14-3-3 and calmodulin control subcellular distribution of Kir/Gem and its regulation of cell shape and calcium channel activity

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Summary

Individual members of the RGK family of Ras-related GTPases, which comprise Rad, Gem/Kir, Rem and Rem2, have been implicated in important functions such as the regulation of voltage-gated calcium channel activity and remodeling of cell shape. The GTPase Kir/Gem inhibits the activity of calcium channels by interacting with the β-subunit and also regulates cytoskeleton dynamics by inhibiting the Rho-Rho kinase pathway. In addition, Kir/Gem interacts with 14-3-3 and calmodulin, but the significance of this interaction on Kir/Gem function is poorly understood. Here, we present a comprehensive analysis of the binding of 14-3-3 and calmodulin to Kir/Gem. We show that 14-3-3, in conjunction with calmodulin, regulates the subcellular distribution of Kir/Gem between the cytoplasm and the nucleus. In addition, 14-3-3 and calmodulin binding modulate Kir/Gem-mediated cell shape remodeling and downregulation of calcium channel activity. Competition experiments show that binding of 14-3-3, calmodulin and calcium channel β-subunits to Kir/Gem is mutually exclusive, providing a rationale for the observed regulatory effects of 14-3-3 and calmodulin on Kir/Gem localization and function.

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Key words: 14-3-3, Calcium channel, Calmodulin, Cytoskeleton, GTPase, Small G protein

Introduction

GTPases of the Ras superfamily control a wide range of signaling pathways and can be grouped into five subfamilies: Ras, Rho, Rab, Arf and Ran (Takai et al., 2001). These proteins switch between inactive GDP and active GTP conformations. The RGK (Rad/Gem/Kir) family belongs to the Ras-related GTPase subfamily, which consists of Kir/Gem (Cohen et al., 1994; Maguire et al., 1994), Rad (also known as Rrad) (Reynet and Kahn, 1993), Rem (Finlin and Andres, 1997) and Rem2 (Finlin et al., 2000). Although the Ras-related core domain is conserved in the RGK family, its members exhibit several unique structural and functional features that differ from other GTPases. These include the lack of lipid modification for membrane anchorage, the presence of N- and C-terminal extensions, and an unconventional G3 motif (DXWE instead of DXXG). Therefore, RGK proteins probably share an exclusive molecular mechanism for GTP hydrolysis. Another distinctive characteristic is their regulation at the transcriptional level (Cohen et al., 1994; Finlin and Andres, 1997; Maguire et al., 1994; Reynet and Kahn, 1993). Aberrant expression of Gem and Rad was observed in neuroblastoma (Leone et al., 2001) and breast cancer cell lines (Tseng et al., 2001), respectively, suggesting a role in tumorigenesis.

The physiological roles of RGK proteins have only recently been explored and include the regulation of Ca^{2+} channel activity and the reorganization of the actin and microtubule cytoskeleton. The Ca^{2+}-transporting α-subunit of voltage-dependent Ca^{2+} channels associates with auxiliary subunits (β, $\alpha_2\delta$ and γ subunits) that have regulatory functions (reviewed by Catterall, 1998). The GTPase Kir/Gem interacts in a GTP-dependent manner with the Ca^{2+} channel β-subunit, resulting in the removal of functional Ca^{2+} channels from the plasma membrane and termination of Ca^{2+}-dependent secretion (Beguin et al., 2001). Although Rad and Rem were subsequently shown also to interact with the β-subunit and suppress Ca^{2+} channel function, the precise mechanism has not been determined (Finlin et al., 2003). Whether Rem2 mediates a similar function is not known.

RGK members are also involved in cytoskeletal reorganization. Overexpression of Gem stimulates neurite extension and flattening of neuroblastoma cells (Leone et al., 2001) and dendrite-like extensions in COS cells (Piddini et al., 2001), whereas Rem1 and its human ortholog Ges induce endothelial cell sprouting (Pan et al., 2000). These activities have been linked to the function of Gem and Rad as negative regulators of the Rho pathway through the inhibition of Rho kinases (Ward et al., 2002). Gem also interacts with Gmip, a

RhoGAP-containing protein (Aresta et al., 2002) and the kinesin-like protein KIF-9 (Piddini et al., 2001).

CaM is a major transducer of Ca^{2+} signaling and, among other functions, regulates Ca^{2+} channels and pumps (reviewed by Vetter and Leclerc, 2003). Rad and Kir/Gem bind CaM through their C-terminal extensions in a Ca^{2+}-dependent manner. CaM inhibits binding of GTP by Kir/Gem (Fischer et al., 1996) and shows a better affinity for the GDP-bound form of Rad (Moyers et al., 1997). The role of CaM binding on RGK-family function is still unclear but, in the case of Kir/Gem, might involve the control of nuclear localization and the regulation of Ca^{2+} channel β-subunit binding (Beguin et al., 2001).

14-3-3 proteins are a family of seven highly conserved isoforms (β, η, σ, ε, τ, γ and ζ) involved in a wide range of signaling pathways. 14-3-3 proteins function by binding to phospho-serine or threonine in the context of a consensus binding motif present in the proteins they interact with (reviewed by Tzivion et al., 2001). The binding of 14-3-3 to their target proteins can either prevent the interaction with other proteins, change the intrinsic catalytic activity of enzymes, regulate the subcellular distribution, protect from proteolysis or dephosphorylation, or provide an adaptor to bridge two proteins (reviewed by Tzivion et al., 2001). In most cases, 14-3-3 exerts its effect by inducing a conformational change in the target protein or through steric hindrance. 14-3-3 proteins can dimerize, and dimers might bind to target proteins that contain two 14-3-3-binding sites (reviewed by Yaffe, 2002). 14-3-3 also interacts with RGK proteins. Rad and Rem, but not Rem2, bind 14-3-3 in a phosphorylation-dependent manner (Finlin and Andres, 1999) and Kir/Gem interacts through two 14-3-3-binding sites (Ward et al., 2004). Although the phosphorylation state of a 14-3-3-binding site in Kir/Gem might regulate cytoskeletal reorganization and stabilize the GTPase (Ward et al., 2004), it is not clear if this reflects the phosphorylation state per se or whether 14-3-3 binding is involved.

Given the growing implication of Kir/Gem on numerous biological processes, we carried out a systematic analysis of the functional effects of CaM and 14-3-3 on Kir/Gem. We demonstrate that Kir/Gem shuttles between the cytoplasm and the nucleus by a mechanism that is tightly regulated by CaM and 14-3-3 binding. In addition, 14-3-3 and CaM modulate Kir/Gem-mediated regulation of cell shape remodeling and Ca^{2+} channel activity. Binding of CaM, 14-3-3 and the Ca^{2+} channel β-subunit to Kir/Gem is mutually exclusive and provides an explanation for the observed effects on the cellular functions of this small G protein.

Materials and Methods

Molecular biology

Point mutations were introduced into the 14-3-3-, CaM- and GTP-binding sites of mouse Kir/Gem protein by a polymerase chain reaction (PCR)-based method. $Ca_v\beta3$ and Kir/Gem were epitope-tagged at their N-terminus as previously described (Beguin et al., 2001). Amino acids K283-H290 of Kir/Gem were substituted by PHCVPRDLSWLDLEANMCLP to generate the R-18 chimera (Wang et al., 1999) and a 14-3-3 ζ dimerization mutant was produced as described (Tzivion et al., 1998). To generate the recombinant proteins, full-length cDNAs for $Ca_v\beta3$ (kindly provided by S. Seino, Kobe University, Japan), Kir/Gem and 14-3-3 were amplified by PCR and introduced into pGEX-6P1 and purified according to the manufacturer's instructions. Mouse Kir/Gem was cloned as described previously (Beguin et al., 2001). cDNAs for human 14-3-3 β, γ, ε, σ, τ, η and ζ isoforms were obtained from IMAGE Consortium (5482228, 5532354, 5502318, 5476961, 5478108, 5531102 and 5492512, respectively). All constructs and purchased cDNAs were verified by automatic DNA sequence analysis (Perkin Elmer).

Cell culture and DNA transfection

COS-1 and PC-12 cells were grown as previously described (Beguin et al., 2001) and were transiently transfected with wt or mutant cDNAs using lipofectamine™, Plus™ and Opti-MEM I reagents (Invitrogen) according to the manufacturer's instructions. Biochemical and immunofluorescence experiments were performed 24-48 hours after transfection.

Biochemistry

Cell homogenates were prepared as follows: cells were lysed into a buffer [50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT and 0.2% Tween-20 supplemented with 5 μg/ml each of proteases inhibitors leupeptin, pepstatin and antipain]. Harvested cells were then subjected to two cycles of freeze/thaw followed by brief sonication and the insoluble material was removed by centrifugation. Analysis of the soluble fraction showed the presence of cytosolic and nuclear proteins. Cellular expression levels of proteins were analyzed by SDS-page (8%) and western blot analysis using monoclonal anti-Myc (Roche), monoclonal anti-Flag (M2; Sigma), rabbit anti-GST (Santa Cruz), and rabbit anti-14-3-3 (Zymed) antibodies. For coprecipitation experiments, cell lysates (300-400 μg protein) were incubated with GST-agarose beads (Sigma), Flag-agarose beads (Sigma) or Myc-agarose beads (Santa Cruz) in lysis buffer (see above) for 3 hours at 4°C. Beads were then extensively washed and eluted protein complexes were subjected to SDS-PAGE and western blot analysis as described above. Pull-down experiments using GST-Kir/Gem proteins were essentially carried out as described (Beguin et al., 2001). Pull-down experiments using GST-$Ca_v\beta3$ were performed in a similar way with the exception that COS-1 cell lysates were prepared and incubated without or with either 0.5 mM GDP-βS or 0.5 mM GTP-γS for 45 minutes at 30°C prior to being used for GST-$Ca_v\beta3$ binding. CaM binding was performed as described (Moyers et al., 1997). Briefly, CaM-Sepharose beads (Amersham Biosciences) were incubated at 4°C for 3 hours with a cell lysate (300-400 μg protein) prepared as described above, in a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 1% Triton X-100. Beads were then extensively washed with the same buffer and CaM-associated Kir/Gem eluted in SDS-PAGE sample buffer prior to SDS-PAGE and western blot analysis. For CaM competition experiments, cell lysates (300-400 μg protein) containing overexpressed N-Flag 14-3-3 and N-Myc Kir/Gem or N-Flag $Ca_v\beta3$ and N-Myc Kir/Gem were first immunoprecipitated using Flag-agarose beads (Sigma) at 4°C for 3 hours. Flag beads were then extensively washed and bound N-Flag 14-3-3/N-Myc Kir/Gem or N-Flag $Ca_v\beta3$/N-Myc Kir/Gem complexes eluted using an excess of Flag peptide (Sigma). Purified complexes were subsequently subjected to either CaM binding or inverse immunoprecipitation using Myc-agarose beads as described above. Subcellular fractionation was performed using the NE-PER kit (Pierce Biotechnology) according to the manufacturer's instructions. Poly-ADP ribose polymerase and α-tubulin were used as nuclear and cytosolic markers, respectively, and detected with corresponding antibodies (Santa Cruz).

Immunocytochemistry

Cells were fixed with 3.7% paraformaldehyde, washed twice with

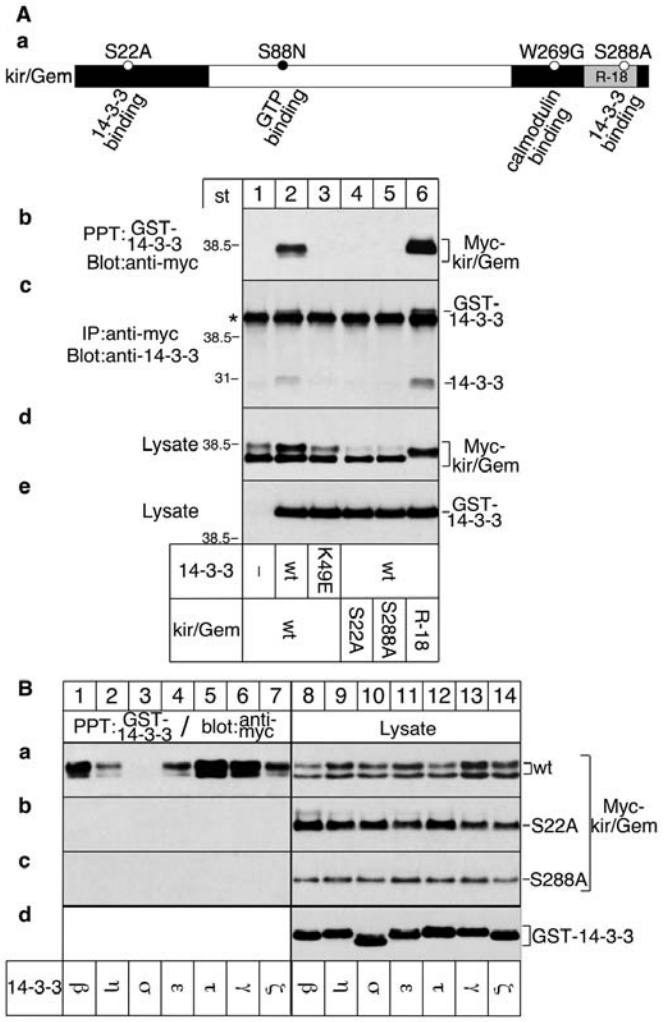


Fig. 1. Association of 14-3-3 and Kir/Gem. (A) 14-3-3 binding to Kir/Gem. (a) The Ras-like core domain (white bar), N- and C-terminal extensions (black bar) and the location of the binding sites for 14-3-3, CaM (white circles) and GTP (black circle) are shown. Mutations that affect the different binding sites are indicated. Kir/Gem-R-18 is a chimera in which the C-terminus (including S288A) was substituted by a stretch of 18 amino acids (R-18) that mediates constitutive 14-3-3 binding. (b) Cells were cotransfected with cDNAs for wt or mutated Myc-Kir/Gem proteins and GST–14-3-3 or GST–14-3-3 K49E ζ isoform. GST–14-3-3 proteins were precipitated and associated Kir/Gem detected by western blot using a Myc antibody. (c) Cells were cotransfected with cDNAs for wt or mutated Myc-Kir/Gem proteins and GST–14-3-3 or GST–14-3-3 K49E. Kir/Gem was immunoprecipitated and associated GST–14-3-3 and endogenous 14-3-3 was detected by western blot using a 14-3-3 antibody. The IgG heavy chain, migrating just below the GST–14-3-3 in (c), is marked by an asterisk. (d and e) Cell lysates were blotted with Myc (d) or GST (e) antibodies to monitor the expression level of Myc-Kir/Gem or GST–14-3-3; st, proteins markers of known molecular mass. (B) Association of Kir/Gem with 14-3-3 isoforms. (a-c, lanes 1-7) Cells were cotransfected with cDNAs for the different GST–14-3-3 isoforms and wt or mutated Myc-Kir/Gem. GST–14-3-3 proteins were precipitated and associated Kir/Gem was detected by western blot using a Myc antibody. (a-c, lanes 8-14) Cell lysates were blotted with Myc antibodies to monitor Kir/Gem expression levels. (d) One example of cell lysates blotted with a GST antibody to monitor expression levels of the GST–14-3-3 isoforms.

phosphate-buffered saline (PBS), quenched with 50 mM NH_4Cl and permeabilized with 0.2% Triton X-100. Cells were then incubated in blocking solution [PBS containing 0.1% Triton X-100, 0.1% bovine serum albumin (BSA) and 250 mM NaCl] for 30 minutes followed by incubation with rabbit anti-Myc (Upstate Biotechnology) and monoclonal anti-GST (Cell Signaling Technology), suitably diluted in blocking solution for 1 hour. After

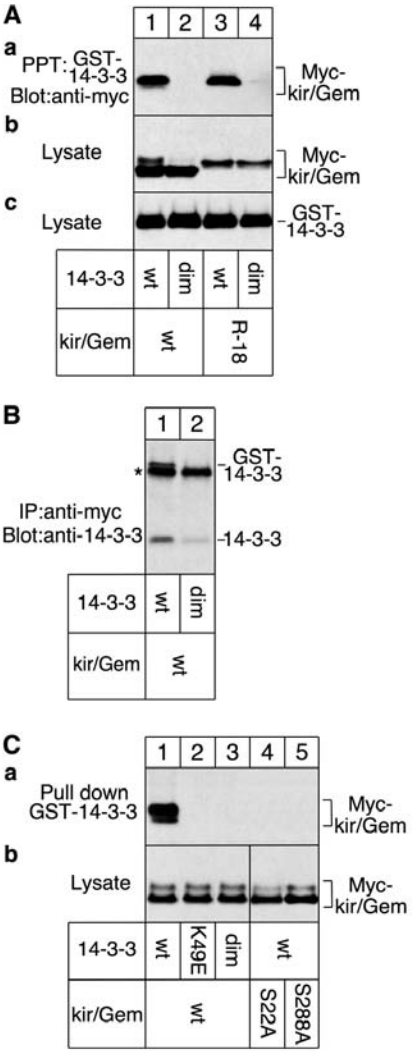


Fig. 2. 14-3-3 dimerization is required for efficient binding to Kir/Gem. (A) Coprecipitation. (a) Cells were cotransfected with cDNAs for wt Myc-Kir/Gem or the R18 chimera and GST–14-3-3 or a dimerization defective mutant (dim). GST–14-3-3 proteins were precipitated and associated Kir/Gem was detected by Western blot using a Myc antibody. (b and c) Cell lysates were blotted with Myc (b) or GST (c) antibodies to monitor Kir/Gem protein and GST–14-3-3 expression levels. (B) Coimmunoprecipitation. Myc-Kir/Gem was immunoprecipitated from cells coexpressing GST–14-3-3 or a GST–14-3-3 dimerization mutant. Overexpressed and endogenous 14-3-3 associated with Myc-Kir/Gem were revealed by western blot analysis using a 14-3-3 antibody. The asterisk indicates the band for IgG heavy chain. (C) Pull-down experiment. (a) Wt and mutated immobilized recombinant GST–14-3-3 were tested for interaction with wt or mutated Myc-Kir/Gem present in homogenates of transfected cells. Kir/Gem proteins were revealed by western blot using Myc antibody. (b) Cell lysates were blotted with a Myc antibody to monitor Myc-Kir/Gem protein expression levels.

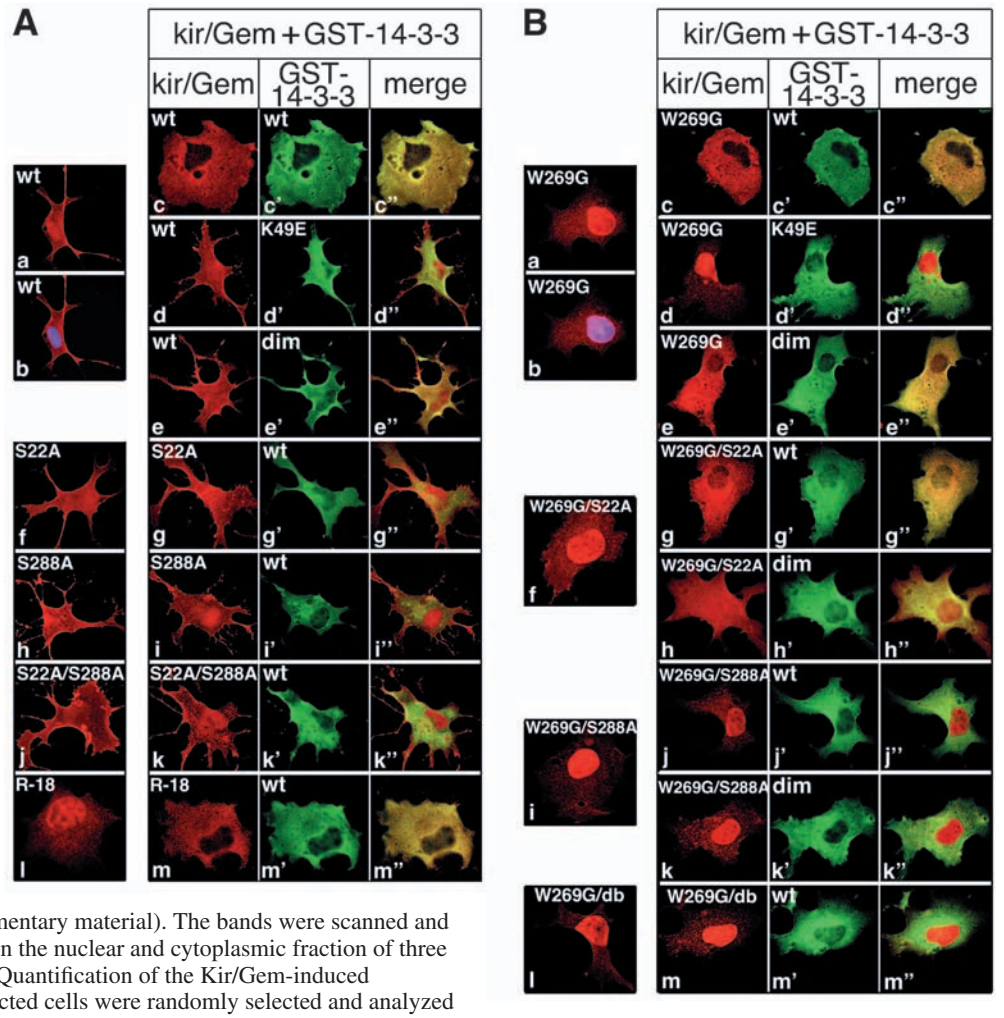


Fig. 3. 14-3-3 and CaM regulate the subcellular distribution of Kir/Gem and Kir/Gem-mediated changes in cell morphology. (A and B) COS-1 cells were transfected with cDNAs for wt or mutated Kir/Gem, either alone, or together with GST–14-3-3, GST–14-3-3 K49E or GST–14-3-3 dimerization mutant. Cells were processed for immunofluorescence microscopy using Myc and GST antibodies to label Kir/Gem (red) and GST–14-3-3 (green), respectively. Areas of colocalization are in yellow in the merged image. Nuclei were stained with Hoechst 2022 (A and B, panel b). (C) Quantification of the 14-3-3-mediated cytosolic relocalization of Kir/Gem. 150-200 transfected cells were randomly selected and analyzed in 3-5 independent experiments. The fraction of cells showing efficient (black bars), partial (gray bar) or no (white bars) nuclear clearance is plotted. (D) Quantification of the 14-3-3-mediated cytosolic relocalization of Kir/Gem. Transfected cells were subjected to subcellular fractionation to obtain nuclear (N) and cytoplasmic (C) fractions. Kir/Gem in the different fractions was detected by SDS-PAGE and western blot analysis (Fig. S1 in supplementary material). The bands were scanned and the fraction of total Kir/Gem recovered in the nuclear and cytoplasmic fraction of three independent experiments is plotted. (E) Quantification of the Kir/Gem-induced morphological changes. 150-200 transfected cells were randomly selected and analyzed in 3-5 independent experiments. The fraction of cells showing one or more dendrite-like extensions, defined as thin protrusions of at least half a cell diameter, is plotted.

washing, the labeled secondary antibodies Cy3-labeled donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories) and AlexaFluor 488 goat anti-mouse IgG (Molecular Probes) were used. Specimens were visualized with an Axiocam microscope (Carl Zeiss) at 100× magnification.

Electrophysiology

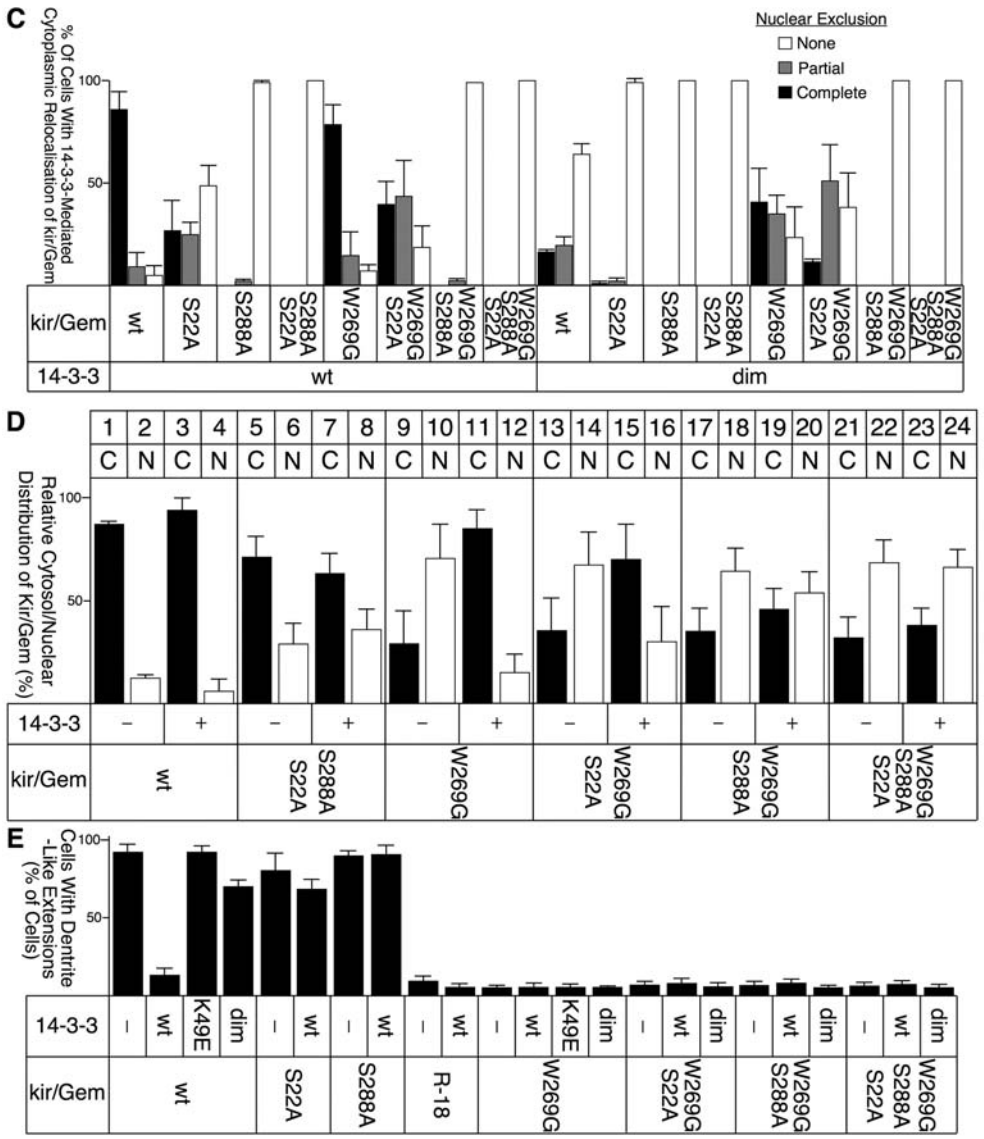
Recordings were made 48 hours after transfection using an Axopatch 200B amplifier (Axon Instruments). The whole-cell VDCC currents in PC12 cells were recorded as described (Beguin et al., 2001). Briefly, Ba^{2+} was used as a charged carrier for measurement of VDCC currents. The extracellular solution contained 40 mmol/l $Ba(OH)_2$, 20 mmol/l 4-aminopyridine, 110 mmol/l tetraethylammonium hydroxide, 10 mmol/l tetraethylammonium chloride, 140 mmol/l methanesulfonate and 10 mmol/l 3-(*N*-morpholino)propanesulfonic acid (pH 7.4). The pipette solution contained 10 mmol/l CsCl, 130 mmol/l cesium aspartate, 10 mmol/l EGTA, 10 mmol/l 3-(*N*-morpholino)propanesulfonic acid and 5 mmol/l Mg-ATP (pH 7.2). Cells were maintained at a holding potential of –60 mV. For recording VDCC currents, square pulses of 400 millisecond duration at potentials between –40 and +60 mV in steps of 10 mV were applied every 4 seconds. For normalization, the currents were divided by the membrane capacitance measured for each cell (Kawaki et al., 1999).

Results

Characterization of 14-3-3 binding to Kir/Gem

To characterize the interaction between Kir/Gem and 14-3-3, Myc-Kir/Gem and GST–14-3-3 ζ were coexpressed in COS-1 cells and tested for their ability to coprecipitate (Fig. 1Ab,c). As controls, we used Kir/Gem mutants in which the N- and C-terminal 14-3-3-binding sites (S22 and S288) (Ward et al., 2004) were mutated (Fig. 1Aa), either alone (Kir/Gem S22A, Kir/Gem S288A) or in combination (Kir/Gem S22A/S288A). A chimera (Kir/Gem-R-18) was also generated in which the eight C-terminal amino acids (including S288) were replaced with the R-18 sequence, which mediates constitutive binding to 14-3-3 (Wang et al., 1999). In addition, we used a 14-3-3 mutant (GST–14-3-3 ζ K49E) that cannot bind to target proteins (Fu et al., 2000).

As shown in Fig. 1A (b and c), Kir/Gem associated with GST–14-3-3 (lanes 2 and 6) but not with GST–14-3-3 K49E (lane 3), confirming a specific interaction. Both 14-3-3-binding sites in Kir/Gem were required for the interaction since Kir/Gem S22A or Kir/Gem S288A (lanes 4 and 5) failed to bind. As expected, Kir/Gem-R18 efficiently bound both GST–14-3-3 (panel c, lane 6). Interestingly, the small amount



of endogenous 14-3-3 associated with Kir/Gem increased following overexpression of GST–14-3-3 (panel c; compare lanes 1 with 2), indicating that in COS-1 cells there is only a limited pool of free 14-3-3 available for binding. Consistent with this interpretation, endogenous 14-3-3 bound more efficiently to Kir/Gem-R18, which binds 14-3-3 constitutively. Analysis of cell lysates of the transfected cells showed similar expression levels for the different Kir/Gem proteins (panel d) and GST–14-3-3 or GST–14-3-3 K49E (panel e).

As previously shown (Leone et al., 2001), Kir/Gem migrated as a doublet in SDS-PAGE (panel d), indicative of a post-translational modification. The ratio between the slower and faster migrating band was affected following mutation of the 14-3-3-binding sites or overexpression of GST–14-3-3 (panel d; compare lanes 1 with 2-5), suggesting that 14-3-3 either induces or stabilizes this modification. Intriguingly, 14-3-3 associated mainly with the slower migrating form of Kir/Gem (panel b, lane 2).

To determine if Kir/Gem associates with 14-3-3 isoforms other than 14-3-3 ζ, coprecipitation experiments with the seven known 14-3-3 isoforms (Fu et al., 2000) were carried out (Fig. 1B). With the exception of 14-3-3 σ, Kir/Gem associated with all 14-3-3 isoforms (panel a, lanes 1-7). In agreement with the data in Fig. 1A using 14-3-3 ζ, mutation of either the N- or C-terminal 14-3-3-binding site in Kir/Gem was sufficient to abolish the interaction with each of the 14-3-3 isoforms (panels b and c, lanes 1-7).

The requirement for two binding sites in Kir/Gem for 14-3-3 binding suggests an association with 14-3-3 dimers. To test this assumption, we used a 14-3-3 ζ mutant that can no longer dimerize but still binds to target proteins (Tzivion et al., 1998). In coprecipitation experiments, the dimerization-defective 14-3-3 failed to associate with either Kir/Gem or Kir/Gem-R-18 (Fig. 2Aa, compare lanes 1 and 3 with lanes 2 and 4, respectively). These results were confirmed in the inverse coprecipitation experiment (Fig. 2B).

Similar data were also obtained in pull-down experiments using immobilized GST fusion proteins (Fig. 2C). GST–14-3-3, but not GST–14-3-3 K49E or the dimerization-defective mutant, bound Kir/Gem (panel a; compare lane 1 to 2 and 3) and binding was abolished if one of the two 14-3-3-binding sites in Kir/Gem was inactivated (lanes 4 and 5).

In conclusion, Kir/Gem associates with all 14-3-3 isoforms except 14-3-3 σ and the concurrent requirement of both the N- and C-terminal site reflects its association with 14-3-3 dimers.

Binding of 14-3-3 and CaM regulates the subcellular distribution of Kir/Gem

To explore the functional role of 14-3-3 binding on Kir/Gem function, we analyzed whether 14-3-3 binding had an effect on the subcellular distribution of the small G protein. COS-1 cells were transfected with cDNAs for Kir/Gem or mutants lacking one (Kir/Gem S22A, Kir/Gem S288A) or both (Kir/Gem S22A/S288A) 14-3-3-binding sites, either alone or together with cDNAs for GST–14-3-3 ζ or GST–14-3-3 ζ K49E. The subcellular localization of Kir/Gem was then analyzed by immunofluorescence microscopy (Fig. 3A,B).

Expression of Kir/Gem in COS-1 cells was distributed throughout the cells and slightly enriched at submembranous and perinuclear locations and in the nucleus (Fig. 3Aa). This distribution was not markedly changed for Kir/Gem S22A, Kir/Gem S288A and Kir/Gem S22A/S288A (panels f, h and j).

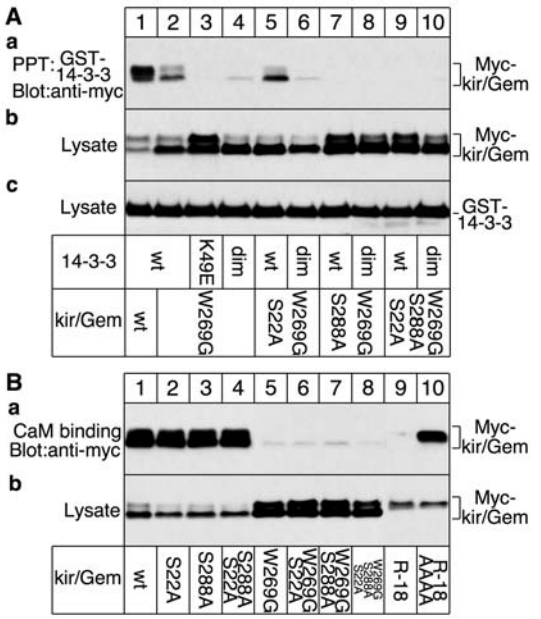


Fig. 4. Binding of 14-3-3 and CaM to Kir/Gem. (A) 14-3-3 binding. Cells were cotransfected with cDNAs for wt or mutated Myc-Kir/Gem and GST–14-3-3, GST–14-3-3 K49E or GST–14-3-3 dimerization mutant. (a) GST–14-3-3 proteins were precipitated and associated Myc-Kir/Gem was detected by western blot using Myc antibody. (b and c) Cell lysates were blotted with Myc (b) or GST (c) antibodies to monitor Kir/Gem and GST–14-3-3 expression levels. (B) CaM binding. (a) Cells were transfected with cDNAs for wt or mutated Myc-Kir/Gem. Cell homogenates were incubated with CaM beads and bound Myc-Kir/Gem detected by western blot using a Myc antibody. (b) Cell lysates were blotted with Myc antibodies to monitor Kir/Gem expression levels.

In comparison, the Kir/Gem-R18 chimera showed a more prominent nuclear localization (panel l).

Interestingly, coexpression of GST–14-3-3 (or Flag–14-3-3; data not shown) and Kir/Gem led to the efficient nuclear exclusion of the small G protein (panels c-c''). By contrast, Kir/Gem S22A (panels g-g''), Kir/Gem S288A (panels i-i'') and Kir/Gem S22A/S288A (panels k'-k'') were not cleared from the nucleus. Remarkably, like Kir/Gem, the Kir/Gem-R18 chimera was efficiently redistributed by exogenous GST–14-3-3 (panels m-m''). These data indicated that, in order to mediate cytoplasmic relocalization, GST–14-3-3 had to bind to Kir/Gem. Consistent with this conclusion, neither GST–14-3-3 K49E (panels d-d'') nor the dimerization-defective 14-3-3 (panels e-e''), both of which do not bind Kir/Gem, showed an effect on Kir/Gem localization.

CaM has been implicated in altering the subcellular distribution of Kir/Gem (Beguin et al., 2001), suggesting a possible interplay between 14-3-3 and CaM in regulating the subcellular distribution of Kir/Gem. We therefore extended our analysis to include Kir/Gem W269G, a mutant that does not bind CaM (Fischer et al., 1996), and derived additional mutants that lacked either the N- (Kir/Gem W269G/S22A) or C- (Kir/Gem W269G/S288A) terminal 14-3-3-binding site, or both (Kir/Gem W269G/S22A/S288A) (Fig. 1Aa).

Similar to HEK 293 cells (Beguin et al., 2001), Kir/Gem W269G showed a predominant nuclear localization in COS-1 cells following either transient (Fig. 3Ba) or stable (data not shown) expression. A comparable distribution was observed for Kir/Gem W269G/S22A (panel f), Kir/Gem W269G/S288A (panel i) and Kir/Gem W269G/S22A/S288A (panel l). If, however, GST–14-3-3 was coexpressed, Kir/Gem W269G (panels c-c''), but not Kir/Gem W269G/S288A (panels j-j'') or Kir/Gem W269G/S22A/S288A (panels m-m''), was efficiently excluded from the nucleus. GST–14-3-3 K49E showed no effect (panels d-d''), confirming that 14-3-3 needs to bind to Kir/Gem to mediate cytoplasmic relocalization of the RGK protein. Surprisingly, overexpression of GST–14-3-3 induced an efficient cytoplasmic relocalization of Kir/Gem W269G/S22A (panels g-g''), suggesting that, in the absence of CaM binding, the C-terminal binding site is sufficient for the effect mediated by 14-3-3. Consistent with this interpretation, the dimerization-defective 14-3-3 was able to relocalize Kir/Gem W269G (panels e-e'') and Kir/Gem W269G/S22A (panels h-h''), but not Kir/Gem W269G/S288A (panels k-k'').

The changes in subcellular distribution of Kir/Gem or mutants defective in 14-3-3 following the overexpression of GST–14-3-3 as observed by immunofluorescence microscopy (Fig. 3A,B) were confirmed biochemically by subcellular fractionation and detection of Kir/Gem to the nuclear and cytosolic fraction of cells (Fig. S1 in supplementary material). Quantification of the immunocytochemical and biochemical data is shown in Fig. 3C and D, respectively.

The above data suggested that, in the absence of CaM binding (i.e. in the context of the W269G mutation), the C-terminal 14-3-3-binding site in Kir/Gem is sufficient for 14-3-3 binding and does not require 14-3-3 dimerization. To test this hypothesis, coprecipitation experiments were carried out to determine which of the different Kir/Gem W269G mutants were able to bind 14-3-3 (Fig. 4A). Indeed, 14-3-3 associated with Kir/Gem W269G (panel a, lane 2) and with Kir/Gem W269G/S22A (panel a, lane 5), but not with Kir/Gem mutants that lacked S288 (panel a, lanes 7 and 9). Similar results were obtained for the dimerization-defective 14-3-3 mutant, although binding was less efficient (panel a, lanes 4, 6, 8 and 10). We consistently observed that the association between Kir/Gem W269G and dimeric or monomeric 14-3-3 was less efficient as compared with wild-type (wt) Kir/Gem and dimeric 14-3-3, probably reflecting a less stable interaction as suggested by the faster dissociation of Kir/Gem W269G (Fig. S2a in supplementary material). As compared with Kir/Gem, overexpression of GST–14-3-3 increased the expression of the slower migrating form of Kir/Gem W269G, which also preferentially bound to 14-3-3 (panel a and b; compare lanes 1 and 2).

Next, we analyzed the ability of the different Kir/Gem mutants to bind CaM by incubating CaM-agarose beads with homogenates from cells expressing the Kir/Gem mutants. As shown in Fig. 4B, Kir/Gem, Kir/Gem S22A, Kir/Gem S288A and Kir/Gem S22A/S288A efficiently associated to CaM-agarose and binding was abolished for the corresponding mutants carrying the W269G substitution (Fig. 4B; panel 1; compare lanes 1 and 5, 2 and 6, 3 and 7 and 4 and 8, respectively). However, owing to the poor binding of endogenous 14-3-3, these experiments do not prove that CaM binding is independent of 14-3-3 binding. It was therefore important to analyze Kir/Gem-R-18, which constitutively binds endogenous 14-3-3. Interestingly, Kir/Gem-R18 did not

associate with CaM, but substitution of four amino acids in R-18 that abolish 14-3-3 binding (R-18AAAA; Wang et al., 1999) restored CaM-binding (panel a, compare lanes 9 and 10), suggesting that CaM and 14-3-3 might compete for binding to Kir/Gem (see below).

In conclusion, the above data reveal a close correlation between the subcellular localization of Kir/Gem and CaM or 14-3-3 binding. The absence of CaM binding favors nuclear localization of Kir/Gem, whereas 14-3-3 binding promotes cytoplasmic redistribution. Furthermore, in the absence of CaM binding, 14-3-3 no longer requires dimerization or the presence of two 14-3-3-binding sites to associate with Kir/Gem, indicating that monomeric 14-3-3 can bind to the C-terminus of Kir/Gem provided CaM is not bound.

14-3-3 and CaM modulate Kir/Gem-mediated changes in cell shape

To explore a possible role of 14-3-3 or CaM binding on different functions of Kir/Gem, we first analyzed if Kir/Gem-induced changes in cell morphology (Leone et al., 2001; Piddini et al., 2001; Ward et al., 2002) were affected by mutating the 14-3-3-binding sites or overexpressing 14-3-3. Kir/Gem or mutants defective in 14-3-3 binding were coexpressed with GST–14-3-3 in COS-1 cells and the induction of dendrite-like extensions was visualized by immunofluorescence microscopy (Fig. 3A,B) and quantified (Fig. 3E) as indicated in the legend to Fig. 3.

Kir/Gem (Fig. 3A, panel a), as well as Kir/Gem S22A (panel f), Kir/Gem S288A (panel h) and Kir/Gem S22A/S288A (panel j) induced dendrite-like extensions. Coexpression with GST–14-3-3 abolished the morphological changes induced by Kir/Gem (panel c-c”) and the inhibitory effect by 14-3-3 required the ability of GST–14-3-3 to bind to Kir/Gem since neither GST–14-3-3 K49E (panel d-d”) nor the dimerization-defective 14-3-3 (panel e-e”) suppressed the Kir/Gem-induced extensions. Consistent with this finding, GST–14-3-3 did not prevent the morphological changes mediated by Kir/Gem S22A (panels g-g”), Kir/Gem S288A (panels i-i”) or Kir/Gem S22A/S288A (panels k-k”) mutants, since they do not associate with 14-3-3.

As might be expected from their predominant nuclear localization, Kir/Gem W269G (Fig. 3B, panel a), Kir/Gem W269G/S22A (panel f), Kir/Gem W269G/S288A (panel i) and Kir/Gem W269G/S22A/S288A (panel l) did not induce dendrite-like extensions. Despite the cytoplasmic relocalization of Kir/Gem W269G (panels c-c” and e-e”) and Kir/Gem W269G/S22A (panels g-g” and h-h”) mediated by the coexpression of GST–14-3-3 or the dimerization-defective 14-3-3 mutant, no morphological changes were observed. This result is expected given the inhibitory effect of 14-3-3 binding on Kir/Gem-induced extensions.

Thus, the effect of Kir/Gem on cell morphology can be inhibited either by 14-3-3 binding or nuclear sequestration.

Binding of 14-3-3 and CaM to Kir/Gem is mutually exclusive

The C-terminal serine (S288) in Kir/Gem critical for 14-3-3 binding is in close proximity to the region where CaM binds to the small G protein, raising the possibility that CaM and

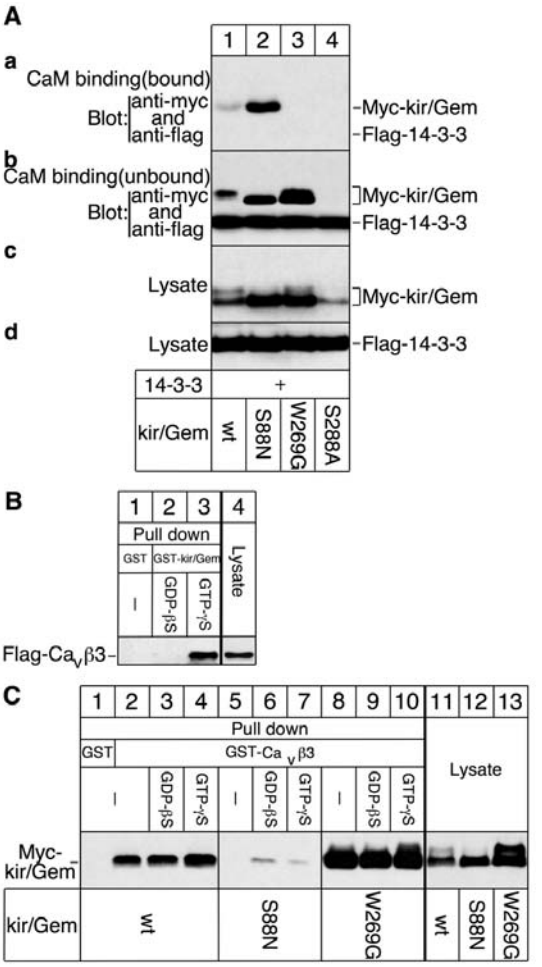


Fig. 5. Binding of CaM and 14-3-3 to Kir/Gem is mutually exclusive (A) and Kir/Gem in COS-1 cells is in a constitutively active, GTP-bound form (B and C). (A) Binding of 14-3-3/Kir/Gem complexes to CaM. (a) Cells were cotransfected with cDNAs for wt or mutated Myc-Kir/Gem and Flag–14-3-3. Flag–14-3-3 was first immunoprecipitated and Flag–14-3-3 (and associated Kir/Gem, if any) was eluted from the beads (under conditions that do not disrupt the 14-3-3/Kir/Gem complex; see Fig. S2a in supplementary material) and incubated with CaM beads. Flag–14-3-3 and Myc-Kir/Gem proteins associated with the CaM beads were detected by western blot by probing with both Flag and Myc antibodies. (b) The supernatant after the CaM pull-down was blotted with both Flag and Myc antibodies to detect unbound Flag–14-3-3 and Myc-Kir/Gem. (c and d) Cell lysates were blotted with Myc (c) and Flag (d) antibodies to monitor Flag–14-3-3 and Myc-Kir/Gem expression levels. (B) $Ca_v\beta3$ binds to the GTP but not GDP form of Kir/Gem. Homogenates from cells expressing Flag-$Ca_v\beta3$ were incubated with recombinant GST-Kir/Gem preloaded with either GDP-βS (lane 2) or GTP-γS (lane 3) and bound Flag-$Ca_v\beta3$ was detected by western blot using a Flag antibody. Recombinant GST served as a control (lane 1). Cell lysates were blotted with a Flag antibody to monitor Flag-$Ca_v\beta3$ protein expression levels (lane 4). (C) Binding of cellular Kir/Gem to $Ca_v\beta3$. Cells were transfected with cDNAs for wt or mutated Myc-Kir/Gem. Cell homogenates were incubated with immobilized recombinant GST-$Ca_v\beta3$ and associated Kir/Gem was detected by western blot using a Myc antibody. During the pull-down experiment, homogenates were preincubated either in the absence or in the presence of exogenous nucleotides. Recombinant GST served as a control (lane 1). Cell lysates were blotted with a Myc antibody to monitor Kir/Gem expression levels (lanes 11-13).

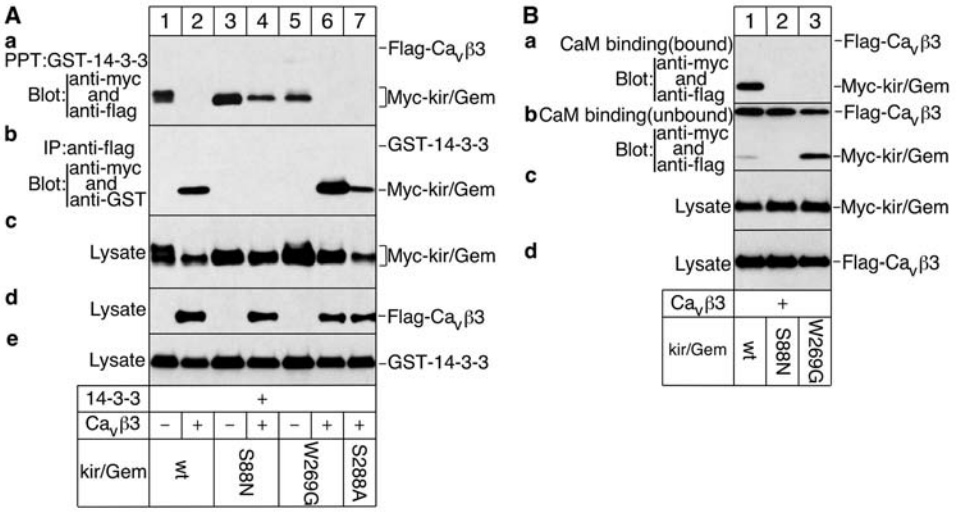


Fig. 6. $Ca_v\beta3$ and 14-3-3 compete for binding to Kir/Gem (A) and CaM displaces $Ca_v\beta3$ from Kir/Gem (B). (A) Binding of 14-3-3, $Ca_v\beta3$ and Kir/Gem. Cells were cotransfected with cDNAs for wt or mutated Myc-Kir/Gem and GST–14-3-3, either with or without Flag-$Ca_v\beta3$. (a) GST–14-3-3 was precipitated and associated Myc-Kir/Gem and Flag-$Ca_v\beta3$ was detected by western blot by sequentially probing with Myc and Flag antibodies, respectively. (b) Flag-$Ca_v\beta3$ subunits were immunoprecipitated and associated Myc-Kir/Gem and GST–14-3-3 were detected by western blot by sequentially probing with Myc and GST antibodies, respectively. (c-e) Cell lysates were blotted with Myc (c), Flag (d) or GST (e) antibodies to monitor expression levels of Myc-Kir/Gem, Flag $Ca_v\beta3$ and GST–14-3-3, respectively. (B) CaM binding. (a) Cells were cotransfected with cDNAs for wt or mutated Myc-Kir/Gem and Flag-$Ca_v\beta3$. Flag-$Ca_v\beta3$ was immunoprecipitated. Flag-$Ca_v\beta3$ (and associated Kir/Gem, if any) was eluted from the beads (under conditions that do not disrupt the Flag-$Ca_v\beta3$/Kir/Gem complex; see Fig. S2b in supplementary material) and incubated with CaM beads. Flag-$Ca_v\beta3$ and Myc-Kir/Gem associated with the CaM beads were detected by western blot by probing with both Flag and Myc antibodies. (b) The supernatant after the CaM pull-down was blotted with both Flag and Myc antibodies to detect unbound Flag-$Ca_v\beta3$ and Myc-Kir/Gem. (c and d) Cell lysates were blotted with Myc (c) and Flag (d) antibodies to monitor Flag-$Ca_v\beta3$ and Myc-Kir/Gem protein expression levels.

14-3-3 might not be able to bind to Kir/Gem simultaneously. To determine if 14-3-3 bound to Kir/Gem interferes with the association of CaM, we carried out sequential binding experiments. Flag–14-3-3 and Myc-Kir/Gem were coexpressed in COS-1 cells, Flag–14-3-3 and associated Myc-Kir/Gem immunoprecipitated and eluted from the beads under conditions that do not disrupt the complex (Fig. S2a in supplementary material). The eluted protein complex was then used for binding assays with CaM beads (see Materials and Methods and Fig. 5A legend).

As shown in Fig. 5A, preformed complexes between Kir/Gem and 14-3-3 inefficiently bound to CaM (panel a, lane 1) and 14-3-3 could not be detected in the bound fraction (panel a) but remained in the unbound supernatant (panel b), suggesting that the presence of 14-3-3 on Kir/Gem interferes with binding to CaM. A mutually exclusive binding of 14-3-3 and CaM was further supported by the observation that only the faster migrating form of Kir/Gem, which binds to 14-3-3 only weakly, was recovered in the bound fraction (panel a, lane 1), whereas the slower migrating form of Kir/Gem, which selectively associates with 14-3-3 (Fig. 1Ab), remained in the unbound fraction (Fig. 5C, panel b, lane 1). Abolishing GTP binding to Kir/Gem (Kir/Gem S88N) enhanced the association with CaM (panel a, lane 2). Since Kir/Gem was initially isolated as a complex with 14-3-3, abolishing GTP binding appears to reduce the affinity between Kir/Gem and 14-3-3 and thereby facilitate the displacement of 14-3-3 by CaM. As expected, Kir/Gem W269G was only recovered in the unbound fraction (panel a, lane 3) and Kir/Gem S288A, defective in 14-3-3 binding, was absent from both fractions (panels a and b, lane 4). Endogenous CaM was not detected in complexes of Flag–14-3-3 and Kir/Gem, further arguing against the presence of a complex containing all three proteins (Fig. S2c in supplementary material). Thus, CaM and 14-3-3 bind to Kir/Gem in a mutually exclusive manner.

Kir/Gem is present in its constitutively active form in COS-1 cells

We next extended our analysis of the binding of 14-3-3 and CaM to Kir/Gem to include a Kir/Gem effector, the β3 subunit of the voltage-gated Ca^{2+} channel ($Ca_v\beta3$). As assessed by pull-down experiments, the GTP-bound form of Kir/Gem selectively interacts with the β-subunit expressed in *Xenopus laevis* oocytes (Beguin et al., 2001). To verify this result for Flag-$Ca_v\beta3$ expressed in COS-1 cells, a pull-down experiment using immobilized GST-Kir/Gem, which was preloaded with either GTP-γS or GDP-βS, was carried out. As shown in Fig. 5B, the β-subunit only bound to the GTP-γS-loaded, activated form (lane 3) but not to the GDP-βS-loaded, inactive form (lane 2) of GST-Kir/Gem.

Having confirmed that $Ca_v\beta3$ is a Kir/Gem effector, we used immobilized GST-$Ca_v\beta3$ to probe the activation state of Kir/Gem expressed in COS-1 cells. As shown in Fig. 5C, Kir/Gem efficiently bound to the β-subunit (lane 2). Unexpectedly, the addition of GDP-βS to cell lysates did not interfere with the binding of Kir/Gem to GST-$Ca_v\beta3$ (lane 3), showing that the non-hydrolyzable GDP analog was unable to replace the cellular GTP presumably bound to Kir/Gem. By contrast, the addition of GTP-γS did not enhance binding efficiency (lane 4), consistent with the bulk of Kir/Gem in COS cells already being in the active form. The interaction of Kir/Gem S88N, which cannot bind GTP, with GST-$Ca_v\beta3$ was dramatically reduced (lanes 5-7), confirming that binding required the active, GTP-bound form, Kir/Gem. Kir/Gem W269G, which predominantly localizes to the nucleus, showed a GST-$Ca_v\beta3$ binding behavior that was similar to Kir/Gem (lanes 8-10), indicating that Kir/Gem found in the nucleus is also in its active state.

In conclusion, the finding that neither GDP-βS nor GTP-γS influenced binding of Kir/Gem to its effector, $Ca_v\beta3$, suggests

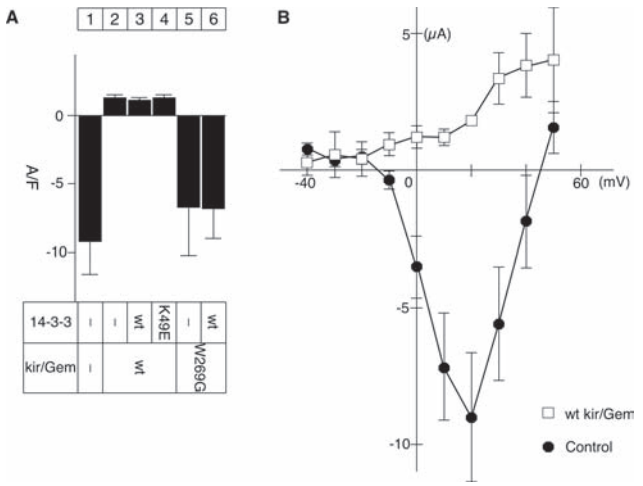


Fig. 7. Kir/Gem downregulates Ca^{2+} channel activity. (A) PC12 cells were cotransfected with a GFP plasmid and cDNAs for wt or mutated Kir/Gem, either with or without 14-3-3 or 14-3-3 K49E. GFP-positive cells were selected for electrophysiology and the average of the maximal current detected at +20 mV for endogenous Ca^{2+} channels was measured. Between 9 and 16 independent experiments were carried out for each condition. (B) An example of the I-V relationship of Ca^{2+} channels in PC-12. A, Ampere; F, Faraday. Cells transfected only with the GFP cDNA served as a control.

that at least in COS-1 cells, Kir/Gem is present in a constitutively active form.

14-3-3 and CaM compete with the Ca^{2+} channel β-subunit for binding to Kir/Gem

We next analyzed whether binding of 14-3-3 or CaM to Kir/Gem regulates its association with its effector, $Ca_v\beta3$. COS-1 cells were cotransfected with GST–14-3-3 and Kir/Gem or the mutants defective in GTP (Kir/Gem S88N), CaM (Kir/Gem W269G) or 14-3-3 (Kir/Gem S288A) binding, either with or without $Ca_v\beta3$. Coprecipitation experiments were then carried out to analyze the association of Kir/Gem to GST–14-3-3 or $Ca_v\beta3$ (Fig. 6A).

The association between GST–14-3-3 and Kir/Gem (panel a, lane 1) or Kir/Gem W269G (lane 5) was abolished in the presence of $Ca_v\beta3$ (lanes 2 and 6) and no complex containing all three proteins was detected, indicating that the effector when bound to Kir/Gem prevents the association of 14-3-3. By contrast, Kir/Gem S88N, which does not associate with $Ca_v\beta3$ (panel b, lane 4), bound GST–14-3-3 both in the absence (panel a, lane 3) and presence (lane 4) of $Ca_v\beta3$. As expected, Kir/Gem S288A failed to coprecipitate with GST–14-3-3 (panel a, lane 7) but, importantly, was able to bind $Ca_v\beta3$ (panel b, lane 7). Expression of $Ca_v\beta3$ led to the disappearance of the slower migrating band of Kir/Gem (panel c, compare lanes 1 and 2) or Kir/Gem W269G (compare lanes 5 and 6), suggesting that $Ca_v\beta3$ associates with the faster migrating form.

To analyze whether CaM affects the binding of $Ca_v\beta3$ to Kir/Gem, we performed sequential binding experiments. $Ca_v\beta3$ was first immnoprecipitated from homogenates of cells coexpressing Kir/Gem and $Ca_v\beta3$ and the isolated complexes between $Ca_v\beta3$ and Kir/Gem were then eluted from the beads under conditions that do not disrupt the association (Fig. S2 in supplementary material). The eluted complexes were then tested for their ability to bind to immobilized CaM (Fig. 6B).

Interestingly, only Kir/Gem and not $Ca_v\beta3$ (panel a, lane 1) was recovered in the fraction that bound to CaM, indicating that the three proteins cannot simultaneously exist in a complex. Since Kir/Gem was isolated in association with $Ca_v\beta3$ before the complex was assayed for CaM binding (panels a and b, lane 1), the above finding suggests that CaM displaced $Ca_v\beta3$ from Kir/Gem. As expected, Kir/Gem W269G, which does not bind CaM, was absent from the bound fraction and remained associated with $Ca_v\beta3$ (panels a and b, lane 3). Kir/Gem S88N, which does not bind GTP and therefore also does not interact with its effector $Ca_v\beta3$, was neither recovered in the CaM-bound or -unbound fractions (panels a and b, lane 2). Control experiments confirmed that, in the absence of CaM, the complex between Kir/Gem and $Ca_v\beta3$ did not dissociate for the duration of the experiment (Fig. S2b in supplementary material) and endogenous CaM was not present in complexes of Flag-$Ca_v\beta3$ and Kir/Gem (Fig. S2c in supplementary material).

In conclusion, Kir/Gem cannot simultaneously bind its effector $Ca_v\beta3$ and 14-3-3 or CaM, and CaM can displace $Ca_v\beta3$ from Kir/Gem.

CaM modulates Kir/Gem-mediated downregulation of Ca^{2+} channel activity

On the basis that CaM and 14-3-3 compete with $Ca_v\beta3$ for binding to Kir/Gem, we explored possible roles of 14-3-3 or CaM on the Kir/Gem-mediated regulation of voltage-dependent Ca^{2+} channel activity (Beguin et al., 2001). Although Kir/Gem S288A, which fails to bind 14-3-3, still downregulated Ca^{2+} channel activity (Ward et al., 2004), the significance of this result is unclear given the limited availability of endogenous 14-3-3 for Kir/Gem binding. We therefore expressed Kir/Gem or Kir/Gem W269G in PC12 cells, either alone or with GST–14-3-3, and measured endogenous Ca^{2+} currents.

As shown in Fig. 7, expression of Kir/Gem abolished endogenous Ca^{2+} currents (compare lane 1 with 2). As previously reported (Beguin et al., 2001; Ward et al., 2004), Kir/Gem W269G, which is defective in binding CaM, failed to inhibit Ca^{2+} currents (lane 5). Overexpression of GST–14-3-3 (or GST–14-3-3 K49E) did not affect Kir/Gem-mediated downregulation of Ca^{2+} channel activity (lanes 3 and 4) nor did it restore the lack of function for Kir/Gem W269G (lane 6).

In conclusion, the inhibitory function of Kir/Gem on Ca^{2+} channel activity is apparently not modulated by 14-3-3 but requires a probably transient interaction with CaM, possibly to prevent nuclear sequestration of the RGK protein.

Discussion

Functions of RGK small G proteins have only recently started to be unraveled and include the regulation of Ca^{2+} channel activity and actin and microtubule dynamics. A characteristic feature of RGK proteins in comparison with other small G proteins is their interaction with the regulatory proteins 14-3-3 and CaM. Here, we characterized the interaction of 14-3-3 and CaM with one of the RGK proteins, Kir/Gem, and showed that

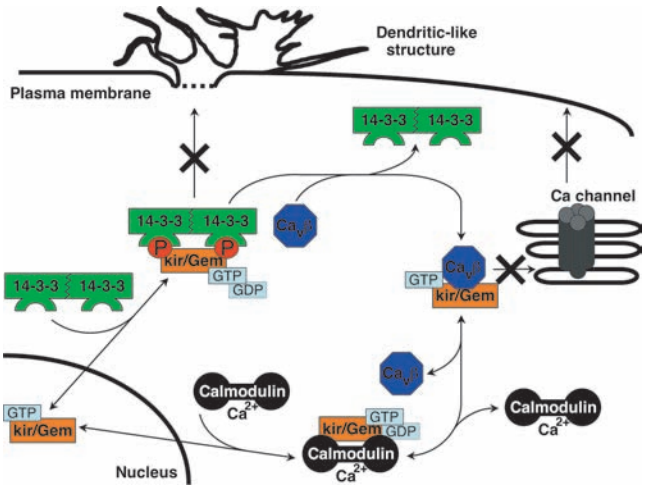


Fig. 8. Working model for the regulatory role of 14-3-3 and CaM on Kir/Gem localization and function. Since CaM and 14-3-3 compete for Kir/Gem binding, two pathways can be considered. Activation of CaM by intracellular Ca^{2+} results in association of CaM with Kir/Gem and retains it in the cytoplasm. Dissociation of CaM allows Kir/Gem to bind the Ca^{2+} channel β-subunit, interfering with plasma membrane expression of the α1-subunit. Alternatively, in the absence of CaM activation, 14-3-3 can relocalize phosphorylated Kir/Gem from a submembranous location to the cytoplasm to block the formation of dendrite-like extensions. 14-3-3 can also relocalize Kir/Gem from the nucleus to the cytoplasm. 14-3-3 bound to Kir/Gem may be exchanged for the β-subunit. Upon activation of CaM by Ca^{2+}, Kir/Gem dissociates from the β-subunit. Kir/Gem is in its active, GTP-bound form when located in the nucleus or bound to the β-subunit; however, the activation state when associated with CaM or 14-3-3 remains to be determined.

14-3-3 and CaM regulate the subcellular localization of Kir/Gem as well as its functions. Analysis of the binding of an effector and the regulatory proteins to Kir/Gem indicate that the regulatory proteins compete among themselves and with the effector for binding to the RGK protein. This provides a possible explanation for the regulatory effects of 14-3-3 and CaM on Kir/Gem localization and function.

Kir/Gem carries one N- and one C-terminal 14-3-3-binding site (S22 and S288, respectively) and binds all 14-3-3 isoforms, except for σ. S22 and S288 are both required for 14-3-3 binding and only 14-3-3 dimers and not monomers bind to Kir/Gem, indicating that the two 14-3-3-binding sites engage one 14-3-3 dimer. If, however, the CaM-binding site in Kir/Gem is mutated (Kir/Gem W269G), the C-terminal 14-3-3-binding site is now sufficient for 14-3-3 binding and Kir/Gem W269G can now bind 14-3-3 monomers. This indicates that, in the absence of bound CaM, 14-3-3 monomers can interact with Kir/Gem probably with low affinity, but that the high-affinity interaction mediated by 14-3-3 dimers may be required to displace CaM from Kir/Gem. 14-3-3 is present in cells both as monomer and dimer, and dimerization is regulated by phosphorylation (Woodcock et al., 2003) and is thought to be required for high-affinity stable binding (Yaffe, 2002). Thus, phosphorylation could regulate 14-3-3 dimerization and hence its ability to displace CaM from Kir/Gem and bind to the RGK protein.

Phosphorylation of S22 and/or S288 represents an additional possibility to regulate binding of 14-3-3 to Kir/Gem. Kir/Gem migrates as a doublet on SDS-PAGE (Leone et al., 2001) with the slower migrating form probably corresponding to a post-translational modification, possibly a phosphorylation of one or both 14-3-3-binding sites. Indeed, the fraction of the higher molecular weight form is increased in response to either 14-3-3 overexpression or inactivation of CaM binding, but is decreased if the 14-3-3-binding sites are mutated. 14-3-3 could affect the post-translational modification state of Kir/Gem either through steric hindrance or masking or through recruitment of enzymes that catalyze this modification, both of which are well-established functions for 14-3-3 (Tzivion et al., 2001). Phosphorylation of the C-terminal 14-3-3-binding site in Kir/Gem could not be blocked using various kinase inhibitors (Ward et al., 2004), suggesting the involvement of more than one or an unknown kinase. In vitro and in vivo, only the faster migrating form of Kir/Gem binds to the Ca^{2+} channel β-subunit; thus, we postulate a crucial role for the post-translational modification in inactivating Kir/Gem.

Kir/Gem W269G was observed in the nucleus of HEK 293T cells (Beguin et al., 2001). Our results in COS-1 cells also show that Kir/Gem W269G shows a more predominant nuclear localization as compared with Kir/Gem, suggesting that CaM binding regulates the subcellular distribution of the RGK protein (Fig. 8). Furthermore, overexpression of 14-3-3 led to the clearance of Kir/Gem from the nucleus (Fig. 8), an effect that was particularly prominent for the CaM-binding mutants, which efficiently relocalized from the nucleus to the cytoplasm. Intriguingly, mutation of the 14-3-3-binding sites in Kir/Gem did not affect its subcellular localization, most probably because of a small pool of free endogenous 14-3-3 in COS-1 cells available for binding to overexpressed Kir/Gem. This interpretation is consistent with the small amount of endogenous 14-3-3 that bound to Kir/Gem and the need to overexpress 14-3-3 for efficient nuclear exclusion of Kir/Gem. Since 14-3-3 and CaM not only compete for binding to Kir/Gem but their association might also be regulated by, for example, phosphorylation and/or dephosphorylation, nuclear localization of Kir/Gem may be tightly controlled. N1E-115 neuroblastoma cells express endogenous Kir/Gem yet they do not display dendrite-like extensions (Leone et al., 2001) and preliminary data from our laboratory indicate that endogenous Kir/Gem might predominantly localize to the nucleus in these cells. Kir/Gem encodes several nuclear localization signals which, when inactivated, prevent nuclear transport of the RGK protein (our unpublished data). Whether Kir/Gem exerts additional functions in the nucleus remains to be analyzed but is an intriguing possibility given that the RGK protein is in its active form when in the nucleus.

The ability of Kir/Gem to coprecipitate from cell lysates with an effector (e.g. the β-subunit) in the absence of GTP-γS, the lack of an effect of either GTP-γS or GDP-βS on this interaction, and the low intrinsic GTPase activity of RGK proteins (Cohen et al., 1994; Finlin et al., 2000) are consistent with the notion that Kir/Gem is present in cells in a constitutively active form. Several members of the Rho family of GTPases, including Rnd1, 2 and 3, RhoH, and RhoBTB1 and 2, are also locked into a GTP-bound conformation and are unable to hydrolyze GTP (Foster et al., 1996; Li et al., 2002; Nobes et al., 1998). The crystal structure of Rnd3 reveals that several amino acids crucial for GTP hydrolysis are absent from

the catalytic site (Fiegen et al., 2002). The corresponding residues are likewise not present in Kir/Gem or the other RGK proteins. In contrast to most other small G proteins, the expression of RGK proteins (Cohen et al., 1994; Finlin and Andres, 1997; Maguire et al., 1994; Reynet and Kahn, 1993) and RhoH (Li et al., 2002) can be regulated at the transcriptional level. Coupled to a short half-life as observed for Kir/Gem (Ward et al., 2004), this might provide a mechanism for long-term regulation of the activity of these small G proteins. Post-translational modifications or, as implied by our results, nuclear sequestration, may be attractive alternative mechanisms to inactivate Kir/Gem rapidly.

Kir/Gem regulates cell morphology by inducing the formation of dendrite-like extensions (Leone et al., 2001; Piddini et al., 2001; Ward et al., 2002). Overexpression of 14-3-3 induced a redistribution of Kir/Gem from a more submembranous to a more cytosolic distribution and abolished the induction of membrane extensions by Kir/Gem. Inhibition of this function of Kir/Gem required 14-3-3 dimers, probably because 14-3-3 needs to displace CaM from the RGK protein (Fig. 8). In a recent study (Ward et al., 2004), disruption of the C- but not the N-terminal 14-3-3-binding site was shown to abolish the ability of Kir/Gem to induce neurite extensions in neuroblastoma cells. Since both the N- and C-terminal 14-3-3-binding sites are required for 14-3-3 binding, the effect of the S288A mutation was attributed to phosphorylation rather than 14-3-3 binding. However, at least in COS-1 cells, little endogenous 14-3-3 is available to associate with Kir/Gem, explaining why mutation of the N- or C-terminal 14-3-3-binding site does not interfere with the ability of Kir/Gem to induce extensions. Exogenous 14-3-3 can abrogate the Kir/Gem-mediated induction of extensions, but only if the two proteins can associate. The correlation between the ability of 14-3-3 to bind to Kir/Gem and its inhibitory effect on Kir/Gem-induced morphological changes, implicates 14-3-3 binding rather than the phosphorylation on S288 in this process. Interestingly, mutation of the CaM-binding site also abrogated the ability of Kir/Gem to induce dendrite-like extensions, most probably due to the nuclear sequestration of these mutants. Since Kir/Gem associates with ROK kinases to regulate actin dynamics, it will be of interest to determine whether 14-3-3 or CaM interferes with this interaction.

Plasma membrane expression of functional Ca^{2+} channels requires the association of the α- and β-subunits (Catterall, 1998). We previously proposed that Kir/Gem prevents Ca^{2+} channel surface expression by binding and sequestering β-subunits, thereby downregulating Ca^{2+} channel activity (Beguin et al., 2001) (Fig. 8). Overexpression of 14-3-3 did not interfere with the ability of Kir/Gem to either suppress Ca^{2+} channel activity or associate with the β-subunit. These results, together with the lack of a functional effect by mutating the 14-3-3-binding site in Kir/Gem (Ward et al., 2004), argue against a role of 14-3-3 on the function of Kir/Gem in regulating Ca^{2+} channel activity (Fig. 8). However, since the molecular mechanisms by which 14-3-3 modulates Kir/Gem-mediated effects on cell shape versus Ca^{2+} channel activity may differ, we cannot rule out a regulatory role of 14-3-3 in certain cell types or under particular conditions where, for example, the phosphorylated form of Kir/Gem is favored.

In summary, 14-3-3 and CaM differentially modulate the effects of Kir/Gem on cell shape and Ca^{2+} channel activity, either by regulating the interaction between Kir/Gem and effectors, or by controlling the spatial distribution of the RGK protein within the cell.

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