RGK Small GTP-binding Proteins Interact with the Nucleotide Kinase Domain of Ca2+ channel β-Subunits via an Uncommon Effector Binding Domain*

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Pascal Bégün1, Yu Jin Alvin Ng1, Carola Krause, Ramasubbu N. Mahalakshmi, Mei Yong Ng, and Walter Hunziker2

From the Epithelial Cell Biology Laboratory, Institute of Molecular and Cell Biology, 61 Biopolis Drive, Singapore 138673, Republic of Singapore

RGK proteins (Kir/Gem, Rad, Rem, and Rem2) form a small subfamily of the Ras superfamily. Despite a conserved GTP binding core domain, several differences suggest that structure, mechanism of action, and functional regulation differ from Ras. RGK proteins down-regulate voltage-gated calcium channel activity by binding in a GTP-dependent fashion to the Caβ subunits. Mutational analysis combined with homology modeling reveal a novel effector binding mechanism distinct from that of other Ras GTPases. In this model the Switch 1 region acts as an allosteric activator that facilitates electrostatic interactions between Arg-196 in Kir/Gem and Asp-194, -270, and -272 in the nucleotide-kinase (NK) domain of Caβ3 and wedging Val-223 and His-225 of Kir/Gem into a hydrophobic pocket in the NK domain. Kir/Gem interacts with a surface on the NK domain that is distinct from the groove where the voltage-gated calcium channel Caα1 subunit binds. A complex composed of the RGK protein and the Caβ3 and Ca1.2 subunits could be revealed in vivo using coimmunoprecipitation experiments. Intriguingly, docking of the RGK protein to the NK domain of the Caβ subunit is reminiscent of the binding of GMP to guanylate kinase.

Small GTPases comprise four major branches, Rab, Ras, Arf, and Rho. The RGK family belongs to the Ras superfamily and includes Rad (1), Rem (2), Rem2 (3, 4), and Kir/Gem (5, 6). Although the core region involved in nucleotide binding is conserved with other Ras superfamily members, RGK proteins show several significant structural and functional differences to Ras (7). These include an unconventional G3 motif indicative of an unusual mechanism for GTP hydrolysis (3, 6), N- and C-terminal extensions that bind 14–3–3 proteins and calmodulin (4, 8–11), and the lack of lipid modification for membrane association.

An important physiological function of RGK proteins is the regulation of VDCC activity (4, 9–16). The calcium transporting Caα1 subunit of VDCCs depends for activity on auxiliary subunits, in particular the Caβ subunit (17, 18). The Caβ subunit is a cytosolic protein with an SH3 and NK (also referred to as guanylate kinase) domain (19, 20), and several isoforms and splice variants have been described (18). RGK proteins bind the Caβ subunit, leading to the down-regulation of channel activity due either to the inhibition of channels present in the plasma membrane (13, 15) or interference with cell surface expression of the Caα1 subunit (4,9–11,21). It was thought that RGK proteins interact through the β-interacting domain of the Caβ subunit, thus preventing association between the Caα1 and Caβ subunits (11, 21). However, this assumption has been challenged since the β-interacting domain appears to be buried in the Caβ subunit and, rather, may play a role in the maintenance of a correct conformation (19, 20). In addition, a complex containing Rem, the Caβ subunit and a glutathione S-transferase–α-interacting domain fusion protein was recently demonstrated (22).

The Caβ subunit interacts with RGK proteins bound to GTP but not GDP, and, thus, represents a bona fide effector (4, 9–11). However, RGK proteins may not be canonical small GTPases that switch between active GTP-bound and inactive GDP-bound forms. RGK proteins lack critical residues in the G2 (i.e. T35) and G3 (i.e. DXXG60) elements that are important for GTP hydrolysis in other Ras superfamily members (7). Indeed, although most other small GTPases show readily detectable intrinsic GTPase activity, GTP hydrolysis by RGK proteins is exceptionally low (3, 6), and they are readily isolated from cells in the GTP-bound form (10). Thus, it is conceivable that in cells RGK proteins are constitutively bound to GTP. In this case inactivation of RGK proteins would have to depend on an atypical mechanism for GTP hydrolysis or actively regulated nucleotide exchange. Alternatively, inactivation could be accomplished by sequestering the RGK protein away from its site of action, an attractive possibility given that RGK proteins undergo highly regulated nuclear transport (4, 9, 10).

Despite these unusual features relative to other small GTPases, little is known about the structure of RGK proteins and how they interact with effectors. Using extensive in vitro mutagenesis combined with in vivo and in vitro binding assays, we identified amino acids in Kir/Gem and the β3 subunit that are critical for their association and used this information to generate a structural model of a complex between the RGK...
protein and its effector. Our data indicate that RGK proteins and the VDCC α-subunit bind to distinct surfaces on the nucleotide kinase (NK) domain of the β-subunit and reveal an unusual effector binding mechanism for these small GTP-binding proteins. Moreover, a complex composed of an RGK protein, the Ca\(_{\beta3}\) subunit, and the intact Ca\(_{1.2}\) subunit was revealed in vivo. These findings provide novel insights into the mechanism by which RGK proteins regulate VDCC activity.

**EXPERIMENTAL PROCEDURES**

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**Yeast Two-hybrid Screen**—The yeast two-hybrid (Y2H) assay was carried out with full-length Kir/Gem W269G and Ca\(_{\beta3}\) (residues 49–379) as described (23).

**Tissue Culture and Transfections**—COS-1 and HEK-293T cells were grown and transiently transfected with different cDNAs as detailed (10) and used for experiments 24–38 h after transfection.

**Molecular Biology and Biochemistry**—Point mutations in mouse Kir/Gem, rat β3, and rat Ca\(_{1.2}\) were created by PCR. A mouse Rho kinase β cDNA was assembled from different IMAGE clones (i.e. 534680, 5008498, 5149049, 30619707). All constructs were verified by DNA sequencing. Epitope-tagged proteins, the preparation of cell homogenates, and coprecipitation and pull-down experiments have been described (4, 10).

For coimmunoprecipitations, cell lysate (400 μg) was prepared in a buffer containing either Tween 0.2% for Ca\(_{\beta3}\)/RGK/Ca\(_{1.2}\) association analysis. The lysate was incubated with 4 μl of anti-FLAG (M2), anti-Myc (Sigma), or anti-HA (Roche Applied Science)-agarose beads and 30 μl of protein A-Sepharose beads (Amersham Biosciences) for 4 h at 4°C. After washing, the complexes were eluted and subjected to SDS-PAGE (7–8%), and Western blot analysis using either mouse monoclonal anti-FLAG (M2; Sigma), anti-Myc (Roche Applied Science), or rat anti-HA (Roche Applied Science) antibody was performed. Wild-type (WT) or mutated glutathione S-transferase-Kir/Gem fusion proteins (residues 20–295) (11) were dialyzed in phosphate-buffered saline and used for GTP/GDP binding assays using the nitrocellulose binding assay (24). 1 μg of fusion protein was incubated with saturating nucleotide concentrations (100 μM GDP and 3 μM GTP) or concentrations approximating the K\(_d\) (10 μM GDP (6) and 0.5 μM GTP), yielding similar results (data not shown).

**Immunocytochemistry**—Analysis of the subcellular localization and cell surface expression of the Ca\(_{1.2}\) was done as described (4, 9, 10). Rabbit anti-Myc (Upstate Biotechnology) and mouse anti-FLAG (Sigma) were used for double labeling, and a rat anti-HA monoclonal (Roche Applied Science) was included for triple-labeling experiments. Specimens were visualized with an Axiocam microscope (Carl Zeiss) at 100× magnification.

**Molecular Modeling**—Molecular modeling, energy calculations, and three-dimensional rendering of protein structures were performed using the Sybyl software package (Tripos, Inc.). Most molecular dynamics simulations were performed at 300 K at an interval of 1000 fs and 1-fs steps using unless otherwise stated, the parameters from Kollman (25) for the force field and charges. Energy minimization was performed using the method by Powell and Fletcher (26). The structure of Kir/Gem was taken from the crystalized structure of Gem bound to GDP (PDB 2G2Y). The Switch 1 region (194GVHDSMDSSCEVLG199) was rebuilt based on the structure of the GTP-bound form of Rap2A as a template (PDB 2RAP) (27). Because Arg-196 is not resolved in PDB 2G2Y, its structure was also rendered. The GTP and Mg\(^{2+}\) were from PDB 2RAP were superimposed onto the structure of Kir/Gem using charges assigned by Gasteiger Huckel parameters for GTP and a formal charge of 2+ for the magnesium ion. Molecular dynamics simulations followed by energy refinement using the Tripos Force Field was performed to render the Switch 1 region in the new GTP-bound form of Kir/Gem. The model for the Ca\(_{\beta3}\) was derived from the crystal structure of the Ca\(_{\beta}\) subunit bound to the interacting fragment of the α-subunit (PDB file 1VYT), and the same secondary structure assignments and nomenclature were used (19). Kir/Gem was docked onto Ca\(_{\beta3}\) taking into account the wet lab experimental data. The largest surface containing the negatively charged residues on the NK domain important for Kir/Gem binding was used as a starting point. This surface showed good shape complementarity with that of the putative G2 domain of Kir/Gem. Modification of the rotamer conformation of the Kir/Gem Arg-196 side chain was required to position the amine group closely enough for it to interact with the carboxyl groups of Asp-194, -270, Asp-272 in Ca\(_{\beta3}\). Molecular dynamics simulations and energy minimization determined the optimal position of the Arg-196 side chain with respect to the three Asp residues of Ca\(_{\beta3}\). The side chains of Val-223 and His-225 in Kir/Gem fit nicely into a large hydrophobic pocket on the NK domain of the Ca\(_{\beta}\) subunit. Molecular dynamics simulations (100-fs interval) were performed on the sequence221AAVQHN226 to investigate the reasons behind the results of the wet lab mutational analysis of Val-223 and His-225. Mutations were introduced in silico into Kir/Gem, and molecular dynamics simulations were carried out to explore the optimal conformations. For the R196K mutation, residues in a sphere radius of 6 Å were subjected to molecular dynamics simulations to explore differences in the conformations of Asp-194, 270, and -272.

**RESULTS AND DISCUSSION**

**Identification of Residues in Kir/Gem Critical for the Interaction with Ca\(_{\beta3}\)**—To identify amino acids important for the interaction with Ca\(_{\beta3}\), we first generated a truncated form of Kir/Gem containing the core Ras homology domain (amino acids 74–251, Fig. 1A) with the G1–G5 motifs involved in GTP binding (Fig. 1A, gray). As assessed by pulldown experiments, this core domain was sufficient for the nucleotide-dependent interaction with the Ca\(_{\beta3}\) subunit (data not shown). We then mutated clusters of three amino acids in the core domain to Ala and tested the interaction of the mutants with Ca\(_{\beta3}\) using a Y2H assay. Individual Ala substitutions in the susceptible regions (Fig. 1B, red) led to the identification of nine residues whose mutation interfered with Ca\(_{\beta3}\) binding (Fig. 1C). The lack of an interaction between the different Kir/Gem mutants and Ca\(_{\beta3}\) was confirmed by communoprecipitation experi-
A Complex Containing RGK Proteins Ca\textsubscript{v}1.2 and Ca\textsubscript{v}3

Gem mutants that bind neither GTP nor GDP (see Fig. 3), indicating that phosphorylation is sensitive to conformation.

The spatial location of the residues critical for Ca\textsubscript{v}3 binding was determined based on a structural model of Kir/Gem generated using the recently solved crystal structure of a GDP-bound form of Kir/Gem (PDB 2G3Y) (Fig. 2A). The solved Kir/Gem-GDP structure is overall very similar to that of the GDP- or GTP-bound Rap2A (28), which has 21% sequence identity with Kir/Gem and is the closest homologue to have been crystallized in its GTP-bound form (27). The Switch 1 region in the GDP Kir/Gem structure was not completely resolved, indicating that this region may be disordered, an observation that is confirmed by the recently solved structure of GDP-bound Rad223 (29). Thus, we used molecular dynamics simulations to render the Switch 1 region of Kir/Gem using the GTP-bound form of Rap2A, including GTP and Mg\textsuperscript{2+}, as a template (Fig. 2A). These calculations indicate that in the GTP-bound form of Kir/Gem, GTP, and Mg\textsuperscript{2+} order the Switch 1 region via the coordination with negatively charged residues in Switch 1. The orientation of the long side chain Arg-196 was also not resolved in the crystal structure of GDP-bound Kir/Gem (Fig. 2A). The backbone structure of this region is assumed to be identical in the GDP- and GTP-bound forms of Kir/Gem based on the absence of major structural variations between the GTP- and GDP-bound forms of Rap2A (Fig. 2C).

Several of the residues that affect Ca\textsubscript{v}3 binding when mutated are buried in the modeled GTP-bound Kir/Gem structure (Fig. 2C), raising the possibility that their mutation alters the conformation of Kir/Gem rather than binding per se. We, therefore, carried out GTP and GDP binding assays to monitor the conformation of the different Kir/Gem mutants. Except for R196A and V223A, the mutants (i.e. L90A, Y156A, L194A, V200A, F231A) displayed a dramatic reduction in both GTP (Fig. 3A, panel a) and GDP (Fig. 3A, panel b) binding, suggesting that they affect the conformation of Kir/Gem. Binding of nucleotides to H225A and V195A was by comparison only marginally affected. Interestingly, Arg-196, Val-223, and His-225 are located on the same surface in the modeled Kir/Gem (Fig. 2C), consistent with them being part of the effector binding domain. This region does not show major conformational differences between the GTP- and GDP-bound structures in Rap2A (Fig. 2B), and Arg-196, Val-223, and His-225 are in close proximity of the G4 and G5 motif. The location and orientation of Arg-196, Val-223, and His-225 is, thus, subject to the conformational constraints for nucleotide binding imposed by the G4 and G5 motifs.

Selected amino acids whose substitution to Ala affected binding (10, 11) were introduced into Kir/Gem W269G, which does not bind calmodulin, and tested for association with Ca\textsubscript{v}3. Except for the W269G/V195A mutant (Fig. 1D, lane 15), no coprecipitation was observed. Because V195A (in either the WT or the W269G context) displayed residual binding to Ca\textsubscript{v}3 in pulldown experiments (supplemental Data 1), we concluded that this residue is less critical for the interaction. Kir/Gem migrates on SDS-PAGE as a doublet, and alkaline phosphatase treatment indicates that the slower migrating band is a phosphorylated form (data not shown). Interestingly, only the faster migrating, non-phosphorylated form of Kir/Gem binds Ca\textsubscript{v}3. Furthermore, only the lower band was detected in Kir/Gem mutants that bind neither GTP nor GDP (see Fig. 3), indicating that phosphorylation is sensitive to conformation.

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Analysis of Val-223 revealed that conservative substitutions (i.e. V223W, V223F, V223I, V223M, V223Y, V223L) did not affect Ca_{\alpha3} binding in communoprecipitation experiments (Fig. 3B), highlighting the requirement of a bulky hydrophobic residue at this position. The only exception was a substitution to Met (see below). Although conservative substitutions of His-225 by Trp and Phe retained binding, the H225R, H225K, and H225I mutants did not associate with Ca_{\alpha3}. Thus, the large planar side chain rather than the positive charge of His-225 accounts for the association of Kir/Gem with Ca_{\alpha3}.

The critical role of Arg-196 and Val-223 of Kir/Gem for Ca_{\alpha3} association was corroborated in vivo. The colocalization of Kir/Gem and Ca_{\alpha3} to dendrite-like elongations and the Kir/Gem W269G-mediated nuclear accumulation of \beta3 were no longer observed in cells expressing the R196A or V223A mutants (supplemental Data II).

Residues in Kir/Gem Critical for Ca_{\alpha3} Binding Are Conserved in Rad, Rem, and Rem2—Because all RGK proteins bind Ca_{\beta} subunits (see (7)), Arg-196, Val-223, and His-225 in Kir/Gem should be conserved if they are indeed critical for the association. The Arg and His corresponding to Arg-196 and His-225 in Kir/Gem are conserved in Rad (Arg-208, His-237), Rem (Arg-200, His-229), and Rem2 (Arg-236, His-265), whereas Val corresponding to Kir/Gem Val-223 encodes a Leu in other RGK proteins (Rad (Leu-235), Rem (Leu-227), and Rem2 (Leu-263)) (Fig. 4A). The compatibility of a Leu at position 223 was confirmed by the finding that WT Kir/Gem and the V223L mutant bind Ca_{\alpha3} with similar efficiencies (Fig. 3B). Consistent with the crucial role of the conserved Arg for the association, its substitution to an Ala prevented Ca_{\alpha3} binding of Rad, Rem, and Rem2 (Fig. 4B), although for Rem2, weak residual binding was occasionally observed. Replacement of the Leu or His with Ala in Rad and Rem abolished or dramatically reduced Ca_{\alpha3} association. In contrast, similar substitutions in Rem2 maintained Ca_{\alpha3} binding, suggesting subtle differences in the way Rem2 associates with Ca_{\alpha3}. The concomitant substitution of the critical Arg and Val or Leu residues was sufficient to completely abolish Ca_{\alpha3} binding to all four RGK proteins (supplemental Data III). As a control, GTP/GDP binding-defective mutants corresponding to L194A in Kir/Gem (i.e. Rad L206A, Rem L198A, and Rem2 L234A; Fig. 4B) strongly interfered with Ca_{\alpha3} association.

As observed for Kir/Gem (supplemental Data II), Rad, Rem, and Rem2 mutants defective in Ca_{\alpha3} binding showed impaired colocalization with Ca_{\beta} when coexpressed in COS-1 cells and also failed to mediate the nuclear translocation of the Ca_{\beta3} subunits (data not shown). Furthermore, the enhanced cell surface expression of the VDCC Ca_{1.2} subunit mediated by Ca_{\beta3} (10, 18, 31) (Fig. 4C), was abrogated by the RGK proteins (4, 9–11); Fig. 4D, panels a–b’’, e–f’’ i–j’’, and m–n’’), consistent with the notion that binding of the RGK protein prevents cell surface expression of coexpressed VDCCs (4,
important for RGK protein binding. N- and C-terminal deletions of Ca_1.2 extending into the SH3 or NK domain, respectively, failed to associate with Kir/Gem (data not shown), indicating the SH3, HOOK, and NK domains are required and that the region containing these domains is sufficient for binding (Fig. 5A). Based on Ala-scan mutagenesis, the HOOK domain was not involved in the interaction with Kir/Gem, but 12 potentially important regions in the SH3 and NK domain were identified (Fig. 5B). Single Ala substitutions in these regions led to a list of 64 residues with a possible role in Kir/Gem binding (Fig. 5C). Using the crystal structure of Ca_1β in complex with a fragment of the α1 subunit (19, 20), we determined which of the identified residues is located on the surface of Ca_3 and may, thus, be accessible for interacting with Kir/Gem (Fig. 5C). Putative surface residues were individually mutated to Ala and tested in coimmunoprecipitation experiments for their interaction with Kir/Gem, leading to the identification of 17 Ala-substitutions that abolished or greatly reduced binding to Kir/Gem (Fig. 5D and supplemental Data VI).

To better understand the relationship between α1-subunit and Kir/Gem docking, residues in Ca_1β3 responsible for α-subunit binding were substituted to Ala, and these mutants were tested for Kir/Gem binding (Fig. 5E). Single or double mutations did not affect the association between Kir/Gem and Ca_3, suggesting that the binding sites on Ca_3 for Kir/Gem and the Ca_1.2 subunit are not identical (see below).

**A Structural Model of Kir/Gem Bound to Ca_1β3**—The crystal structure of the Ca_1β subunit in complex with the interacting helix of the α-subunit (19, 20) served as a template to model Ca_1β3. Interestingly, mutations in Ca_1β3 that affected the interaction with Kir/Gem clustered either to a patch on the NK domain, to the SH3 domain, or the interface of the two domains (Fig. 6A). The putative structure of Kir/Gem in its GTP-bound form, derived by homology to GTP-bound Rap2 (27) (see Fig. 2A), was used for in silico docking renditions. In the computed model, Kir/Gem binds to a region on the NK domain that is distinct from that interacting with Ca_3 (Fig. 6, A and B). In the rendered model the interacting surfaces on
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**A**

![Diagram showing Kir/Gem and GTP-binding regions]

**B**

*Panel a*

IP: anti-FLAG
Biot: anti-myc

*Panel b*

Lysate

*Panel c*

Lysate

| Ca\textsubscript{v}\beta3 | Rad | Rem | Rem2 |
|------------------------|-----|-----|------|
| wt                     | wt  | wt  | wt   |
| L206A                  |     |     |      |
| V227A                  |     |     |      |
| R208A                  |     |     |      |
| L235A                  |     |     |      |
| L198A                  |     |     |      |
| R200A                  |     |     |      |
| L227A                  |     |     |      |
| L234A                  |     |     |      |
| L236A                  |     |     |      |
| L263A                  |     |     |      |

**C**

![Images of Ca\textsubscript{v}1.2 and Ca\textsubscript{v}\beta3 expression and labeling]

**D**

| RGK/CaCN Cell Expression | Ca\textsubscript{v}\beta3 | Ca\textsubscript{v}1.2 | Ca\textsubscript{v}1.2 |
|--------------------------|--------------------------|-----------------------|-----------------------|
| **RGK**                  | a                        | a'                    | a''                   |
| **Ca\textsubscript{v}\beta3** | b                        | b'                    | b''                   |
| **Ca\textsubscript{v}1.2** | c                        | c'                    | c''                   |
| **CaCN Cell Surface Labeling** | d                        | d'                    | d''                   |

| Rad | Rem | Rem2 |
|-----|-----|------|
|     |     |      |
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**Journal Information**

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Kir/Gem and Ca₁β₃ show excellent shape complementarities. Consistent with the biochemical data presented above, binding is mediated by three electrostatic interactions between Arg-196 of Kir/Gem and Asp-194, Asp-270, and Asp-272 of β3 (Fig. 6C), and the insertion of Val-223 and His-225 of Kir/Gem into a hydrophobic pocket on the NK domain of Ca₁β₃ (Fig. 6D). Based on the structure of the GDP-bound and the rendered GTP-bound Kir/Gem, the side chain of Arg-196 points outward, facilitating the interactions with Asp-194, Asp-270, and Asp-272 (computed distances of ~1.65 Å each) of Ca₁β₃. Experimentally, R196K binds Ca₁β₃ less efficiently, likely because its side chain can only engage with Asp-194 and Asp-272 (supplemental Data VII), indicating that the electrostatic bond between Arg-196 and Asp-270 is critical for efficient binding.

The above model was tested using charge reversal experiments. The positively charged Arg-196 in Kir/Gem was replaced with a negatively charged Glu, and the three negatively charged Asp residues in Ca₁β₃ were individually changed to a positively charged Arg. Although Kir/Gem R196E no longer interacted with WT Ca₁β₃ (Fig. 7A, lanes 1 and 3), the association was weakly restored for Ca₁β₃ D194R and D270R and partially restored for D272R (lanes 3–5). The interaction of the mutant proteins was then analyzed by molecular modeling (Fig. 7B). For the WT proteins, Arg-196 of Kir/Gem and Asp-194, Asp-270, and Asp-272 in Ca₁β₃ are ideally positioned to establish three strong interactions (Fig. 7B, panel a), and interfering with one of the three possible interactions is sufficient to weaken or abolish the association (see above). In the case of Ca₁β₃ D194R and Ca₁β₃ D270R, the Glu residue in Kir/Gem R196E could interact with the mutated Asp residue and Arg-216 in Ca₁β₃, but these interactions are likely to be weak due to the engagement of D194R or D270R in intramolecular interactions with Arg-216 and other Asp residues in the pocket (Fig. 7B, panels b and c). In the case of Ca₁β₃ D272R, in contrast, the intramolecular association of D272R with Asp-194 and Asp-270 facilitates an ideal positioning of the Glu in Kir/Gem R196E to interact with Arg-216 of Ca₁β₃ D272R (Fig. 7B, panel d), providing a stronger interaction. If this interpretation is correct, the mutation of Arg-216 to Ala in Ca₁β₃ D272R is expected to affect the interaction. Indeed, the interaction between Kir/Gem R196E and Ca₁β₃ D272R/D216A was abolished (Fig. 7A, lanes 5 and 6). As expected, binding of Ca₁β₃ R216A to WT Kir/Gem was not altered (lane 8), but similar to WT Ca₁β₃, Ca₁β₃ R216A failed to associate with Kir/Gem R196E (Fig. 7A, lanes 2 and 7). The relatively weak interactions between Kir/Gem R196E and the Ca₁β₃ mutants are comparable with that between Kir/Gem R196K and Ca₁β₃ (see above). In conclusion, these findings confirm the interaction of Arg-196 in Kir/Gem with the hydrophilic pocket of Ca₁β₃ and, hence, support the model proposed in Fig. 6.

His-225 and Val-223 of Kir/Gem associate with Ca₁β₃ through hydrophobic interactions with Leu-187 and Asn-275, respectively (Fig. 5D and supplemental Data VII; a computed distance of ~2 Å). His-225, conserved in all RGK proteins, fills the large hydrophobic pocket on the NK domain, where it interacts with its large planar side chain of Leu-187. At the position corresponding to Val-223, Rad, Rem, and Rem2 carry a Leu, and Kir/Gem V223L binds β3 with similar efficiency as WT Kir/Gem. The side chains of either a Val or a Leu at this position fit into the hydrophobic pocket of Ca₁β₃, which is deep enough to accommodate the slightly longer side chain of a Leu or the bulkier one of a Trp. Although the side chain of a Met can also insert into the hydrophobic pocket, its high degree of flexibility predicts a weaker interaction (supplemental Data VII), and the V223M substitution indeed affects the association with Ca₁β₃ (see Fig. 3B, panel a, lane 8).

On Ca₁β₃, most of the residues that affect Kir/Gem binding when substituted to Ala are located in a patch on the surface of the NK domain that includes the critical amino acids Asp-194, Asp-270, and Asp-272 (Fig. 6, A and B). Many of the substitutions in the NK domain that interfere with Kir/Gem binding are predicted to alter the structure of the NK domain either by altering the flexibility of turns, the shape of the interacting surface, the ideal distance between Asp-194, Asp-270, and Asp-272, important for the coordinated interaction with Arg-196 of Kir/Gem, or the ideal depth and size of the hydrophobic pocket that accepts Val-223 and His-225 of Kir/Gem (supplemental Data VII). Consistent with the association of all Ca₁β₃ isoforms with Kir/Gem (11), the residues in Ca₁β₃ critical for binding are conserved among the different isoforms (data not shown).

Interestingly, mutation of several residues in the SH3 domain of Ca₁β₃ also affected binding to Kir/Gem (Fig. 5C) but not to α-subunit (supplemental Data V). These mutations are likely to affect the stability of the SH3 domain and/or the integrity of the conformation of the SH3/NK interphase, critical for Kir/Gem binding. Kir/Gem occludes a much larger surface on the NK domain than the α-subunit (~2600 versus 1600 Å²) (32), and

FIGURE 4. Amino acids in Kir/Gem important for Ca₁β₃ binding are conserved in RGK proteins. A, the N- and C-terminal extensions (black) and the Ras-like core domain (white) with the G1–G5 motifs involved in GTP binding (gray), and the identified G2 effector binding motif (red) of Kir/Gem is shown. The G1–G5 domains of Kir/Gem, Rad, Rem, and Rem2 are aligned, and a dash indicates identical amino acids. Critical residues of the effector binding motif are shown in red, with Arg-196 and His-225 of Kir/Gem conserved and Val-223 substituted in Leu in the other RGK family members. B, effect of mutating critical residues in Rad, Rem, and Rem2 on Ca₁β₃ binding. a, lysate of COS-1 cells expressing WT or mutated Myc-Rad, Rem or Rem2, and FLAG-Ca₁β₃ were immunoprecipitated with FLAG antibodies to isolate Ca₁β₃, and associated RGK proteins were revealed by Western blot using Myc antibody. b and c, cell lysate blotted with Myc or FLAG antibody to monitor RGK and Ca₁β₃ expression levels, respectively. C, the Ca₁β₃ subunit facilitates surface expression of Ca₁α₁. HEK-293T cells transfected with a CDNA for Ca₁,2 carrying an extracellular HA tag (a and c) or an internal ribosome entry site-based vector carrying the cDNAs for HA-Ca₁,2 and FLAG-Ca₁β₃ subunits (b–b’ and d–d’), were fixed, permeabilized, and processed for immunofluorescence microscopy using HA and FLAG antibodies to detect Ca₁,2 (green) and Ca₁β₃ (blue), respectively (calcium channel cell expression). Alternatively, live cells were first incubated with HA antibodies to selectively label surface-exposed Ca₁,2 before fixation, permeabilization, and labeling with Myc and FLAG antibodies (calcium channel cell-surface labeling).
FIGURE 5. Residues in Ca_{β3} important for its interaction with Kir/Gem. A, diagram of Ca_{β3} and amino acid sequence of the SH3 (blue), HOOK (pink), and NK (green) domains. B, clusters of three residues were substituted to Ala and the mutant proteins screened for their interaction with Kir/Gem in a Y2H assay. Regions where substitutions affect Kir/Gem binding are shaded in red. C, individual residues from the regions identified in B were substituted to Ala and tested for their interaction with Kir/Gem using the Y2H assay. 64 critical residues were identified and classified as being exposed on the surface (green) of Ca_{β3} or buried (pink), taking the crystal structure of the Ca_{β3}-subunit in complex with a fragment of the Ca_{α1} subunit as reference. D, coimmunoprecipitation of Ca_{β3} and Kir/Gem. a, lysate of COS-1 cells expressing WT or mutated FLAG-Ca_{β3} and Myc-Kir/Gem were immunoprecipitated with FLAG-antibodies to isolate Ca_{β3}, and associated Kir/Gem was revealed by Western blot using Myc antibody. b and c, cell lysate was blotted with Myc or FLAG antibody to monitor Kir/Gem and Ca_{β3} expression levels, respectively. E, effect of the mutation of residues in Ca_{β3} involved in Ca_{α1} binding on the association with Kir/Gem. a, lysate of COS-1 cells expressing WT or mutated FLAG-Ca_{β3} and Myc-Kir/Gem were immunoprecipitated with FLAG-antibodies to isolate Ca_{β3}, and associated Kir/Gem was revealed by Western blot using Myc antibody. b and c, cell lysate was blotted with Myc or FLAG antibody to monitor Kir/Gem and Ca_{β3} expression levels, respectively.
The SH3 domain could be important to maintain an ordered NK surface for interaction with Kir/Gem. In a recent paper, in vitro translated Cav/H9252 truncation mutants (as opposed to point mutations in the present study) were used to show that the SH3 domain is dispensable for Rem binding and that binding occurs with the GTP-as well as the GDP-bound forms of the RGK protein (22). Consistent with this data, we similarly observed that in contrast to in vivo expressed Caβ3, the in vitro translated protein has lost its selectivity for the GTP-bound form of Kir/Gem (data not shown), indicating that additional cellular factors or modifications may be required for nucleotide selectivity.

Evidence for a Complex Containing the RGK Protein, Caβ3, and Ca1.2—In our computed model, Kir/Gem and Ca1.2 of VDCC interact with distinct surfaces on the NK domain of Caβ (Fig. 6, A and B). Whether the Caα1 subunit and RGK proteins compete for Caβ or whether both proteins can bind simultaneously is controversial. Although Kir/Gem and a Caα1 fragment have been thought to compete for an interaction with the β-interacting domain of Caβ (10, 21), Caβ3 has recently been shown to simultaneously bind Rem and a glutathione S-transferase-α-interacting domain fusion protein (22). Although the conflicting results may reflect the use of α-interacting domain peptides or fusion proteins in these studies, they also do not take into account that an intact Caα1 may induce steric hindrance effects or subtle conformational changes after binding to Caβ.

To experimentally determine whether a complex composed of Caβ3, Ca1.2, and Kir/Gem can exist in vivo, Myc-tagged RGK proteins, FLAG-tagged Caβ3, and HA-tagged Ca1.2 were coexpressed in COS-1 cells. To verify the presence of a tri-complex, either the RGK protein or Ca1.2 was immunoprecipitated, and the association of the other two proteins was determined. As controls, mutants of Ca1.2 (i.e. Y449A/W452A/I453A) (33, 34) and the RGK proteins (i.e. Kir/Gem R196A/V223A, Rad R208A/L235A, and Rem R236A/L263A) that do not bind to Caβ3 were used. As expected, the Caβ3 coprecipitated with the WT (Fig. 8, panel a, lanes 1, 3, 5, 7, 9, and 11) but not the mutant (lanes 2, 4, 6, 8, 10, and 12) RGK proteins, both in the presence of WT or mutated Ca1.2. In contrast, Caα1 only coprecipitated with the RGK protein if both the RGK protein and Caα1 were able to associate with Caβ3 (Fig. 8, panel b, lanes 1, 5, and 9), consistent with a complex containing all three proteins. The presence

FIGURE 6. Proposed model of Kir/Gem bound to Caβ3. A, rotations of a molecular surface model of the SH3 (blue) and NK (green) domains of Caβ3 based on the crystal structure of the β-subunit bound a fragment of the Caα1 subunit (pink). Residues implicated in Kir/Gem binding are in red. B, molecular surface models of the SH3 (blue) and NK (green) domains of Caβ3 in association with Kir/Gem (ribbon structure, cyan) bound to GTP. Residues implicated in Kir/Gem binding are in red, and the Switch 1 motif is in yellow. Note the excellent surface complementarities, in particular of the Switch 1 motif. C, view of close-ups depicting the electrostatic interactions between Arg-196 of Kir/Gem (cyan) and Asp-194, Asp-270, and Asp-272 (red) of the NK domain of Caβ3 (green). D, view from a different angle illustrating how Val-223 and His-225 of Kir/Gem (cyan) are wedged into the hydrophobic pocket of the NK domain (green) containing Leu-187 and Asn-275 (red).

the SH3 domain could be important to maintain an ordered NK surface for interaction with Kir/Gem. In a recent paper, in vitro translated Caβ truncation mutants (as opposed to point mutations in the present study) were used to show that the SH3 domain is dispensable for Rem binding and that binding occurs with the GTP- as well as the GDP-bound forms of the RGK protein (22). Consistent with this data, we similarly observed
FIGURE 7. Charge reversal experiments and molecular modeling of these interactions. A, immunoprecipitation of WT Kir/Gem or Kir/Gem R196E and WT or mutated Ca\textsubscript{v}3.3. a, lysate of transfected COS-1 cells were immunoprecipitated with FLAG antibodies to isolate WT or mutated FLAG-Ca\textsubscript{v}3.3, and associated WT or mutated Myc-Kir/Gem was revealed by Western blot using Myc antibodies. b and c, cell lysate was blotted with Myc or FLAG antibody to monitor Kir/Gem and Ca\textsubscript{v}3.3 protein expression levels, respectively. B, molecular modeling of the interactions between Arg-196 or R196E of Kir/Gem (cyan) and Asp-194, Asp-270, and Asp-272 of Ca\textsubscript{v}3.3 (red) (a) or Ca\textsubscript{v}3.3 D194R (b), Ca\textsubscript{v}3.3 D270R (c), and Ca\textsubscript{v}3.3 D272R (d). Arrows indicate possible inter (arrows with solid line)- or intramolecular (arrows with dashed line) interactions. Arg-196 of Kir/Gem and Asp-194, Asp-270, and Asp-272 of Ca\textsubscript{v}3.3 are ideally positioned to establish three interactions with computed distances of $1.65$, $1.67$, and $1.64$ Å, respectively (a). The Glu residue in Kir/Gem R196E could interact with D194R ($1.63$ Å) and weakly with Arg-216 ($1.73$ Å) of Ca\textsubscript{v}3.3 D194R. D194R could also engage Asp-270 in an intramolecular interaction ($1.68$ Å) (b). The Glu residue in Kir/Gem R196E could interact with D270R ($1.64$ Å) or Arg-216 ($1.68$ Å) of Ca\textsubscript{v}3.3 D270R. Both D272R and Arg-216 could also form intramolecular associations with Asp-272 (c). In the absence of Asp-272 in Ca\textsubscript{v}3.3 D272R, both oxygen atoms of the carboxyl group of the Glu in Kir/Gem R196E could now optimally engage Arg-216 of Ca\textsubscript{v}3.3 D272R ($1.63$ and $1.64$ Å). This interaction was facilitated by intramolecular associations of D272R with Asp-194 and Asp-270 ($1.71$ and $1.73$ Å, respectively) (d). Molecular modeling indicated that replacing more than one Asp residue in the hydrophilic pocket of Ca\textsubscript{v}3.3 with an Arg results in the collapse of the pocket, and biochemically, no interaction between these Ca\textsubscript{v}3.3 mutants and Kir/Gem R196E or R196D were detected (data not shown).
of this complex was corroborated by the inverse coimmunoprecipitation experiment, showing coprecipitation of the RGK proteins with Ca\textsubscript{1,2} (Fig. 8, panels c and d, lanes 1, 5, and 9). This association was also abolished for mutants of Ca\textsubscript{1,2} (Fig. 8, panel d, lanes 3, 4, 7, 8, 11, and 12) or RGK proteins (panel d, lanes 3, 7, and 11) that are defective for Ca\textsubscript{\beta3} binding. Western blot analysis of cell lysates expressing WT or mutated proteins confirmed similar expression levels for Ca\textsubscript{1,2}, Ca\textsubscript{\beta3}, and the RGK proteins (Fig. 8, panels e–g). Consistent with the above results, Ca\textsubscript{1,2} and the RGK proteins coprecipitated with Ca\textsubscript{\beta3} (supplemental Data VIII), although in this case the presence of two Ca\textsubscript{\beta3} populations, one bound to Ca\textsubscript{\alpha1} and the other to the RGK protein, cannot be ruled out. In conclusion, these findings provide compelling biochemical evidence for a triprotein complex containing the two VDCC subunits and the RGK protein.

Conclusions—In summary, we identified residues in Kir/Gem and Ca\textsubscript{\beta3} that are critical for their biochemical and functional interaction and used in silico homology modeling combined with molecular dynamics simulations to obtain a rendered model for the complex between the small GTP-binding protein and one of its effectors, the VDCC \beta3-subunit. The effector binding domain of Kir/Gem is located in the vicinity of the G4 and G5 motifs of the core GTP binding domain and differs from that used by other members of the Ras superfamily, where it is located between the G1 and G3 motifs (30). We propose that the main difference between the GTP- and GDP-bound forms of Kir/Gem is the conformation of the Switch 1 region and that its ordered structure in the GTP-bound form provides optimal shape complementarity between the RGK protein and the NK domain of the Ca\textsubscript{\beta3} subunit. Validation of this hypothesis will have to await the crystal structure of a complex between an RGK protein and a Ca\textsubscript{\beta3} subunit. The rendered model also shows Kir/Gem and Ca\textsubscript{\alpha} binding to distinct surfaces on the NK domain of Ca\textsubscript{\beta3}, indicating that the two proteins do not compete for the same binding site on the Ca\textsubscript{\beta3} subunit. Consistent with the model, a tri-complex containing Ca\textsubscript{\alpha1}, Ca\textsubscript{\beta3}, and the RGK protein can be isolated from cells. Whether this complex assembles in the endoplasmic reticulum and/or the plasma membrane and how it regulates VDCC function remains to be determined.

Ca\textsubscript{\alpha1} associates through a helix with a groove on the NK domain of Ca\textsubscript{\beta3} and the corresponding surface in guanylate kinase, which is highly homologous to the NK domain, is involved in nucleotide binding (32). Thus, docking of Ca\textsubscript{\alpha1} on the NK domain appears to correspond to the binding of ADP in guanylate kinase (Fig. 9). Intriguingly, a sim-
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ilar analogy can be observed for the docking of Kir/Gem to the NK domain, which appears to occur to the analogous region in guanylate kinase that binds GMP (35) (Fig. 9). In conclusion, our findings provide novel insights into the interaction between RGK proteins and VDCC subunits and functional relevance of this interaction with respect to the regulation of VDCC activity by these GTP binding proteins.

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