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Nuclear Sequestration of β-Subunits by Rad and Rem is Controlled by 14-3-3 and Calmodulin and Reveals a Novel Mechanism for Ca^{2+} Channel Regulation

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Voltage-gated Ca^{2+} channels (VDCCs) are heteromultimeric proteins that mediate Ca^{2+} influx into cells upon membrane depolarization. These channels are involved in various cellular events, including gene expression, regulation of hormone secretion and synaptic transmission. Kir/Gem, Rad, Rem, and Rem2 belong to the RGK family of Ras-related small G proteins. RGK proteins interact with the β-subunits and downregulate VDCC activity. Kir/Gem was proposed to prevent surface expression of functional Ca^{2+} channels, while for Rem2 the mechanism remains controversial. Here, we have analyzed the mechanism by which Rad and Rem regulate VDCC activity. We show that, similar to Kir/Gem and Rem2, 14-3-3 and CaM binding regulate the subcellular distribution of Rad and Rem, which both inhibit Ca^{2+} channel activity by preventing its expression on the cell surface. This function is regulated by calmodulin and 14-3-3 binding only for Rad and not for Rem. Interestingly, nuclear targeting of Rad and Rem can relocalize and sequester the β-subunit to the nucleus, thus providing a novel mechanism for Ca^{2+} channel downregulation.

Keywords: 14-3-3; calcium channel; calmodulin; Rad; Rem

Introduction

Voltage-dependent Ca^{2+} channels (VDCCs) allow Ca^{2+} entry into cells upon membrane depolarization, triggering intracellular events such as gene expression, muscle contraction, hormone secretion and synaptic transmission.[1] VDCCs are composed of the pore-forming α_1 subunit, which associates with auxiliary subunits that have regulatory functions. The cytosolic β subunit is thought to facilitate plasma membrane expression and modulate gating properties of VDCCs, while the functions of the $\alpha_2\delta$ and γ subunits remain to be determined.[2,3] Due to their central role in calcium signaling, VDCCs are tightly regulated. For instance, heterotrimeric G-proteins, protein kinases and calmodulin (CaM) control several channel properties.[2] Recently, the β-subunit was shown to interact with all members of the RGK family of small G proteins.[4,5,14,16]

The ras-related RGK proteins include Kir/Gem,[6,7] Rad,[8] Rem[9] and Rem2.[10,16] Despite a conserved Ras-related core domain, RGK proteins exhibit unique structural and functional features that differ from other GTPases, including the lack of a lipid modification for membrane anchorage, the presence of N-terminal and C-terminal extensions, and an unconventional G3 motif. Furthermore, RGK proteins are regulated at the transcriptional level.[6–9]

The physiological roles of RGK proteins include the regulation of Ca^{2+} channel activity,[4,5,14,16] and remodeling of the actin[11,12] and microtubule cytoskeletons.[13] Through their effects on VDCCs and Ca^{2+} influx, RGK proteins regulate Ca^{2+}-triggered secretion of hormones. For instance, Kir/Gem and Rem2 have been shown to regulate

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Abbreviations used: IRES, internal ribosomal entry site; RGK, Rad/Gem/Kir; wt, wild-type; GFP, green fluorescent protein; VDCC, voltage-gated Ca^{2+} channels; GST, glutathione-*S*-transferase; CaM, calmodulin.

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the secretion of insulin.[4,14] Rad is overexpressed in skeletal muscle of type II diabetic patients.[8] and its overexpression in muscle and fat cells inhibits glucose uptake.[15]

Kir/Gem and Rem2 interact in a GTP-dependent manner with the Ca^{2+} channel β-subunits, resulting in the removal of functional Ca^{2+} channels from the plasma membrane.[4,16,17] For Rem2, it has been suggested that inactivated Ca^{2+} channels may be present at the plasma membrane.[14] As for Rad and Rem, although they were shown to interact with the β-subunit and to suppress Ca^{2+} channel function, the precise mechanism was not determined.[5]

CaM, a major transducer of Ca^{2+} signaling, regulates VDCCs and associates with Kir/Gem and Rad *via* their C-terminal extensions in a Ca^{2+}-dependent manner. CaM inhibits binding of GTP by Kir/Gem,[18] and shows a better affinity for the GDP-bound form of Rad.[19] The role of CaM binding in RGK family function is unclear but, at least in the case of Kir/Gem and Rem2, may involve the control of their subcellular localization.[4,16,17]

The 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. The 14-3-3 proteins bind to phosphoserine or threonine to prevent the interaction with other proteins, regulate the subcellular distribution of proteins or protect proteins from proteolysis.[20] The 14-3-3 proteins can dimerize, and dimers may bind to target proteins that contain two 14-3-3 binding sites.[21] Kir/Gem and Rem2 interact *via* two 14-3-3 binding sites.[15,16,23] The 14-3-3 proteins modulate the subcellular distribution of Kir/Gem and Rem2, and their effects on cell shape remodeling.[16,17] Although Rad and Rem bind 14-3-3 in a phosphorylation-dependent manner,[22] the 14-3-3 binding sites were not identified and the role of 14-3-3 binding was not analyzed.

Here, we analyze the regulatory roles of 14-3-3 and CaM binding on the functions of Rad and Rem. We show that Rad and Rem exert similar effects on cell shape and calcium channel activity, and that 14-3-3 and CaM associate with both RGK proteins to regulate their subcellular distribution. Furthermore, through their interaction with the two RGK proteins, Ca^{2+} channel β-subunits can be translocated to the nucleus, providing a novel mechanism to regulate surface expression of VDCCs.

Results

Binding of 14-3-3 to Rad and Rem

Amino acid residues S23 and S289 in human Kir/Gem, S22 and S288 in mouse Kir/Gem and S69 and S334 in mouse Rem2 are known 14-3-3 binding sites.[16,17,23] While C-terminal serine is conserved in Rad and Rem, sequence analysis indicated that two alternative N-terminal serine residues could act as 14-3-3 binding sites in these RGK proteins (Figure 1(a)). To test if these serine residues are functional 14-3-3 binding sites, Myc-tagged Rad and Rem, or mutants where the N-terminal or C-terminal serine residues were mutated to alanine (Rad S38A and Rad S300A, Rem S18 and Rem S290; Figure 1(a)), were co-expressed with glutathione-*S*-transferase (GST)-14-3-3 ζ in COS-1 cells and tested for their ability to coprecipitate. GST-14-3-3 ζ K49E, a 14-3-3 mutant defective in target protein binding, was used as a control.[24]

As shown in Figure 1(b), Rad and Rem co-precipitated with GST-14-3-3 (lanes 2 and 7), but not with GST-14-3-3 K49E (lanes 3 and 8). GST-14-3-3 failed to associate with Rad S38A or Rad S300A (lanes 4 and 5), demonstrating that these serine residues are 14-3-3 binding sites. In the case of Rem, Rem S18A and Rem S290A still associated with GST-14-3-3 and only mutation of both serine residues (Rem S18A/S290A) prevented the interaction (lanes 9–11). Mutation of the other putative N-terminal 14-3-3 binding sites (S25 in Rad and S38 in Rem) did not affect 14-3-3 binding (data not shown), indicating that these serine residues are not involved in the interaction with 14-3-3. Western blot of the cell lysates confirmed that the transfected cells expressed similar levels of the wild-type (wt) or mutated RGK proteins (Figure 1(d)) and GST-14-3-3 or GST-14-3-3 K49E (Figure 1(e)), respectively.

Similar results were obtained for the inverse experiment, where the RGK proteins were first immunoprecipitated and the associated over-expressed GST-14-3-3 and endogenous 14-3-3 were detected using an anti-14-3-3 antibody (Figure 1(c)). Rem bound endogenous 14-3-3 more efficiently than Rad (Figure 1(c), lanes 1 and 6) and the interaction of endogenous 14-3-3 with Rad was increased following over-expression of GST-14-3-3 (Figure 1(c), compare lane 1 with 2), likely reflecting differences in binding affinities for a limiting pool of free 14-3-3. Rad and Rem bound all seven 14-3-3 family members *via* the identified 14-3-3 binding sites, although apparently with different efficiencies (see Supplementary Data I).

In conclusion, Rad and Rem bind all seven 14-3-3 isoforms. Whereas Rad requires the concurrent presence of an N-terminal and C-terminal site for 14-3-3 binding, the two sites in Rem can function independently as 14-3-3 binding sites.

Dimerization of 14-3-3 and binding to Rad and Rem

The presence of two 14-3-3 binding sites in Rad and Rem, and the requirement of both sites for binding of 14-3-3 to Rad suggests that the latter RGK protein may associate with 14-3-3 dimers. To test this hypothesis, we took advantage of a mutation in 14-3-3 ζ that has been shown to abolish dimerization without affecting binding to target proteins.[25]

On the basis of co-precipitation experiments as described above, the 14-3-3 dimerization-defective mutant did not associate with Rad (Figure 1(f),

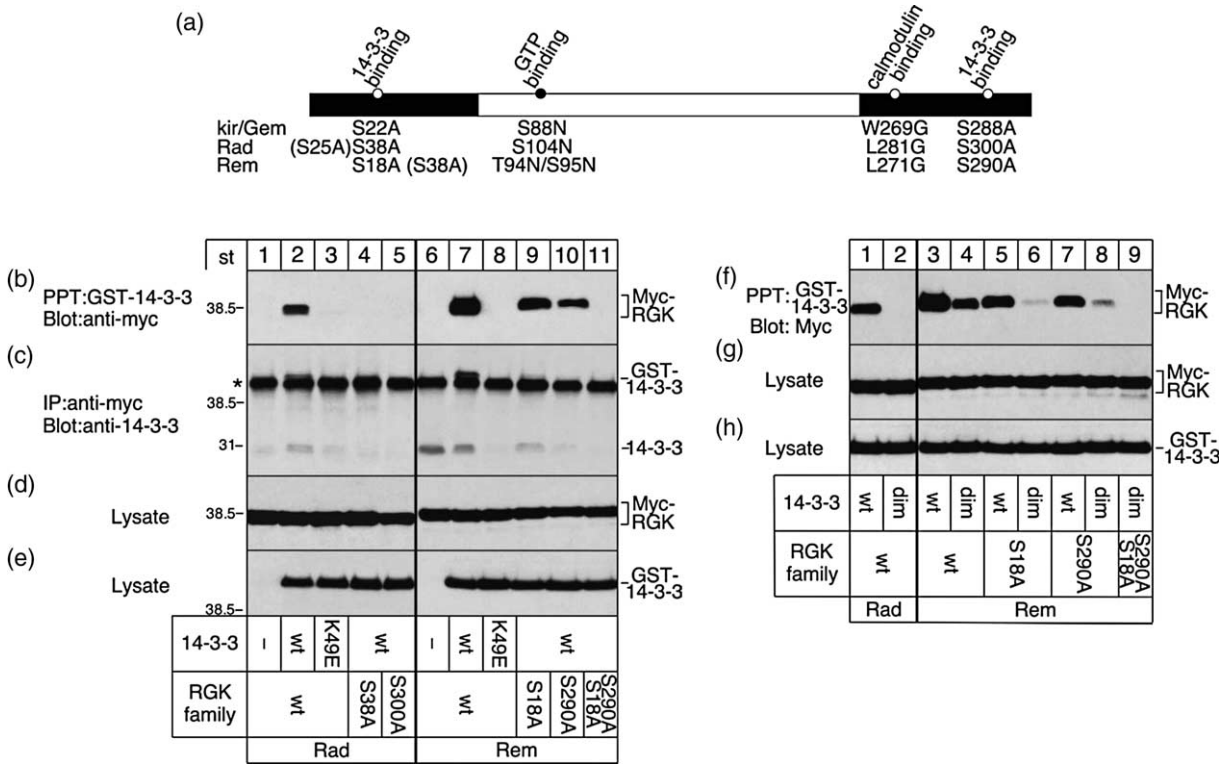

Figure 1. Identification of 14-3-3 binding sites in RGK proteins and characterization of binding. (a) The Ras-like core domain (open bar), N and C-terminal extensions (filled bar) and the location of the 14-3-3, CaM (open circles) and GTP (filled circle) binding sites are shown. Mutations that affect the different binding sites are indicated, with non-functional putative 14-3-3 binding sites in parentheses. (b) Cells were cotransfected with cDNAs for wt or mutated Myc-RGK proteins and GST-14-3-3 or GST-14-3-3 K49E ζ isoforms. GST-14-3-3 proteins were precipitated and associated RGK proteins detected by Western blot using Myc antibodies. (c) Cells were cotransfected with cDNAs for wt or mutated Myc-RGK proteins and GST-14-3-3 or GST-14-3-3 K49E. The RGK proteins were immunoprecipitated and associated GST-14-3-3 and endogenous 14-3-3 were detected by Western blot using 14-3-3 antibodies. The IgG heavy chain, migrating just below the GST-14-3-3 in (c), is marked by an asterisk (*). (d) and (e) Expression levels. Cell lysates were blotted with (d) Myc or (e) GST antibodies to monitor the expression level of the Myc-RGK proteins or GST-14-3-3, respectively; st, protein markers of known molecular mass. (f) 14-3-3 dimerization is required for efficient binding to RGK proteins. Cells were cotransfected with cDNAs for wt or mutated Myc-RGK proteins and GST-14-3-3 or a dimerization defective mutant (dim). GST-14-3-3 proteins were precipitated and associated RGK proteins were detected by Western blot using Myc antibody. (g) and (h) Cell lysates were blotted with (g) Myc or (h) GST antibodies to monitor RGK protein and GST-14-3-3 expression levels. Two or three independent experiments were performed and data from a representative experiment are shown.

compare lane 1 with lane 2), consistent with the notion that Rad requires two functional 14-3-3 binding sites and associates only with 14-3-3 dimers. As for Rem, the dimerization-defective 14-3-3 mutant bound to wt Rem (Figure 1(f), lane 4) and showed residual binding to Rem S18A and Rem S290A (Figure 1(f), lanes 6 and 8). Only the inactivation of both 14-3-3 binding sites (Rem S18A/S290A) abolished the interaction completely (Figure 1(f), lane 9). The ability of Rem to interact with 14-3-3 monomers thus correlates with the requirement of only one of its two 14-3-3 binding sites for the interaction.

Similar results were obtained for the inverse co-immunoprecipitation experiments (data not shown). Western blot analysis of cell lysates confirmed that the transfected cells expressed similar levels of the different Myc-RGK proteins (Figure 1(g)) and GST-14-3-3 (Figure 1(h)).

In conclusion, Rad associates with 14-3-3 dimers and thus requires the presence of two 14-3-3 binding sites. Rem can associate with 14-3-3 monomers *via* either an N-terminal or a C-terminal 14-3-3 binding site, although this interaction is less efficient as compared to dimeric 14-3-3.

CaM and 14-3-3 regulate the subcellular distribution of Rad and Rem

Since one function of 14-3-3 is to regulate the subcellular distribution of proteins it interacts with,[20] we used immunofluorescence microscopy to analyze the subcellular distribution of Rad and Rem in COS-1 cells expressing different combinations of wt RGK proteins or mutants defective in 14-3-3 binding and GST-14-3-3 ζ (Figure 2(a) and (b)).

Expression of wt Rad and Rem in COS-1 cells induced dendrite-like extensions, and both RGK

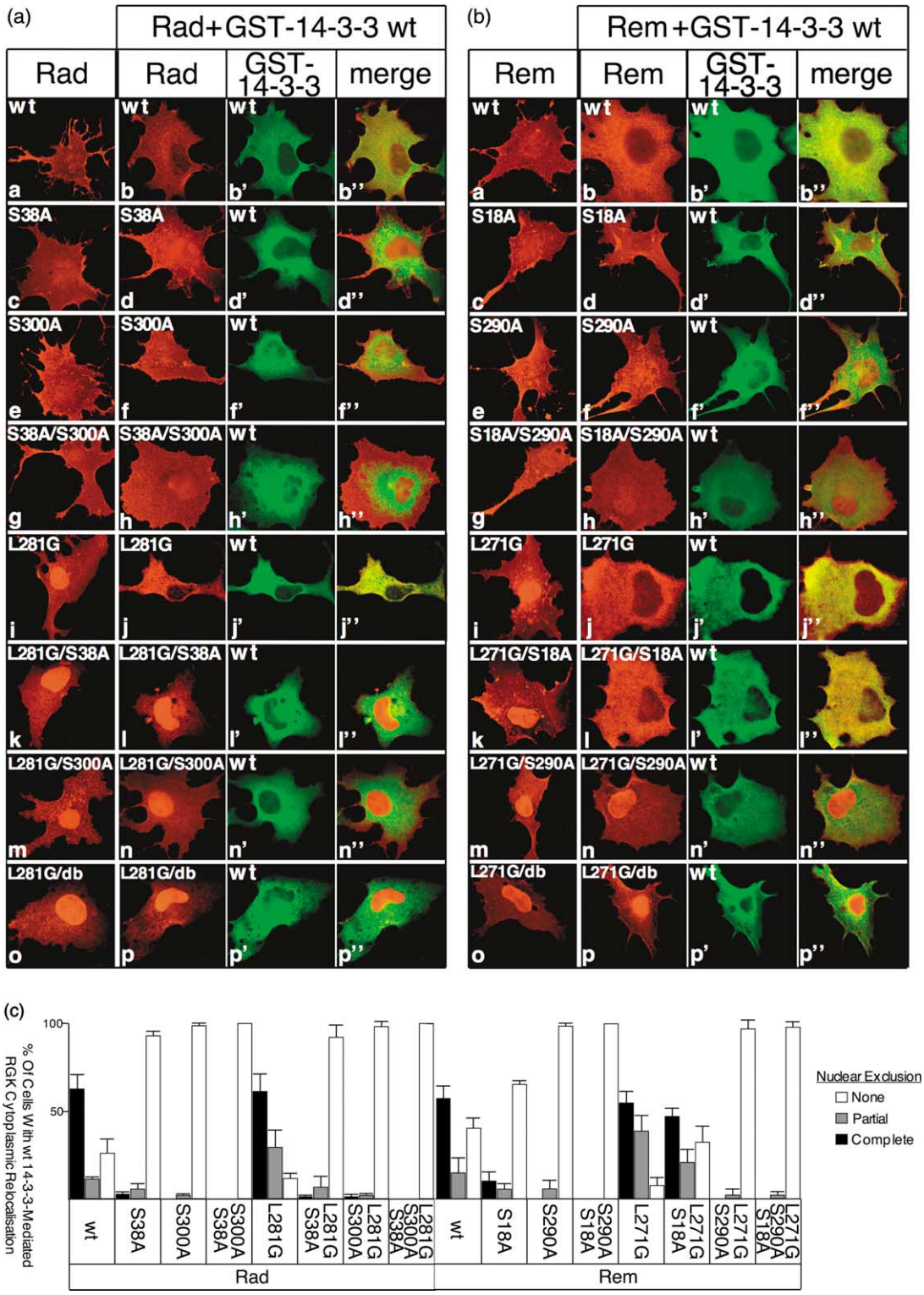

Figure 2. 14-3-3 and CaM regulate the subcellular distribution of Rad and Rem and RGK proteins mediate changes to cell morphology. (a) and (b) COS-1 cells were transfected with cDNAs for wt or mutated RGK proteins, either alone or together with GST-14-3-3. Cells were processed for immunofluorescence microscopy using Myc and GST antibodies to label (a) Rad (red), (b) Rem (red) and (a) and (b) GST-14-3-3 (green), respectively. Areas of colocalization are in yellow in the merged image; db, double mutant. (c) Quantification of the 14-3-3-mediated cytosolic relocalization of Rad and Rem. 150–200 transfected cells were selected randomly and analyzed in three to five independent experiments (see Materials and Methods). The fraction of cells showing efficient (black bars), partial (grey bar) or no (white bars) nuclear clearance is plotted. Statistical analysis of the data using Student's unpaired *t*-test showed a significant 14-3-3-mediated relocalization of wt or CaM binding-deficient RGK proteins as compared to the 14-3-3 binding-deficient mutants ($p < 0.01$). The only exception was Rem L271G/S18A, which showed no significant difference from wt Rem or Rem L271G ($p > 0.05$).

proteins were present at submembranous/cytoplasmic regions and in the nucleus (Figure 2(a)a and (b)a). The distribution of the RGK mutants defective in 14-3-3 binding was not changed markedly (Figure 2(a)c, e and g and (b)c, e and g). Overexpression of GST-14-3-3 (or Flag-14-3-3; data not shown) led to a reduction of the dendrite-like extensions induced by both RGK proteins, and a clearance of Rad and Rem from the nucleus (Figure 2(a) and (b), b–b″). This effect was abolished if one or both 14-3-3 binding sites were mutated (Figure 2(a) and (b), d–d″, f–f″ and h–h″). As a control, GST-14-3-3 K49E showed no effect (data not shown). While reduction of dendrite-like extensions and subcellular relocalization of Rad and Rem by 14-3-3 correlated with the ability of 14-3-3 to bind the RGK proteins, one exception was noted: 14-3-3 monomers did not affect Rem localization, suggesting that only dimeric 14-3-3 is able to regulate the subcellular distribution and function of RGK proteins efficiently. This interpretation is supported by the observation that the dimerization-defective 14-3-3 mutant did not affect the localization or function of Rad and Rem (data not shown).

The C-terminal 14-3-3 binding site in RGK proteins is in close proximity to the domain that interacts with CaM (see Figure 1(a)). In addition, CaM has been implicated in affecting the subcellular distribution of Kir/Gem and Rem2.[4,16] To explore the possibility that CaM and 14-3-3 binding could play distinct roles in Rad and Rem localization, we generated mutations corresponding to the CaM-binding deficient mutant Kir/Gem W269G in Rad (Rad L281G) and Rem (Rem L271G). Similar mutations were introduced also into RGK proteins with mutated 14-3-3 binding sites (see Figure 1(a)). These RGK proteins were then co-expressed with GST-14-3-3 and their subcellular distribution analyzed by immunofluorescence microscopy.

Similar to what had been found for Kir/Gem W269G,[4] and Rem2 L317G,[16] Rad L281G and Rem L271G showed a more predominant nuclear localization (Figure 2(a)i and (b)i). Like the wt RGK proteins, no redistribution was observed if the N-terminal or C-terminal, or both, 14-3-3 binding sites were mutated in the CaM binding-deficient mutants (Figure 2(a)k, m and o and (b)k, m and o).

Overexpression of GST-14-3-3 led to the efficient translocation of Rad L281G and Rem L271G from the nucleus to the cytosol (Figure 2(a)j–j″ and (b)j–j″). No nuclear clearance of the RGK proteins was observed if either the C-terminal or both the N-terminal and C-terminal 14-3-3 binding sites were mutated (Figure 2(a)n–n″ and p–p″, and (b)n–n″ and p–p″). As for the N-terminal 14-3-3 binding site, its mutation abolished the GST-14-3-3-induced nuclear clearance of Rad L281G (Figure 2(a)l–l″) but not that of Rem L271G/S18A (Figure 2(b)l–l″). Quantification of these data is shown in Figure 2(c). As expected, GST-14-3-3 K49E did not affect the distribution of any of the three RGK proteins with a mutated CaM binding site (data not shown).

These data therefore show that binding of 14-3-3 and CaM to Rad and Rem modulate their nuclear localization.

Binding of 14-3-3 and CaM to Rad and Rem

Given the differences in the effect of GST-14-3-3 on nuclear exclusion of Rad L281G/S38A as compared to Rem L271G/S18A, we carried out co-precipitation and binding experiments to determine if their ability to bind 14-3-3 or CaM correlates with their subcellular localization.

As expected, Rad L281G and Rem L271G associated efficiently with GST-14-3-3 (Figure 3(a)a, lanes 1 and 6) but not with GST-14-3-3 K49E (Figure 3(a)a, lanes 2 and 7). Mutation of the N-terminal or C-terminal, or both, 14-3-3 binding sites abolished the interaction of Rad L281G with GST-14-3-3 (Figure 3(a)a, lanes 3–5). Similar to wt Rem (see Figure 1(b)), mutation of the N-terminal 14-3-3 binding site in Rem L271G did not affect the interaction with GST-14-3-3 (Figure 3(a)a, lane 8). In contrast, inactivation of the C-terminal site severely affected the interaction with GST-14-3-3 (Figure 3(a)a, lane 9). Western blot analysis of cell lysates confirmed that the transfected cells expressed similar levels of the different Myc-RGK proteins and GST-14-3-3 (Figure 3(a)b and c). Thus, in the absence of CaM, S290 is the unique 14-3-3 binding site in Rem, whereas both 14-3-3 binding sites are required in Rad for efficient binding.

Altogether, the ability of the different RGK protein mutants to bind 14-3-3 correlated well with the capacity of 14-3-3 to induce their nuclear exclusion (see Figures 2 and 3). A similar correlation between subcellular RGK protein distribution and ability to bind 14-3-3 and CaM was observed in PC12 ands Hela cells (data not shown).

While CaM has been shown to interact with Rad,[19] Kir/Gem,[4,17,18,23] and Rem2,[16] its association with Rem has not been established. We therefore carried out CaM binding experiments to analyze the ability of the RGK proteins to bind CaM. The wt Rad and Rem and mutants lacking one or both 14-3-3 binding sites bound CaM (Figure 3(b)a, lanes 1–4 and 9–12). In contrast, binding of Rad and Rem mutants containing the L281G and L271G substitutions, respectively, was reduced significantly (Figure 3(b)a, lanes 5–8 and 13–16),[4] demonstrating that the respective mutations indeed interfere with CaM binding. Western blot analysis of cell lysates confirmed that the transfected cells expressed similar levels of the different Myc-RGK proteins (Figure 3(b)b).

In conclusion, the above biochemical data together with the subcellular localization shown in Figure 2, indicate that 14-3-3 and CaM regulate the subcellular localization of Rad and Rem by a similar mechanism.

Interaction of Rad and Rem with the Ca^{2+} channel β-subunit

Rad, Rem, Rem2 and Kir/Gem are known to downregulate calcium channel activity by

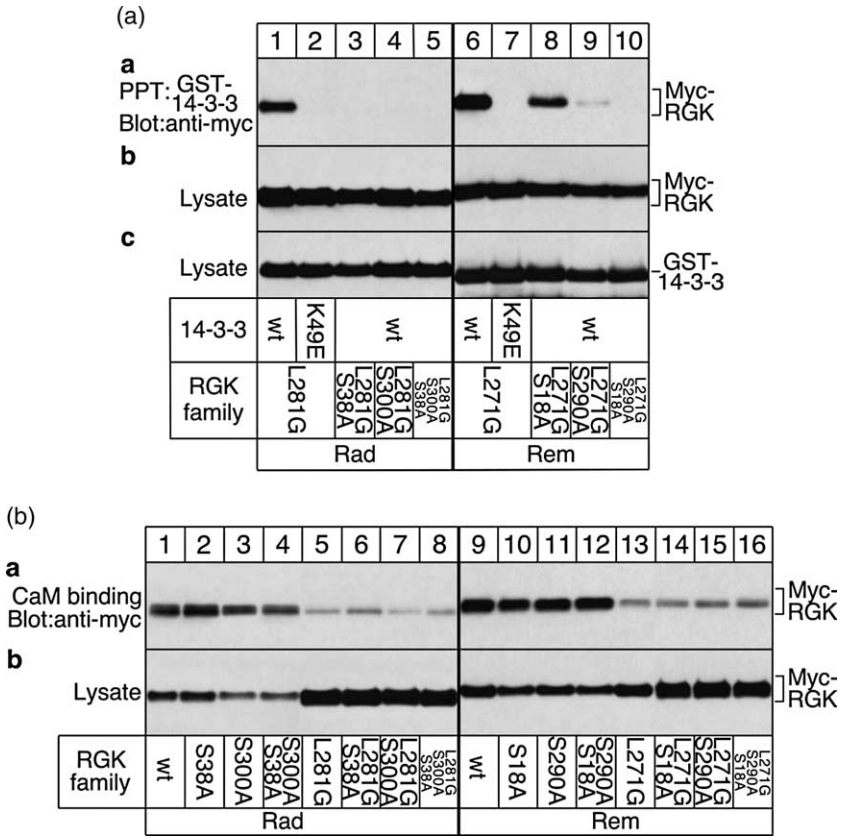

Figure 3. Binding of 14-3-3 and CaM to RGK proteins. (a) Cells were cotransfected with cDNAs for wt or mutated Myc-RGK proteins and GST-14-3-3 or GST-14-3-3 K49E. a, GST-14-3-3 proteins were precipitated and the associated Myc-RGK proteins detected by Western blot using Myc antibody. b and c, Cell lysates were blotted with b Myc or c GST antibodies to monitor Rad, Rem or GST-14-3-3 expression levels, respectively. (b) CaM binding. a, Cells were transfected with cDNA for wt or mutated Myc-RGK proteins. Cell homogenates were incubated with CaM beads and bound Myc-RGK proteins detected by Western blot using Myc antibody. b, Cell lysates were blotted with Myc antibodies to monitor Rad and Rem expression levels. Two or three independent experiments were performed and data from a representative experiment are shown.

interacting with the β-subunit.[4,5,14,16,17,23] In the case of Kir/Gem and Rem2, it has been shown that the β-subunit is a *bona fide* effector that interacts selectively with the GTP-bound form.[4,16,17] To determine if the β-subunit can interact with Rad and Rem mutants defective in 14-3-3 and CaM binding, we carried out co-immunoprecipitation experiments from COS-1 cells co-expressing Flag-$Ca_v\beta3$ and the respective wt or mutated RGK proteins (Figure 4(a)). Rad and Rem co-immunoprecipitated with the β-subunit in the absence of exogenous nucleotide (Figure 4(a)a, lanes 1 and 5). To confirm that binding of Rad and Rem to the β-subunit requires their GTP-bound activated form, we generated mutants that can no longer bind GTP (Rad S104N[19] and Rem T94N/S95N; see Figure 1(a)). The association of Rad S104N or Rem S95N/T94N with the β-subunit was reduced dramatically or abolished (lanes 2 and 6), indicating that the β-subunit is indeed an effector of the two RGK proteins. Mutants defective in CaM binding (lanes 3 and 7) or carrying inactivated 14-3-3 binding sites (lanes 4 and 8) were not affected in their ability to associate with the β-subunit. Since the CaM binding mutants localize predominantly to the nucleus (see above), their interaction with the β-subunit indicates that the nuclear pool of these RGK proteins is also in the active form. Western blot analysis of cell lysates confirmed that the transfected cells expressed similar levels of the different RGK proteins and Flag-$Ca_v\beta3$ (Figure 4(a)b and c).

To confirm the tested associations *in vitro* (Figure 4(b)), lysates of COS cells expressing similar amounts of wt or mutated RGK proteins (Figure 4(b), lanes 5–7) were incubated with immobilized β-subunits (GST-$Ca_v\beta3$) and bound RGK proteins were detected by Western blot. Rad (Figure 4(b)a) and Rem (Figure 4(b)b) bound to the β-subunits and the interaction was abolished if the GTP-binding site was eliminated (compare lanes 2 and 3) but the interaction was not affected for the CaM binding mutants (lane 4). Binding of Rad and Rem to the β-subunit did not require the addition of the non-hydrolyzable GTP analog GTPγS, consistent with the low rate of GTP hydrolysis of this family of small G proteins.[7,10,19]

In conclusion, the β-subunit is an effector for Rad and Rem, and their interaction with the β-subunit did not require the ability of the two RGK proteins to bind 14-3-3 or CaM.

Roles of 14-3-3 and CaM in Rad and Rem-mediated downregulation of Ca^{2+} channel activity

Kir/Gem,[4,17,23] Rem2,[14,16] Rad and Rem[5] have been shown to downregulate the VDCC activity. To analyze whether 14-3-3 or CaM binding play a regulatory role in this function of Rad and Rem, we expressed the two RGK proteins and their corresponding CaM or 14-3-3 binding-deficient mutants in PC12 cells, either alone or together with GST-14-3-3, and measured endogenous VDCC Ca^{2+} currents.

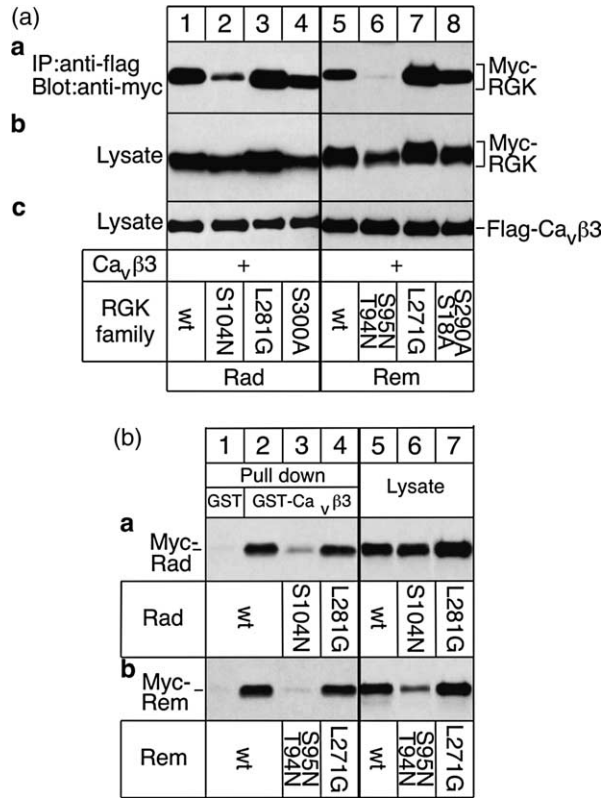

Figure 4. Binding of RGK proteins to the β-subunit of VDCCs. (a) Co-immunoprecipitation. Cells were cotransfected with cDNAs for wt or mutated Myc-RGK proteins and Flag-Ca_vβ3 subunits. a, Flag-Ca_vβ3 subunits were immunoprecipitated and associated Myc-RGK proteins were detected by Western blot by probing with Myc antibodies. b and c, Cell lysates were blotted with (b) Myc and (c) Flag antibodies to monitor expression levels of Myc-RGK proteins and Flag Ca_vβ3, respectively. (b) Pull down. Cells were transfected with cDNAs for wt or mutated Myc-RGK proteins. Cell homogenates were incubated with immobilized recombinant GST-Ca_vβ3 and associated Rad (a) or Rem (b) was detected by Western blot using Myc antibody. Recombinant GST served as a control (lane 1). Cell lysates were blotted with Myc antibodies to monitor RGK protein expression levels (lanes 5–7). Two or three independent experiments were performed and data from a representative experiment are shown.

As shown in Figure 5, expression of both RGK proteins repressed Ca^{2+} channel activity (lanes 2 and 10). In the case of Rad, the mutant defective in CaM binding (lane 4) no longer showed an effect on Ca^{2+} channel activity, whereas Rem did not require CaM binding for their function (lane 12). Mutation of either one or both 14-3-3 binding sites, or overexpression of GST-14-3-3, did not interfere with RGK function (lanes 6, 7 and 14 and 3, 5 and 11, 13, respectively). Surprisingly, however, although Rad L281G did not downregulate Ca^{2+} channel function, mutation of the N-terminal or C-terminal 14-3-3 binding site in Rad L281G restored its activity (lanes 8 and 9), suggesting that in the absence of bound CaM, 14-3-3 also interferes with Rad function.

These data thus show that Rad and Rem downregulate Ca^{2+} channel activity, and that this function is regulated differently by CaM and 14-3-3. While CaM binding was required for Rad function, it was dispensable for Rem. Furthermore, in the absence of bound CaM, 14-3-3 binding showed an inhibitory effect on Rad.

Nuclear sequestration of the β-subunit correlates with Rad and Rem-mediated downregulation of Ca^{2+} channel activity

To determine the mechanism by which Rad and Rem regulate calcium channel activity in PC12 cells, and to understand how CaM and 14-3-3 binding modulate RGK protein function, we analyzed the subcellular distribution of the Ca^{2+} channel α and β-subunits in PC12 cells expressing either wt or mutated RGK proteins. PC12 cells were cotransfected with cDNAs encoding a Cav1.2 α-subunit in which the HA-tag was inserted into an external loop,[26] a Flag-tagged Cavβ3 and one of the Myc-tagged wt or mutated RGK proteins. To ensure homogenous expression of the Ca^{2+} channel subunits, the α and the β-subunit were expressed from an internal ribosomal entry site (IRES)-containing vector (see Materials and Methods). The expression of the RGK proteins and the α and the β-subunit was verified in permeabilized cells. Alternatively, live cells were incubated with HA antibodies prior to permeabilization to selectively detect the Ca^{2+} channels present at the cell surface (surface labeling).

As shown in Figure 6(a), the α-subunit expressed alone showed a cytoplasmic localization (Figure 6(a)a) and could not be detected at the cell surface (Figure 6(a)c). Only in the presence of the β-subunit, the α-subunit was detected at the surface of unpermeabilized cells as a diffused staining (Figure 6(a)d′), confirming the importance of the β-subunit in facilitating surface transport of the α-subunit.[27] The β-subunit was diffused in the cytoplasm and, to a lesser extent, present in the nucleus (Figure 6(a)b and d).

Rad and Rem (Figure 6(b)a and b, and i and j, respectively), as well as the mutants in which both N-terminal and C-terminal 14-3-3 binding sites were mutated (Figure 6(b)c and d, and k and l, respectively) showed a subcellular distribution similar to that in COS-1 cells. However, the nuclear localization of the RGK proteins in PC12 cells was less pronounced than in COS-1 cells. The CaM binding-defective mutants of Rad and Rem (Figure 6(b)e and f, and m and n, respectively) showed only partial nuclear localization. Mutation of one (data not shown) or both 14-3-3 binding sites in the CaM binding-deficient Rad and Rem mutants resulted in an efficient nuclear localization (Figure 6(b)g and h, and o and p, respectively), consistent with a role of endogenous 14-3-3 in the regulation of the subcellular distribution of RGK

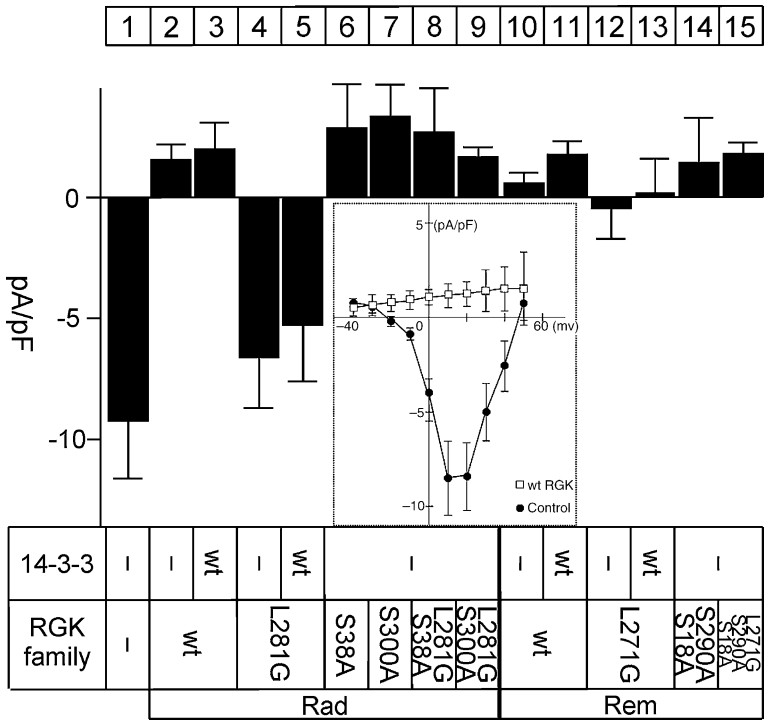

Figure 5. RGK proteins downregulate Ca^{2+} channel activity. PC12 cells were cotransfected with a GFP plasmid and cDNAs for wt or mutated RGK proteins, either with or without 14-3-3. GFP-positive cells were selected for electrophysiology and the average of the maximal current detected at +20 mV for endogenous Ca^{2+} channels was measured. Between nine and 16 independent experiments were carried out for each condition. An example of the I–V relationship of Ca^{2+} channels in PC12 cells is shown in the inset. A, Ampere; F, Farad. Cells transfected only with the GFP cDNA served as a control. Statistical analysis of the data using Student's unpaired *t*-test showed that, with the exception of Rad L281G ($p > 0.05$), the RGK proteins significantly inhibited channel activity ($p < 0.01$).

proteins in PC12 cells. Similar distributions were obtained for HEK293 cells (see Supplementary Data II). Compared to HEK293 cells, the different RGK proteins expressed in COS-1 or PC12 cells showed a bias for either a more nuclear or a more cytoplasmic distribution, respectively.

The β-subunit colocalized predominantly with the RGK proteins. Where the RGK proteins were in the cytoplasm, the β-subunit was also cytoplasmic (Figure 6(b)a′–d′ and i′–l′). If the RGK proteins were in the nucleus, the β-subunit also translocated into the nucleus (Figure 6(b)e′–h′, and m′–p′), reflecting closely the extent of nuclear localization of Rad and Rem. The nuclear colocalization of the Cavβ3 subunit by the RGK proteins in which the two 14-3-3 and the CaM binding sites were mutated was observed also in COS-1 cells (data not shown).

Cell surface transport of the α-subunit in response to the expression of the β-subunit (Figure 6(a), compare c with d′) was blocked upon the expression of either the wt RGK proteins or the different mutants (Figure 6(b), b″–p″). The only exception was cells expressing Rad L281G, where the α-subunit was readily detected at the cell surface (Figure 6(b), f″). Thus, surface transport of the α-subunit in cells expressing the different RGK proteins and their mutants correlated with their functional effect on Ca^{2+} channel activity (see above; and see Figure 5). Similar results were obtained for HEK293 cells (Supplementary Data II).

In summary, binding of 14-3-3 to Rad is not required to repress Ca^{2+} channel surface expression. In the absence of bound CaM, however, 14-3-3 can inhibit the Rad-mediated downregulation of Ca^{2+} channels by preventing the nuclear translocation of the Rad/β3 subunit complex. At least in PC12 cells, 14-3-3 and CaM are apparently not involved in regulating the function of Rem on Ca^{2+} channel surface expression.

Discussion

The functions of members of the RGK small G protein family, Kir/Gem, Rad, Rem and Rem2, have started to be unraveled only recently. We carried out a comparative analysis of the role of 14-3-3 and CaM binding on the functions of Rad and Rem. We show that Rad and Rem exert similar effects on cell shape and calcium channel activity. 14-3-3 and CaM associate with both RGK proteins and regulate their subcellular distribution between the cytoplasm and the nucleus. In the absence of 14-3-3 and CaM binding, the β-subunit can be sequestered, through its interaction with the RGK proteins, to the nucleus, thus preventing cell-surface expression of VDCCs. The inhibitory effect of Rad, but not that of Rem, on VDCC activity is modulated by 14-3-3 and CaM binding.

Rad and Rem carry one N-terminal and one C-terminal 14-3-3 binding site and interact with all 14-3-3 isoforms. Rad requires both 14-3-3 binding sites and binds only 14-3-3 dimers, whereas each 14-3-3 binding site in Rem can individually bind 14-3-3 and 14-3-3 dimerization is not required. However, functional effects of 14-3-3 on Rad and Rem were found only when 14-3-3 was dimerized. After mutating the CaM binding site to prevent CaM association with the RGK proteins, the C-terminal 14-3-3 binding site in Rem became sufficient for 14-3-3 association, whereas Rad still required both binding sites. Since both the

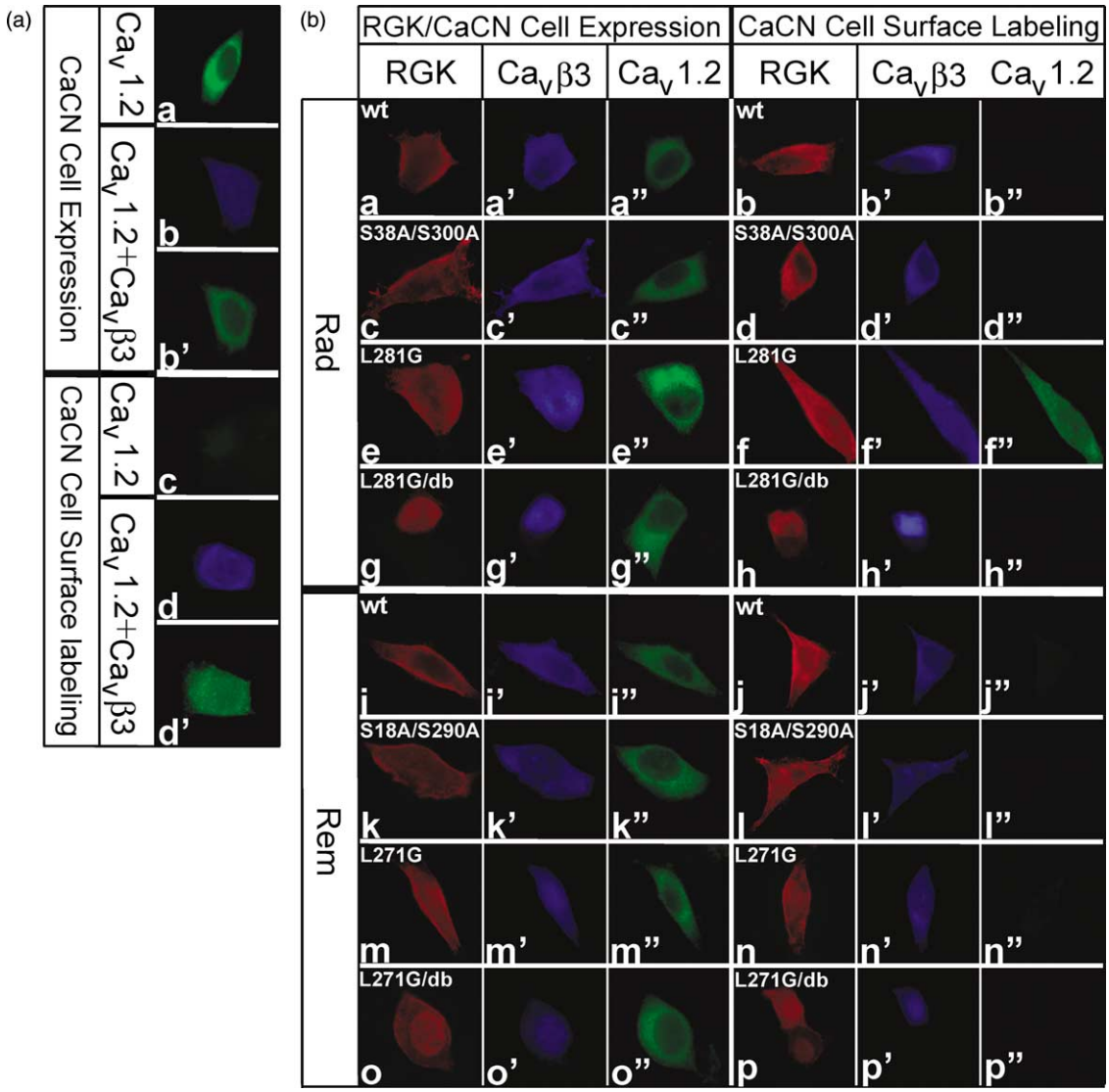

Figure 6. RGK-mediated nuclear sequestration of VDCC β-subunits and inhibition of cell-surface expression of α-subunits in PC12 cells. (a) The β-subunit facilitates surface expression of the α-subunit. PC12 cells were transfected with a cDNA for $Ca_v1.2$ carrying an extracellular HA tag or with an IRES-based vector carrying the cDNAs for HA-$Ca_v1.2$ and Flag-$Ca_v\beta3$ subunits. Cells were fixed, permeabilized and processed for immunofluorescence microscopy using HA and Flag antibodies to detect $Ca_v1.2$ (green) and $Ca_v\beta3$ (blue), respectively (CaCN cell expression). Alternatively, live cells were first incubated with HA antibodies to selectively label surface-exposed $Ca_v1.2$ prior to the fixation, permeabilization and labeling with Flag antibodies (CaCN cell-surface labeling). (b) Effect of RGK proteins on β-subunit localization and cell-surface expression of α-subunits. PC12 cells were transfected with cDNAs for wt or mutated Myc-RGK proteins, together with an IRES-based vector carrying the cDNAs for HA-$Ca_v1.2$ and Flag-$Ca_v\beta3$. Cells were fixed, permeabilized and processed for immunofluorescence microscopy using Myc, HA and Flag antibodies to detect the RGK protein (red), $Ca_v1.2$ (green) and $Ca_v\beta3$ (blue), respectively (RGK/CaCN cell expression). Alternatively, live cells were first incubated with HA antibodies to selectively label surface-exposed $Ca_v1.2$ prior to the fixation, permeabilization and labeling with Myc and Flag antibodies (CaCN cell-surface labeling). db, double mutant with both 14-3-3 binding sites mutated. Four or five independent experiments were performed with 15–20 cells expressing the Ca^{2+} channels and the RGK proteins. Statistical analysis of the data was performed as outlined in Materials and Methods.

N-terminal and C-terminal 14-3-3 binding sites in Rad and Rem are relatively conserved, and thus probably phosphorylated by the same kinase(s), the differential requirement of dimerization for the interaction of 14-3-3 with Rad and Rem may provide a mechanism for the differential regulation of the two RGK proteins. Both monomeric and dimeric forms of 14-3-3 are found in cells, and dimerization is regulated by phosphorylation,[28] and is thought to be required for a high-affinity, stable binding.[21] Thus, while 14-3-3 monomers may be able bind to RGK proteins in the absence of CaM (our unpublished data), high-affinity interaction as provided by 14-3-3 dimers may be required for 14-3-3 to displace CaM from the RGK protein.

Kir/Gem W269G and Rem2 L317G have been observed in the nucleus in various cells, raising the possibility that CaM binding might regulate the subcellular localization.[4,16,17] RGK proteins usually show cytoplasmic, submembranous and nuclear localization, and our results for Rad and Rem confirm that nuclear translocation in the absence of CaM binding is a common feature of RGK proteins. However, a direct link between the subcellular distribution of Rad or Rem and the change in intracellular concentrations of Ca^{2+} could not be established, since compounds such as ionomycin or BAPTA-AM affect nuclear transport in living cells.[29] Over-expression of 14-3-3 led to the exclusion of RGK proteins from the nucleus and this effect was particularly prominent for the CaM binding mutants. In COS-1 cells, mutation of the 14-3-3 binding site in RGK proteins did not affect their subcellular localization and, together with the observation that only a small amount of endogenous 14-3-3 was bound to RGK proteins, suggests that most of the endogenous 14-3-3 is sequestered, and that nuclear exclusion of RGK proteins requires over-expression of 14-3-3. In PC12 cells, however, the subcellular localization of Rad and Rem, and their CaM-deficient mutants, was shifted towards a more cytoplasmic localization and the abolition of the 14-3-3 association was required for efficient nuclear translocation. This was observed also for Rem2,[16] and is consistent with a more prominent role of endogenous 14-3-3 in regulating the subcellular localization of the RGK proteins in PC12 cells, possibly reflecting a larger pool of available free 14-3-3. The variations in different cell types[16] probably reflect differences in the pools of free 14-3-3 and CaM in these cell lines available to interact with the over-expressed RGK proteins. Alternatively, since binding of 14-3-3 to RGK proteins is likely to be regulated by phosphorylation and/or dephosphorylation, the expression level of kinases and phosphatases that are involved in the post-translational modification of RGK proteins may differ. Indeed, preliminary evidence indicates that substitution of a serine residue in the C-terminal extension of Rem (and Kir/Gem, but not Rad) to alanine results in nuclear translocation independent of CaM binding (our unpublished data). The mechanisms by which 14-3-3 and CaM binding interfere with nuclear translocation of Rad and Rem are unclear. Kir/Gem has several nuclear import signals (NLS) that are conserved in Rad, Rem and Rem2 (our unpublished data). Since one of these NLSs is in proximity to the CaM and C-terminal 14-3-3 binding sites, it is conceivable that 14-3-3 and/or CaM binding mask this NLS and thereby reduce the rate of nuclear import. Such a function is well established for 14-3-3.[30]

Kir/Gem,[12,13,17,31] Rad,[31] Rem,[11] and Rem2[16] have been shown to regulate cell morphology. Over-expression of exogenous 14-3-3, but not a 14-3-3 mutant defective in target protein binding reduced Rad and Rem-mediated induction of extensions, and a similar effect was observed for Kir/Gem[17] and Rem2.[16] The change in cell morphology by Kir/Gem was attributed to the function of Gem and Rad as negative regulators of the Rho-Rho kinase pathway.[31] Whether 14-3-3 binding to RGK proteins modulates this pathway remains an open question.

Rad and Rem bind the β-subunit of VDCCs. Similar to Kir/Gem and Rem2, the association with the β-subunit requires the GTP-bound form of the RGK proteins, since binding to Rad S104N, which is locked into the GDP-bound form,[19] as well as to the corresponding Rem mutants (i.e. Rem T94N/S95N) was affected severely. Thus, the β-subunit appears to be a *bona fide* effector of all RGK proteins. By binding the β-subunit, Kir/Gem, Rad, Rem and Rem2 downregulate Ca^{2+} channel activity.[4,5,14,16,17]

Ca^{2+} influx through VDCCs triggers intracellular events such as gene expression, muscle contraction, hormone secretion and synaptic transmission.[1] Given that all RGK proteins regulate VDCCs, they may mediate similar functions in different tissues. Rad[8] and Rem[9] are expressed in muscle cells and have been implicated in muscle cell Ca^{2+} signaling.[5,19,32] Kir/Gem is present in pituitary, adrenal and pancreatic islet cells, and may regulate VDCC activity in neuroexocrine cells;[4] and Rem2 has been implicated in regulating insulin secretion.[14] VDCCs are also present in high density in the presynaptic nerve terminals, where they couple Ca^{2+} influx in neuronal excitation to neurotransmitter release.

Cell surface transport of VDCCs is thought to require the association of the α-subunit with the β-subunits,[3,33] and Kir/Gem and Rem2 have been proposed to interfere with cell-surface transport of VDCCs by associating with the β-subunit.[4,16] Using an α-subunit carrying an extracellular epitope tag, we confirmed that the downregulation of VDCC activity in the presence of Rad and Rem correlates with the absence of Ca^{2+} channels at the cell surface. Although both RGK proteins downregulate Ca^{2+} channel activity by inhibiting cell-surface transport of the α-subunit, regulation by CaM and 14-3-3 differs for Rad and Kir/Gem,[17] as compared to Rem and Rem2.[16] Mutating the CaM binding site prevented the Rad-mediated downregulation of VDCC activity but did not interfere with Rem function. Intriguingly, however, abolishing 14-3-3 binding in Rad L281G (i.e. Rad L281G/S38A, Rad L281G/S300A or Rad L281G/S38A/S300A) restored the repression of Ca^{2+} channel activity. Conceivably, the different requirement for CaM binding could reflect differences in the relative binding affinities of CaM, 14-3-3 and the β-subunit for Rad and Rem. Indeed, CaM, 14-3-3 and the β-subunits bind in a mutually exclusive fashion to Kir/Gem.[17] Thus, it is possible that a transient association of CaM is required for the β-subunit to displace the bound 14-3-3 from Rad, but not from Rem.

Analysis of the distribution of β-subunits in cells expressing Rad L281G S38A/S300A revealed that both proteins localized to the nucleus. Indeed, a

close co-distribution of the β-subunit with Rad and Rem was observed. Thus, cell-surface expression of VDCCs may be regulated by two distinct mechanisms, one involving the association of the RGK protein with the β-subunit in the cytosol, the other a nuclear sequestration of the β-subunit by the RGK protein. The extent to which one or the other mechanism is utilized may be determined, in turn, by signaling events that control the association of CaM and 14-3-3 with the RGK proteins. The β3 and β4-subunits were detected in the nucleus of heart myocytes,[34] and a splice variant of the β4-subunit was shown to interact with a nuclear protein and attenuate its gene-silencing activity.[35] Whether RGK proteins modulate this novel function of the β-subunit will be of interest to explore.

In conclusion, 14-3-3 and CaM binding regulate the subcellular distribution of Rad and Rem between the cytoplasm and the nucleus. The two RGK proteins exert similar effects on Ca^{2+} channel activity by blocking surface expression of VDCCs, either by binding the β-subunit in the cytosol or by sequestering it into the nucleus.

Materials and Methods

Molecular biology

Point mutations were introduced into the putative 14-3-3, CaM and GTP binding sites of mouse RGK proteins by a polymerase chain reaction (PCR)-based method. RGK and $Ca_v\beta3$ were Myc and Flag epitope tagged respectively at their N terminus as described.[4] For Ca^{2+} channel cell surface expression experiments, $Ca_v1.2$ was hemagglutinin (HA)-tagged by inserting the epitope into the extracellular S5-H5 loop of domain II as reported,[26] and introduced into a pIRES vector containing the $Ca_v\beta3$ subunit; 90% of the cells transfected by the pIRES vector expressed both subunits (data not shown). GST-14-3-3 was generated as follows: 14-3-3 cDNA was first subcloned into the pGEX-6P1 then the GST-14-3-3 chimera was introduced into a mammalian expression vector (pME18S). The 14-3-3 ζ dimerization mutant was produced as described.[25] To generate the recombinant proteins, full length cDNA for $Ca_v\beta3$ (kindly provided by S. Seino (Kobe University, Japan)), was amplified by PCR and introduced into pGEX-6P1, and purified according to the manufacturer's instructions. The cDNAs for mouse Rad and Rem, and human 14-3-3 β, γ, ε, σ, τ, η, ζ isoforms were obtained from I.M.A.G.E. Consortium (numbers 5149047, 5389715, 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, PC-12 and HEK-293T cells were grown as described,[4] and 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 h 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, 0.2% (v/v) Tween-20 supplemented with protease inhibitors, leupeptin, pepstatin and antipain (5 μg/ml each)). 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% polyacrylamide) and Western blot analysis using monoclonal anti-c-Myc (Roche), monoclonal anti-Flag (M2; Sigma), rabbit anti-GST (Santa Cruz), and rabbit anti-14-3-3 (Zymed) antibodies. For co-precipitation experiments, cell lysates (300–400 μg of protein) were incubated with GST-agarose beads (Sigma), Flag-agarose beads (Sigma) or c-Myc agarose beads (Santa Cruz) in lysis buffer (see above) for 3 h at 4 °C. Beads were then washed extensively and eluted protein complexes were subjected to SDS-PAGE and Western blot analysis as described above. Pull-down experiments using GST-$Ca_v\beta3$ proteins were carried out essentially as described.[4] CaM binding to RGK proteins was performed as reported.[19] Briefly, CaM-Sepharose beads (Amersham Biosciences) were incubated at 4 °C for 3 h with a cell lysate (300–400 of μg protein) prepared as described above, in 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1% (v/v) Triton X-100. Beads were then washed extensively with the same buffer and CaM-associated RGK proteins eluted in SDS-PAGE sample buffer prior to SDS-PAGE and Western blot analysis.

Immunocytochemistry

Cells were fixed with 3.7% (w/v) paraformaldehyde, washed twice with 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% (w/v) bovine serum albumin (BSA) and 250 mM NaCl) for 30 min followed by incubation with rabbit anti-Myc (Upstate biotechnology) and monoclonal anti-GST (Cell Signaling Technology), suitably diluted in the blocking solution for 1 h. After washing, the labeled secondary antibodies Cy3-labeled donkey anti-rabbit IgG (Jackson Immuno Research Laboratories) and Alexafluor 488 goat anti-mouse IgG (Molecular Probes) were used. For triple labeling experiments, N-myc RGK proteins, N-flag $Ca_v\beta3$ and HA-$Ca_v1.2$ proteins were detected as described above by a first incubation with rabbit anti-Myc (Upstate biotechnology), monoclonal anti-Flag (M2; Sigma) and rat anti-HA (Roche) antibodies, following by a second incubation with the labeled secondary antibodies Cy3-labeled donkey anti-rabbit IgG (Jackson Immuno Research Laboratories), Alexafluor 350 goat anti-mouse IgG and Alexafluor 488 goat anti-rat IgG (Molecular probes), respectively. Cell-surface expression studies in PC12 and HEK-293T cells were carried out 48 h after transfection. Cells were first incubated with 2 μg/ml of rat anti-HA (Roche) for 1 h at 37 °C then washed twice in ice-cold PBS prior to fixation. Specimens were visualized with an Axiocam microscope (Carl Zeiss) at a magnification of 100×. For quantification of the subcellular localization, 150–200 cells from three or four independent experiments were analyzed and the localization of the RGK proteins grouped into different categories. To avoid any bias, two

independent researchers did the quantification and obtained similar results. Statistical significances of the combined results were determined using unpaired Student's *t*-tests.

Electrophysiology

Recordings were made 48 h after transfection using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). The whole-cell VDCC currents in PC12 cells were recorded as described.[4] Ba^{2+} was used as a charged carrier for measurement of VDCC currents. Cells were maintained at a holding potential of −60 mV. For recording VDCC currents, square pulses of 400 ms duration at potentials between −40 and +60 mV in steps of 10 mV were applied every 4 s. For normalization, the currents were divided by the membrane capacitance measured for each cell.[36] Statistical significances were determined using unpaired Student's *t*-tests and results are expressed as mean ± SE.

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at 10.1016/j.jmb.2005.10.013

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