terminus of Rem, contribute to Rem:CCT association and suggest that CCT binding is necessary for effective Rem-mediated channel regulation.

Ca\textsuperscript{2+}-calmodulin inhibits Rem:CCT binding. Since calmodulin (CaM) is known to interact with the Ca\textsubscript{1} C-terminus in the same region required for Rem association\(^7\) (Figs. 1B and 2B), and also associates with the C-terminus of Rem,\(^2,8,35,50\) we next examined whether CaM binding might regulate Rem:CCT association. Compared to GST-Rem:CCT-FL binding in the presence of EGTA, CCT-FL binding was modestly decreased by the addition of 2 mM Ca\textsuperscript{2+} (Fig. 5A, top, compare lanes 2 and 4). However, while the addition of CaM to the in vitro binding reaction had no obvious effect on Rem:CCT-FL association in the presence of EGTA, the interaction between Rem and CCT-FL was almost completely inhibited upon the addition of Ca\textsuperscript{2+}/CaM (Fig. 5A, top, compare lanes 6 and 8). Similar results were seen using recombinant GST-Rem and PCT (Fig. 5A and bottom). Addition of high concentrations of Ca\textsuperscript{2+} also disrupted Rem:PCT association in TsA201 cell lysates. As seen in Figure 5B, Rem displayed PCT binding in lysis buffer containing EGTA but Rem/PCT association was abolished in the presence of excess Ca\textsuperscript{2+} (Fig. 5B, top, compare lanes 1 and 2). Transfected cells expressed similar levels of Rem (Fig. 5B and bottom) and PCT (Fig. 5B, middle), indicating that the loss of binding was not the result of Ca\textsuperscript{2+}-dependent proteolysis or a transfection artifact. The addition of exogenous CaM did not further alter the relative binding affinity between Rem and PCT either in the presence of Ca\textsuperscript{2+} or EGTA (data not shown). Taken together, these data suggest that Ca\textsuperscript{2+}/CaM acts to inhibit Rem and PCT association.

Ca\textsuperscript{2+}/CaM-mediated inhibition of Rem:PCT association is not due to CaM binding to the Rem C-terminus. To determine whether CaM binding to Rem disrupts Rem:CCT association, we examined the effect of CaM on PCT binding to Rem-(1-265)-KCAAX and Rem-(1-265)-HCAAX.\(^28\) Whereas the K-Ras4B-CAAX motif shares several properties with the Rem C-terminus, including a polybasic domain capable of PIP lipid-mediated plasma membrane targeting and CaM association,\(^27,51,52\) the H-Ras-CAAX domain contains a prenylation/palmitoylation membrane localization signal and does not bind CaM.\(^3\) CaM sepharose pulldown assays were used to confirm these properties. As expected, only WT-Rem and Rem-(1-265) -HCAAX bound PCT (Fig. 5C), indicating that the loss of binding was not the result of Ca\textsuperscript{2+}-dependent proteolysis or a transfection artifact. The addition of exogenous CaM did not further alter the relative binding affinity between Rem and PCT either in the presence of Ca\textsuperscript{2+} or EGTA (data not shown). Taken together, these data suggest that Ca\textsuperscript{2+}/CaM acts to inhibit Rem and PCT association.

Overexpression of CaM partially blocks Rem-mediated I\textsubscript{Ca,L} inhibition. Since association of Rem with the Ca\textsubscript{1} C-terminus appears to play a role in the blockade of I\textsubscript{Ca,L} (Fig. 4D) and Ca\textsuperscript{2+}/CaM inhibits in vitro Rem:PCT association (Fig. 5A and B), we reasoned that CaM may serve to modulate Rem-mediated Ca\textsuperscript{2+} channel blockade. Peak currents elicited from tsA201 cells expressing Ca\textsubscript{1,2} + Flag-β\textsubscript{II} + GFP + HA-CaM (Fig. 6A and filled squares, -18.6 ± 6.1 pA/pF, n = 11) using 30 mM Ba\textsuperscript{2+} as the charge carrier were not significantly different from those obtained in the absence of co-expressed CaM [Ca\textsubscript{1,2} + Flag-β\textsubscript{II} + GFP + pKH3] (Fig. 6A, open diamonds, -12.3 ± 2.4 pA/pF, n = 11). As expected, co-expression of GFP-Rem was capable of generating complete current blockade in the presence of 30 mM Ba\textsuperscript{2+} (Fig. 6A, open
Figure 4. For figure legend, see page 6.
Figure 4. Plasma membrane targeting is necessary for Rem:CCT association. (A) TsA201 cells were transiently co-transfected with the indicated Rem and CCT expression vectors. Co-immunoprecipitation was performed with anti-HA antibody and interaction with Rem examined by immunoblotting with biotinylated FLAG antibody. Results are representative of four independent experiments. (B) TsA201 cells were transiently co-transfected with the indicated plasmids and co-immunoprecipitation performed with HA antibody as described in (A). Results are representative of three independent experiments. (C) TsA201 cells were transfected with the indicated plasmids. Co-immunoprecipitation was performed with Flag antibody and interaction with Rem proteins examined by immunoblotting with biotinylated anti-HA antibody. Immunoprecipitates were blotted for β1 subunit using biotinylated Flag antibody. Results are representative of three independent experiments. (D) TsA201 cells were transfected with plasmids expressing CaV1.2, Rem-(1-265)-aX, GFp-Rem-(1-265)-aX, or unfused GFP as control. Current was examined using the whole-cell patch clamp configuration in the presence of 30 mM Ba2+. (E) TsA201 cells were transiently co-transfected with GST alone, GST-Rem, or GST-Rem(266-297) and PCT. GST fusion proteins were isolated using glutathione-Sepharose resin and interaction with PCT examined by immunoblotting. Results are representative of three independent experiments.

Figure 5. Ca2+/CaM inhibits in vitro Rem:CCT binding. (A) Recombinant GST or GST-Rem proteins bound to glutathione-Sepharose beads were preloaded with GDP, incubated for 3 h at 4°C with 35S-labeled CCT-FL (top) or PCT (bottom) in the presence of 2 mM Ca2+ or 5 mM EGTA with or without CaM (2 μg). The glutathione-Sepharose beads were then pelleted and washed as described under “Materials and Methods”. Bound proteins were subjected to SDS-PAGE, and the dried gel was exposed to film for 16–72 h. Results are representative of four independent experiments. (B) TsA201 cells were transiently co-transfected with expression vectors encoding HA-PCT and Flag-Rem, Flag-Rem-(1-265)-KCAAX, or Flag-Rem-(1-265)-HCAAX. The ability of PCT to interact with either Rem or the indicated Rem chimeric proteins was determined by co-immunoprecipitation analysis as described under “Materials and Methods”. Results are representative of three independent experiments. (C) TsA201 cells were transfected with plasmids expressing 3xFlag-Rem, 3xFlag-Rem-(1-265), 3xFlag-Rem-(1-265)-KCAAX, 3xFlag-Rem-(1-265)-HCAAX or empty p3xFlag-CMV10 as control. Cell lysates (1 mg) were pulsed down using CaM-Sepharose beads in the presence of 2 mM CaCl2, bound proteins were released with two washes with assay buffer (containing 5 mM EGTA), and the ability to associate with CaM was examined by immunoblotting with anti-Flag antibody. Results are representative of three independent experiments.
+ GFP + HA-CaM (Fig. 6B and filled squares, -6.9 ± 1.6 pA/pF, n = 20 and D) was not statistically different from those seen following expression of CaV1.2 + Flag-β2a + GFP + pKH3 (Fig. 6B, open diamonds, -6.3 ± 1.9 pA/pF, n = 11, and D). As expected, Rem expression resulted in a complete blockade of current expression in this system (Fig. 6B, open circles, -0.1 ± 0.2 pA/pF, n = 8, and D). However, co-expression of Rem and CaM resulted in >3 pA/pF peak inward current (Fig. 6B and filled triangles, -3.0 ± 0.7 pA/pF, n = 16, and D), which was significantly different from the average current density elicited from the CaV1.2 + Flag-β2a + GFP-Rem + pKH3 transfection (Fig. 6D) (p < 0.01). CaM co-expression resulted in a partial restoration of ICa,L (43% of the peak current elicited in 30 mM Ca2+ in the absence of Rem) (Fig. 6B and D). Immunoblotting of whole cell lysates indicate that overexpression of CaM does not alter the expression of Rem or channel subunits (Suppl. Fig. S2). Together these data indicate that CaM overexpression partially antagonizes Rem-mediated Ca2+ channel current inhibition.

Rem alters the kinetics of calcium-dependent channel inactivation. It is well established that CaM bound to the CaV1 C-terminus plays a critical role in the generation of calcium dependent inactivation.9 Because Rem interacts with PCT and overexpression of CaM partially relieves Rem-mediated L-type Ca2+ channel inhibition (Fig. 6A and B), we next asked whether Rem alters the kinetics of voltage-dependent inactivation (VDI) or calcium-dependent inactivation (CDI). Figure 7A shows the superimposed representative time course of the Ipeak generated during 300 ms test pulses to +5 mV from Vh = -80 mV (holding potential) from the indicated transfection experiments; all of the current traces were normalized in order to facilitate the kinetics comparison. The Ba2+-current restored by CaM overexpression had a partial restoration of Ipeak (43% of the peak current elicited in 30 mM Ca2+ in the absence of Rem) (Fig. 6B and D). Immunoblotting of whole cell lysates indicate that overexpression of CaM does not alter the expression of Rem or channel subunits (Suppl. Fig. S2). Together these data indicate that CaM overexpression partially antagonizes Rem-mediated Ca2+ channel current inhibition.

Figure 6. Calmodulin expression blunts Rem-mediated Ca2+ channel inhibition. (A) TSA201 cells were transfected with the indicated plasmids, and the current was examined in the presence of 30 mM Ba2+ using the whole-cell patch clamp configuration. (B) TSA201 cells were transfected as in (A), and current was examined in the presence of 30 mM Ca2+ using the whole-cell patch clamp configuration. (C) Current density at +5 mV from (A). (D) Current density at +20 mV from (B). A significant difference (p < 0.01, Kruskal-Wallis test followed by a post hoc Dunn’s test) between treatments is denoted by asterisks.
the peak current elicited in the absence of Rem. Given that CDI is dependent on Ca$^{2+}$-entry we were concerned that the apparent loss of CDI with Rem $+$ CaM co-expression simply was secondary to a decreased absolute degree of Ca$^{2+}$-entry. To evaluate this possibility, we plotted the macroscopic decay time constant $\tau_{\text{fast}}$ against the peak $I_{Ca,L}$ density measured at $+20$ mV from individual cells in the presence or absence of Rem. Macroscopic $I_{Ca,L}$ decay was slower for Rem $+$ CaM compared to CaM overexpression even in fortuitous instances of similar $I_{Ca,L}$ density (Suppl. Fig. S3). These data suggest that Rem significantly slows the kinetics of CDI.

**Discussion**

All members of RGK GTPases have been found to act as potent inhibitors of high voltage-activated (HVA) Ca$^{2+}$ channel function when overexpressed.\(^{26,30,31,33-35,39-44}\) While binding to accessory Ca$\beta$ subunits contributes to RGK-mediated HVA Ca$^{2+}$-channel regulation,\(^{10,40-44}\) this interaction alone is not sufficient for Rem-dependent current blockade.\(^{28,54}\) In the present study, we provide new insight into the mechanisms of RGK-mediated Ca$^{2+}$ channel regulation through the identification of an interaction between the Ca$_{1.2}$-CCT and RGK GTPases and find that Rem:CCT association appears to contribute to Rem-dependent regulation of L-type Ca$^{2+}$ channel activity. Ca$^{2+}$/CaM is able to disrupt Rem:CCT binding in vitro (Fig. 5A) and overexpression of CaM partially reverses Rem-mediated VDCC inhibition (Fig. 6). Finally, the Rem:CCT interaction site is implicated in CDI because Rem-CaM co-expression was found to significantly alter the kinetics of CDI (Fig. 7B and D). Based upon these findings we propose that RGK proteins can associate with Ca$\beta$ and CCT, and that these interactions promote reorganization of Ca$_{\alpha,\beta}$ intracellular domains to generate the blockade of current expression which is the hallmark of RGK-dependent Ca$^{2+}$-channel regulation upon overexpression.\(^{26,30,33-35,39-44}\)

Our studies also pointed out the possibility that Rem may function as an endogenous regulator for Ca$^{2+}$/CaM-mediated channel inactivation.

The L-type Ca$^{2+}$-channel exists in most cells as a multi-protein complex. Although heterologous expression studies have led to great insight into Ca$^{2+}$-channel function, overexpression studies increase selected components of the channel complex and must be cautiously interpreted. For example, the bulk of RGK-Ca$^{2+}$-channel studies never overexpress CaM, yet CaM is known to be an integral component of the L-type channel complex\(^{10,12}\) and even without CaM overexpression, CDI occurs. The observation of CDI led us and others to conclude that endogenous (in this case tsA201 cell) CaM is sufficiently abundant to modulate $I_{Ca,L}$. However, this assumes that the overexpressed protein does not compete with CaM for access to the Ca$^{2+}$-channel complex. A second reason we previously overlooked CaM co-expression was that based on western blotting CaM abundance was relatively high in tsA201 cells. This overlooks the important finding that CaM has numerous interacting proteins in a cell. Taken together, our data now suggest that RGK proteins via interactions with Ca$_{1.2}$ proximal C-terminus effectively compete with CaM. Although, CaM is not limiting for $I_{Ca,L}$ carried by Ca$_{1.2}$ (+Ca$\beta_h$) expression, the presence of excess Rem reveals the limiting nature of endogenous CaM. This new interpretation logically leads to a provocative suggestion—that a function of RGK proteins is to not only govern $I_{Ca,L}$, but also to influence CaM-channel regulation.
Our studies indicate that Rem overexpression only partially inhibits Ca\textsuperscript{2+} current amplitude in the presence of overexpressed CaM (Fig. 6A and B), and that co-expression of CaM and Rem slows the inactivation kinetics of CDI (Fig. 7B and D). Altering CDI has a strong effect on action potential duration in cardiac myocytes.\textsuperscript{5} These data suggest that RGK proteins may serve to increase Ca\textsuperscript{2+} flux under physiological conditions. Moreover, the complete channel blockade observed in earlier studies may be an artifact of in vitro overexpression resulting from a cellular imbalance between Rem and CaM. These results also address a vexing issue in RGK signaling—namely if RGK proteins potentially inhibit Ca\textsuperscript{2+} channel function, and endogenous RGK proteins are expressed in excitable cells, why are L-type Ca\textsuperscript{2+} currents maintained? These data indicate that rather than serving as endogenous inhibitors of Ca\textsuperscript{2+} channel activity, RGK proteins may play a more nuanced role, regulating Ca\textsuperscript{2+} currents via modulation of Ca\textsuperscript{2+}/CaM-mediated channel inactivation kinetics. It has been reported that levels of Rad mRNA and protein are decreased significantly in failing human hearts and that Rad expression has been reported to serve as docking sites for both apocammodulin and Ca\textsuperscript{2+}/CaM.\textsuperscript{14} Our data indicate that Rem and CaM could compete for interaction—namely if RGK proteins potentiate association and Rem:CaM association which appears capable of modulating Ca\textsuperscript{2+}/CaM-mediated CDI kinetics. Additional studies will be needed to identify the cellular signaling pathways that control Rem-mediated Ca\textsuperscript{2+} channel activity and to characterize the interplay between Rem:CCT and Rem:Ca\textsubscript{1.2} binding in Ca\textsuperscript{2+} channel regulation.

Materials and Methods

Protein expression. Mammalian expression vectors for Ca\textsubscript{1.2}, Flag-\(\beta\), and Flag-\(\beta\)-subunits have been described previously.\textsuperscript{10} GFP-Ca\textsubscript{1.2} (rabbit) was kindly provided by K. Beam (University of Colorado Health and Sciences Center, Aurora, CO). Truncation mutants for Ca\textsubscript{1.2} were generated using the Quickchange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA), while Rem(266-297) was generated by PCR. Rem containing a CAAX box have been described previously, and the SAAX mutants produced by mutagenesis.\textsuperscript{28} Full length and truncated Ca\textsubscript{1.2}-CCT cDNAs were subcloned into pCite4 (Novagen) for production of in vitro translated proteins. pKH3-CaM was kindly provided by Dr. Daniel Noonan (Dept. of Biochemistry, Univ. of Kentucky). All site-directed mutants and PCR reaction products were verified by DNA sequencing.

In vitro GST pulldown assay. GST-Ren and GST-Ren(1-265) were produced as described previously.\textsuperscript{11} Radiolabeled proteins were prepared by in vitro transcription and translation in the presence of \textsuperscript{35}S methionine using the Single Tube Protein System 3 (STP3) kit (Novagen). Binding of radiolabeled CCT to Rem was assessed as follows. Glutathione-Sepharose beads (GE Healthcare) (10 \(\mu\)l) were incubated for 3 h at 4°C with end-over-end rotation (100 ml). The beads were washed and then resuspended in EDTA buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 0.05% Tween 20, 1 mM DTT, 1 mM EDTA), and GST or GST-Gem (10 \(\mu\)g) added. The beads were incubated for 5 min with end-over-end rotation at 4°C, washed with ice-cold EDTA buffer (1 ml), and then with either ice-cold EDTA buffer (1 ml), GDP buffer (1 ml) (50 mM Tris, pH 7.5, 100 mM NaCl, 0.05% Tween 20, 1 mM DTT, 10 mM MgCl\textsubscript{2}, 20 \(\mu\)M GDP), or GTP buffer (GDP buffer with 20 \(\mu\)M GTP[S]) (1 ml) to promote nucleotide exchange. Binding was initiated by the addition of \textsuperscript{35}S-labeled CCT protein (4 \(\mu\)l) and incubated for 3 h at 4°C with end-over-end rotation (100 ml total volume). Resin was washed three times and bound fraction eluted using assay buffer containing 25 mM glutathione (40
µl total). Fractions were resolved on 10% SDS-PAGE gels, the gels dried and exposed to film for 16–72 h. Purified bovine CaM protein was kindly provided by Dr. Thomas Vanaman, Dept. of Biochemistry, Univ. of Kentucky.

Co-immunoprecipitation analysis. Expression vectors expressing Ca1.2, RGK GTases, or various mutants, were co-transfected into tsA201 cells by the calcium phosphate method.48 Forty-eight hours post-transfection, cells were suspended in ice-cold immunoprecipitation (IP) buffer [20 mM Tris, pH 7.5, 250 mM NaCl, 1% TX-100, 0.5 mM DTT, 1x protease inhibitor mixture (Calbiochem)], 10 mM MgCl2, 10 µM GTPγS], lysed by sonication, subjected to centrifugation (100,000 x g), and 0.5 mg of the cleared supernatant incubated with Protein G Sepharose (12.5 µl) (Pharmacia) and anti-HA antibody (5 µg) for 3 h with rotation at 4°C (500 µl total volume). The beads were pelleted and washed three times with IP buffer (1 ml). Fractions were resolved on SDS-PAGE, transferred to nitrocellulose, and subjected to immunoblot analysis using biotinylated-Flag antibody (1 µg/ml) and streptavidin horseradish peroxidase (Pierce) (1:40,000 dilution). Expression of recombinant proteins was examined by immunoblotting using either anti-Flag (Sigma) or anti-HA monoclonal antibodies (1 µg/ml)

Confocal imaging. Confocal imaging of green fluorescent protein (GFP)-tagged Rem protein was performed as previously described.30 Images displayed are representative of the cells observed.

β subunit association assay. Co-immunoprecipitation of Rem with Flag-Caβsb in tsA201 cells was performed as previously described.59

Calmodulin binding assay. TsA201 cells were transfected with Rem, Rem-(1-265), Rem-(1-265)-KCAAX, Rem-(1-265)-HCAAX or empty p3xFlag-CMV10 using the calcium phosphate method.48 48 h post-transfection, cells were harvested and binding of Rem or Rem chimeric proteins to calmodulin was performed as previously described.28 The eluted proteins were resolved on 10% SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-Flag M2 monoclonal antibody (1 µg/ml) (Sigma) to detect bound Rem.

Electrophysiology. TsA201 cells were transfected with the indicated plasmids using the calcium phosphate method, and whole-cell patch clamp experiments were performed as described previously.28 Transfected cells were identified by GFP expression. The selection of Caβ subunit was motivated by the desire to compare results in the present study with our earlier work,28 while studies exploring CDI used Caβ2α2 because of the larger current densities generated. The whole cell configuration of the patch-clamp technique was used to measure ionic current. Patch electrodes with resistances of 1–2 mΩ contained (in mM) 150 CsCl, 3 EGTA, 1 MgCl2, 5 Mg-ATP, and 5 HEPES, pH 7.36. The bath solution consisted of (in mM) 112.5 CsCl, 30 BaCl2, or CaCl2, 1 MgCl2, 10 tetraethylammonium chloride, 5 glucose, 5 HEPES, pH 7.4. The I-V relationship was determined by applying 1,000 ms voltage pulses at 0.25 Hz to potentials between -90 and 80 mV in 5 mV increments from a holding potential of -80 mV. Signals were amplified with an Axopatch 200B amplifier and 333 kHz A/D system (Axon Instruments, Union City, CA). All recordings were performed at room temperature (20–22°C). Data was analyzed with Clampfit 9 (Axon Instruments) software. In Figure 6C and D, values are reported as normalized mean ± SE for Ca2+ currents and as normalized mean ± SE for Ba2+ currents, and significance was determined using Kruskal-Wallis test followed by a post hoc Dunn’s test with p-values of <0.01 (Graphpad Prism 5). For the data analysis in Figure 7C, the degree of voltage-dependent inactivation was analyzed using analyzed using rslow, the ratio of the residual current values at 600 ms and at the initial peak. For the data analysis shown in Figure 7D, Ca2+ currents recorded during a typical 1,000 ms test pulse applied ranging from 0 mV to +40 mV from a holding potential of -80 mV at indicated experimental conditions were analyzed using pCLAMP10 software and the time constants of inactivation were derived from fitting the decay phases of the time courses with the following biexponential function equation: is the time; A1, A2, tau slow and tau fast are the amplitudes and time constants of the fast or slow exponential components; and C is the fraction of noninactivating current. The fast component of inactivation was used for the analysis of Ca2+-dependent channel inactivation. For the data analysis shown in Figure 7D, statistical significance was determined using Student’s t-test (OriginLab, Northampton, MA) denoted by single (p < 0.05) or double (p < 0.0005) asterisks.

Acknowledgements
We wish to thank Dr. Carole Moncman for her assistance with the confocal imaging studies and members of the Andres lab for critical reading of this manuscript. This work was supported by Public Health Service Grants HL072936 (to Douglas A. Andres), HL074091 (to Jonathan Satin), and P20 RR020171 from the National Center for Research Resources, National Institutes of Health (to Douglas A. Andres), an American Diabetes Junior Faculty Award 7-05-JF-16 to (Brian S. Finlin), and an American Heart Association pre-doctoral fellowship and an NIH Interdisciplinary Cardiovascular Training Grant T32 HL072743 (to Robert N. Correll and Shawn M. Crump).

Note
Supplementary materials can be found at: www.landesbioscience.com/supplement/PangCHAN4-3-Sup.

References
1. Catterall WA. Structure and regulation of voltage-gated Ca2+ channels. Annu Rev Cell Dev Biol 2000; 16:521-55.
2. Walker D, De Waard M. Subunit interaction sites in voltage-dependent Ca2+ channels: role in channel function. Trends Neurosci 1998; 21:148-54.

3. Pragnell M, De Waard M, Nosi Y, Tanabe T, Snutch TP, Campbell KP. Calcium channel beta-subunit binds to a conserved motif in the I-II cytoplasmic linker of the alpha1-subunit. Nature 1994; 368:67-70.
4. Richards MW, Boucher AJ, Dolphin AC. Ca2+ channel beta-subunits: structural insights AID our understanding. Trends Pharmacol Sci 2004; 25:626-32.
5. Bichet D, Corner V, Gehl S, Carlier E, Volso S, Hoshi T, et al. The I-II loop of the Ca2+ channel alpha1 subunit contains an endoplasmic reticulum retention signal antagonized by the beta subunit. Neuron 2000; 25:177-90.
6. Dolphin AC. Beta subunits of voltage-gated calcium channels. J Bioenerg Biomembr 2003; 35:599-620.
7. Kamp TJ, Hell JW. Regulation of cardiac L-type calcium channels by protein kinase A and protein kinase C. Circulation rReseach 2000; 87:1095-1102.
channel by its proteolytically processed distal C subunit associate with and regulate L age of the components controlling inactivation of voltage-gated Ca2+ channel inhibition. J Biol Chem 2007; 282:28431-40.

Correll RN, Botzet GJ, Satin J, Andrews DA. Finlin BS. Analysis of the Rem2—voltage dependent calcium channel beta subunit interaction and Rem2 interaction with phosphorylated phosphatidylinositol lipids. Cell Signal 2008; 20:400-8.

Finlin BS, Crump SM, Satin J, Andrews DA. Regulation of voltage-gated calcium channel activity by the Rem and Rad GTPases. Proc Natl Acad Sci USA 2003; 100:14469-74.

Chen H, Puhl HL, 3rd, Niu SL, Mitchell DC, Beda SR. Expression of Rem2, an RGK family small GTPase, reduces N-type calcium current without affecting channel surface density. J Neurosci 2005; 25:7672-82.

Kelly K. The RGK family: a regulatory tail of small GTP-binding proteins. Trends Cell Biol 2005; 15:640-3.

Begun P, Mahalakshmi RN, Nagashima K, Cher DH, Takahashi K, Vakurama N, Yamada Y, et al. 14-3-3 and calmodulin control subcellular distribution of KirGem and its regulation of cell shape and calcium channel activity. J Cell Sci 2005; 118:1933-44.

Begun P, Mahalakshmi RN, Nagashima K, Cher DH, Ikeda H, Yamada Y, et al. Nucleotide sequestration of beta-subunits by Rad and Rem is controlled by 14-3-3 and calmodulin and reveals a novel mechanism for Ca2+ channel regulation. J Mol Biol 2006; 355:34-46.

Zhu J, Reynert C, Caldwell JS, Kahn CR. Characterization of Rad, a new member of the RGK family: a regulatory tail of small GTPase. J Biol Chem 2005; 280:41864-71.

Splain Andreaty, Kerer M, Cocolides A, Charalambous E, Rettori F, et al. Biochemical and structural characterization of the gem GTPase. J Biol Chem 2008; 283:1905-15.

Opazo-Poyosky Y, Sasson Y, Shaked I, Ward Y, Chomsvsky-Hecht O, Livik Y, et al. Structure-function studies of the G-domain from human gem, a novel small G-protein. FEBS Lett 2006; 580:5959-64.

Finlin BS, Mosley AL, Crump SM, Correll RN, Ozcan S, Satin J, et al. Regulation of L-type Ca2+ channel activity and insulin secretion by the Rem2 GTPase. J Biol Chem 2005; 280:41864-71.

Beguin P, Nagashima K, Gono T, Shibasaki T, Takahashi K, Kashima Y, et al. Regulation of Ca2+ channel expression at the cell surface by the small G-protein kiri/Gem. Nature 2001; 411:701-6.

Finlin BS, Correll RN, Pang C, Crump SM, Satin J, Andrews DA. Analysis of the complex between Ca2+ channel beta-subunit and the Rem GTPase. J Biol Chem 2006; 281:23577-66.

Crump SM, Correll RN, Schroder EA, Lester WC, Finlin BS, Andrews DA, et al. L-type calcium channel alpha-subunit and protein kinase inhibitors modulate Rem-regulated current of cardiac myocytes. J Physiol Heart Circ Physiol 2006; 291:1599-71.

Seu L, Pirt GS. Non-dependent and isoform-specific modulation of Ca2+ channels by RGG GTPTases. J Gen Physiol 2006; 128:605-13.

Yada H, Murata M, Shimoda K, Yasua S, Kawaguchi H, Ieda M, et al. Dominant negative suppression of Rad leads to QT prolongation and cause ventricular arrhythmias via modulation of L-type Ca2+ channels in the heart. Circulation Research 2007; 101:69-77.

Beguin P, Ng YJ, Krause C, Mahalakshmi RN, Ng MY, Hunchiker W. Small GTP-binding proteins interact with the nucleotide kinase domain of Ca2+ channel beta-subunits via an uncommon effecter binding domain. J Biol Chem 2007; 282:11509-20.

Dolphin AC. A short history of voltage-gated calcium channels. Br J Pharmacol 2006; 147:56-62.

Hancock JE, Magee AI, Childs JE, Marshall CJ. All ras proteins are polyisoprenylated but only some are palmitoylated. Cell 1989; 57:1167-77.

Willemsen BM, Norris K, Pagapeorge AG, Hubbert NL, Lowy DR. Harvey murine sarcoma virus p21 ras protein: biological and biochemical significance of the cysteine near the carboxy terminus. EMBO J 1984; 3:2581-5.

Gutierrez L, Magee AI, Marshall CJ, Hancock JE. Post-translational processing of p21ras is two-step and involves carboxyl-methylation and carboxyl-terminal prenylation. EMBO J 1989; 8:1093-9.

Vilallonga P, Lopez-Alcaza B, Bosch M, Chioleches A, Rocamora N, Gil J. et al. Calmodulin binds to K-Ras, but not to H- or N-Ras, and modulates its downstream signaling. Mol Cell Biol 2001; 21:7454-54.

Ploorman SJ, Hancock JE. Ras signaling from plasma membrane and endomembrane microdomains. Biochem Biophys Acta 2005; 1746:274-83.

de Leon M, Wang Y, Jones L, Perez-Reyes E, Wei X, Soong TW et al. Essential Ca2+-binding motif for Ca2+-sensitive inactivation of L-type Ca2+ channels. Science 1995; 270:1502-6.

Yang T, Suhail Y, Dalton S, Kerman T, Colecraft HM. Genetically encoded molecules for inducibly inactivating L-type Ca2+ channels. Nat Neurosci 2007; 10:573-80.

Alseikhan BA, DeMaria CD, Colecraft HM, Yue DT, Engeretin calmodulin reveal the unexpected eminence of Ca2+ channel inactivation in controlling heart excitation. Proc Natl Acad Sci USA 2002; 99:17185-90.

Chang L, Zhang J, Tieng YH, Cie CQ, Iany J, Bruning JC, et al. Rad GTPTase deficiency leads to cardiac hypertrophy. Circulation 2007; 116:2796-83.

Soldarov NM, Ca2+ channel moving tail: link between Ca2+-induced inactivation and Ca2+ signal transduction. Trends Pharmacol Sci 2003; 24:167-71.

Andres DA, Shao H, Crick DC, Finlin BS. Expression cloning of a novel farnesylated protein, RDJ2, encoding a DnaJ protein homologue. Arch Biochem Biophys 1997; 346:113-24.

Andres DA, Crump SM, Correll RN, Satin J, Finlin BS. Analyses of Rem/GK signaling and biological activity. Methods Enzymol 2005; 407:484-98.