Plasma Membrane Targeting Is Essential for Rem-mediated Ca\textsuperscript{2+} Channel Inhibition

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The small GTPase Rem is a potent negative regulator of high voltage-activated Ca\textsuperscript{2+} channels and a known interacting partner for Ca\textsuperscript{2+} channel accessory \(\beta\) subunits. The mechanism for Rem-mediated channel inhibition remains controversial, although it has been proposed that Ca\textsubscript{\(\alpha\)}\textsubscript{\(\beta\)} association is required. Previous work has shown that a C-terminal truncation of Rem (Rem\textsuperscript{(1–265)}) displays reduced \textit{in vivo} binding to membrane-localized \(\beta\)2a and lacks channel regulatory function. In this paper, we describe a role for the Rem C terminus in plasma membrane localization through association with phosphatidylinositol lipids. Moreover, Rem\textsuperscript{(1–265)} can associate with \(\beta\)2a \textit{in vitro} and \(\beta\)1b \textit{in vivo}, suggesting that the C terminus does not directly participate in Ca\textsubscript{\(\beta\)}\textsubscript{2} association. Despite demonstrated \(\beta\)1b binding, Rem\textsuperscript{(1–265)} was not capable of regulating a Ca\textsubscript{\(\alpha\)1.2–\(\beta\)1b channel complex, indicating that \(\beta\) subunit binding is not sufficient for channel regulation. However, fusion of the CAAX domain from K-Ras\textsubscript{4B} or H-Ras to the Rem\textsuperscript{(1–265)} C terminus restored membrane localization and Ca\textsuperscript{2+} channel regulation, suggesting that \(\beta\) binding and membrane localization are independent events required for channel inhibition.

High voltage-activated Ca\textsuperscript{2+} channels (Ca\textsubscript{\(\alpha\)1 and Ca\textsubscript{\(\alpha\)2} families) transduce electrical activity into increased intracellular calcium that mediates a diverse array of essential cellular processes, including hormone secretion, neurotransmitter release, and excitation-contraction coupling in muscle systems (1). The cardiac L-type Ca\textsuperscript{2+} channel is a multiprotein complex consisting of the pore-forming Ca\textsubscript{\(\alpha\)1.2} \(\alpha\) subunit and auxiliary subunits, including Ca\textsubscript{\(\beta\)} and \(\alpha\)\textsubscript{\(\delta\)} subunits (1). The Ca\textsubscript{\(\alpha\)}\textsubscript{\(\alpha\)} subunit determines the ion selectivity and single channel conductance of the mature channel, whereas \(\alpha\)\textsubscript{\(\delta\)} co-expression of Ca\textsubscript{\(\alpha\)}\textsubscript{\(\beta\)} or \(\alpha\)\textsubscript{\(\delta\)} facilitates cell surface trafficking of the \(\alpha\)\textsubscript{\(\alpha\)} subunit, increases Ca\textsuperscript{2+} current amplitude, and alters channel gating properties (1, 2). Ca\textsubscript{\(\alpha\)}\textsubscript{\(\beta\)} subunits are encoded by four genes (\(\beta\)1–\(\beta\)4), each subject to complex splicing (3). Ca\textsubscript{\(\alpha\)}\textsubscript{\(\beta\)}\textsubscript{2}a, a \(\beta\) isoform found in the heart, is subject to post-translational palmitoylation, which directs plasma membrane localization, whereas other \(\beta\) isoforms are predominantly localized to the cytosol when not bound to Ca\textsubscript{\(\alpha\)}\textsubscript{\(\alpha\)} (3).

Recently, members of the RGK\textsuperscript{3} family of Ras-related GTPases, including Rem (4), Rem2 (5), Rad (6), and Gem/Kir (7), have been identified as potent regulators of HVA Ca\textsuperscript{2+} channel function (8–10). Although all RGK GTPases associate with Ca\textsubscript{\(\alpha\)}\textsubscript{\(\beta\)} subunits and prevent \textit{de novo} expression of L-type \(I\)\textsubscript{\(\text{Ca}\)} (8–10), the mechanism of RGK protein-mediated Ca\textsuperscript{2+} channel inhibition remains controversial. It was originally hypothesized that RGK protein binding blocked Ca\textsubscript{\(\alpha\)}\textsubscript{\(\alpha\)1/\(\alpha\)2} association, leading to a reduction of functional channels at the cell surface (8, 11–14). However, a series of recent studies suggests instead that the majority of RGK proteins inhibit the activity of the preassembled channel complex at the plasma membrane (10, 15, 16), although Ca\textsubscript{\(\alpha\)}\textsubscript{\(\beta\)} association still appears critical (16, 17). Moreover, RGK-mediated channel regulation appears more complex than simple Ca\textsubscript{\(\alpha\)}\textsubscript{\(\beta\)} sequestration (16, 17) and may include contributions from both the Ca\textsubscript{\(\alpha\)}\textsubscript{\(\alpha\)} C terminus and protein kinase A signaling pathways (18).

The conserved RGK C terminus plays a crucial role in Ca\textsuperscript{2+} channel regulation. Deletion of the Rem, Rem2, and Rad C terminus inhibits plasma membrane localization of the proteins, greatly reduces Ca\textsubscript{\(\alpha\)}\textsubscript{\(\beta\)}\textsubscript{2}a subunit binding, and eliminates Ca\textsuperscript{2+} channel regulation (9, 15, 19). Recent work has described mutations to the C-terminal domain that alter CaM and 14-3-3 binding in all RGK proteins (12–14, 20), and research by Beguin \textit{et al.} (12–14) suggests that loss of CaM binding leads to nuclear localization, whereas overexpression of 14-3-3 proteins promotes the clearance of RGK proteins from the nucleus. Mutations that prevent 14-3-3 and CaM binding in Rad result in the redistribution of Rad and Ca\textsubscript{\(\alpha\)}\textsubscript{\(\beta\)}\textsubscript{3} to the nucleus (14). A corresponding loss of Rad-mediated Ca\textsuperscript{2+} channel regulation for these mutants has led to the suggestion that RGK-mediated channel inhibition involves nuclear targeting of Ca\textsubscript{\(\alpha\)}\textsubscript{\(\beta\)} subunits (14). Thus, although it is clear that the conserved RGK C ter-

\textsuperscript{3} The abbreviations used are: RGK, Rem, Rem2, Rad, and Gem/Kir GTPases; GST, glutathione S-transferase; GFP, green fluorescent protein; HA, hemagglutinin; WT, wild type; PI, phosphatidylinositol; RFP, red fluorescent protein; PtdIns, phosphatidylinositol; PIP, phosphorylated phosphatidylinositol; PIP\textsubscript{2}, diphasphorylated phosphatidylinositol; PIP\textsubscript{3}, PtdIns(3,4,5)P\textsubscript{3}.

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Plasma Membrane Targeting of Rem GTPase

minus plays a role in channel regulation, the exact mechanism of action remains to be determined.

Here, we analyze the contribution of the Rem C terminus to Ca\(^{2+}\) channel regulation. We find that Rem is trafficked to the plasma membrane and associates with phosphatidylinositol lipids and that truncation of the C-terminal results in redistribution to the cytosol, accompanied by a loss of calmodulin binding and Ca\(^{2+}\) channel inhibition. These truncation mutants display a reduction in Ca\(_{\alpha}\)β2a but not Ca\(_{\alpha}\)β1b association in vivo, and loss of the C terminus does not affect in vitro β2a subunit binding, indicating that β subunit interaction does not require the Rem C terminus. In addition, the Rem-(1–265) truncation mutant that binds Ca\(_{\alpha}\)β1b does not inhibit current expression from the heterologously expressed Ca\(_{\alpha}\)1.2-Ca\(_{\alpha}\)β1b channel, indicating that Rem does not inhibit channel function solely through β subunit sequestration. Anchoring of Rem-(1–265) to the plasma membrane using the CAAX motif from H-Ras or K-Ras4B restores Ca\(^{2+}\) channel inhibition, suggesting that plasma membrane localization is critical for Rem-mediated Ca\(^{2+}\) channel regulation.

EXPERIMENTAL PROCEDURES

Plasmids—Mammalian expression vectors for Ca\(_{\alpha}\)1.2 α subunit, FLAG epitope-tagged β2a subunit, FLAG epitope-tagged β1b subunit, and HA epitope-tagged Rem have been described previously (9). Rem truncation mutants were generated by PCR using HA-tagged Rem as the template and fully sequenced. RFP-Rem-(266–297) was generated by PCR and inserted behind RFP in pDsRed vector (Clontech). Chimeric Rem proteins were generated by ligation of oligonucleotides corresponding to the C terminus of human K-Ras4B (residues 171–188) or mouse H-Ras (residues 171–189) to the C terminus of pcDNA3.1 + zeo 3×HA-Rem-(1–265) utilizing XbaI/Apal sites.

Confocal Imaging—Confocal imaging of green fluorescent protein (GFP)-tagged Rem truncations, chimeric Rem proteins, RFP-Rem-(266–297) and Rem\(^{WT}\) was performed as previously described (18). Images displayed are representative of the cells observed. Quantification was performed using Leica LCS software. Plasma membrane localization was quantified by four line scan intensity measurements through each cell beginning in the central cytoplasm, avoiding the nucleus, and ending at the cell periphery. GFP intensity at the cell periphery in each scan was divided by the mean intensity over the entirety of the scanned line to monitor GFP cell periphery intensity over that of the GFP-tagged protein in the cytosol. Line scans were averaged for each cell, and the mean values of the averaged cell measurements are reported as mean ± S.E. Significance was determined using Student’s t test with a p value of <0.05. To examine the localization of GFP-Rem-(1–276) at the cell periphery, a double-blind study was performed. From the line scan analysis above, 32 cells expressing Rem-(1–276) and 33 cells expressing Rem-(1–265) were randomized and examined by three individuals, who were asked to score each cell for the presence of increased punctate GFP fluorescence at the cell periphery. Scored cells were then matched to their appropriate treatment, and the percentage of cells from each treatment displaying localized increases of GFP fluorescence at the cell boundary was determined. Values are reported as mean ± S.D., and significance was determined using Student’s t test with a p value of <0.05.

PIP Binding Assay—3× FLAG-tagged Rem truncations or empty 3× FLAG vector was expressed in tsA201 cells using the calcium phosphate transfection method as described previously (21). 48 h post-transfection, cells were harvested and lysed in PIP binding buffer (50 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0), 100 mM NaCl, 0.5% Triton X-100, 1× protease inhibitor mixture I (Calbiochem)), sonicated, and centrifuged at 100,000 × g. PIP strips (Molecular Probes) were blocked in TBS-T + 3% fatty acid-free bovine serum albumin for 1 h and incubated with total cell lysate from the appropriate treatment in TBS-T + 3% fatty acid-free bovine serum albumin at 4 °C overnight with gentle rocking. Membranes were then washed with TBS-T supplemented with 3% fatty acid-free bovine serum albumin and probed with biotinylated FLAG antibody and horseradish peroxidase-conjugated streptavidin. Binding of Rem truncations was detected using enhanced chemiluminescence reagent (Pierce).

β Subunit Association Assays—Co-immunoprecipitation of 3× HA-tagged Rem truncations, chimeric Rem proteins, and Rem\(^{WT}\) with Ca\(_{\alpha}\)β2a and Ca\(_{\alpha}\)β1b in HEK293 and tsA201 cells were performed as previously described (9, 21).

Calmodulin Binding—TsA201 cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and transfected with the indicated plasmids using the calcium phosphate method as previously described (21). 48 h post-transfection, cells were harvested and lysed in calmodulin immunoprecipitation buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% Nonidet P-40, 1× phosphatase inhibitor mixture II (Calbiochem), 1× protease inhibitor mixture I (Calbiochem), 1 mM phenylmethylsulfonyl fluoride), sonicated, and centrifuged at 100,000 × g. Calmodulin-Sepharose beads (GE Healthcare) were washed twice with immunoprecipitation buffer and incubated with 1 mg of total lysate in the presence of 2 mM CaCl\(_{2}\) or 2.5 mM EGTA. Beads were washed three times in immunoprecipitation buffer containing 2 mM CaCl\(_{2}\) or 2.5 mM EGTA as appropriate, and proteins were released from the beads by boiling for 5 min in 20 μl of 2× SDS-PAGE loading buffer. Associated proteins were resolved on 10% SDS-PAGE minigels and transferred to nitrocellulose membranes. Interaction of Rem proteins with calmodulin was examined by immunoblot with Rem polyclonal antibody (9).

In Vitro Rem Binding Assay—Generation of GST-tagged Rem-(1–265) vector as well as protein production and purification have been previously described (4, 9). Generation of in vitro transcribed/translated \(^{35}\)S-labeled Ca\(_{\alpha}\)β2a and the in vitro binding assay with GST-tagged Rem have been previously described (16).

Electrophysiology—HEK293 cells were transfected using Effectene (Qiagen) according to the manufacturer’s instructions. TsA201 cells were transfected with the indicated plasmids using the calcium phosphate method as previously described (21), and whole-cell patch clamp experiments were performed as described previously (21). Pipette solutions consisted of 150 mM CsCl, 1 mM MgCl\(_{2}\), 5 mM Mg-ATP, 3 mM
EGTA, 5 mM Hepes (pH 7.36). The bath solution for Ba2+ recordings consisted of 112.5 mM CsCl, 30 mM BaCl2, 1 mM MgCl2, 10 mM tetraethylammonium chloride, 5 mM glucose, 5 mM Hepes (pH 7.4). The bath solution for Ca2+ recordings consisted of 112.5 mM CaCl2, 30 mM CaCl2, 1 mM MgCl2, 10 mM tetraethylammonium chloride, 5 mM glucose, 5 mM Hepes (pH 7.4). Traces were analyzed using Origin statistical software. Values are reported as normalized mean at 5 mV ± S.E. for Ba2+ currents and as normalized mean at 15 mV ± S.E. for Ca2+ currents, and significance was determined using Student’s t test with a p value of <0.05. Voltage curves were fit to the Boltzmann form.

\[
I(V) = G_{\text{max}} \times (V - E_{\text{rev}})/(1 + \exp(V_{1/2} - V)/k).
\] (Eq. 1)

Electrophysiological parameters of the analyzed currents are reported in Table 1.

**RESULTS**

The Rem C Terminus Is Required for Plasma Membrane Trafficking—Previous studies have shown that Rem has a complex subcellular distribution, since it is found in both the cytosol and in association with the plasma membrane when expressed in a variety of cells (5, 18, 22, 23). Since Rem has been shown to directly interact with Ca3β subunits, and this association appears to be required for Rem-mediated blockade of surface-localized Ca2+ channels, we used confocal microscopy to examine whether Ca3β2a subunit expression modulates Rem trafficking to the plasma membrane. As seen in Fig. 1B, GFP-RemWT displayed a border-enriched fluorescence pattern, consistent with localization to the plasma membrane. Fluorescence was also observed in the cytosol but was excluded from the nucleus. The distribution of GFP-RemWT co-expressed with pCMV7/F2 (control vector) was statistically indistinguishable from GFP-RemWT co-expressed with either FLAG-Ca3β2a, Ca3.1, or FLAG-Ca3β2a + Ca3.1 (Fig. 1B). Thus, plasma membrane localization of Rem probably involves an intrinsic membrane targeting domain and is not greatly influenced by interactions with Ca2+ channel subunits.

To identify the structural domain in Rem responsible for plasma membrane trafficking, we generated a series of Rem C-terminal truncation mutants fused to GFP and examined their subcellular distribution using confocal microscopy in the presence of co-expressed empty FLAG vector control, FLAG-Ca3β2a, Ca3.1, or FLAG-Ca3β2a + Ca3.1 (Fig. 1C). Once again, co-expression of Ca3 subunits had no measurable effect on Rem mutant localization (Fig. 1C). Intensity profiling analysis (Fig. 1D) revealed that both GFP-Rem-(1–282) (5.05 ± 0.36, n = 40) and GFP-RemWT (2.89 ± 0.30, n = 28) were prominently localized to the cell periphery in a manner consistent with plasma membrane localization and, surprisingly, that Rem-(1–282) displayed significantly stronger targeting than RemWT (p < 0.001), perhaps suggesting that the distal C terminus plays a regulatory role in Rem localization. GFP-Rem-(1–276) (1.22 ± 0.02, n = 43) displayed only a slight enrichment at the cell periphery using this analysis; however, this truncation did show a statistically significant increase in membrane localization when compared with Rem-(1–270) (1.03 ± 0.01, n = 58) or Rem-(1–265) (0.99 ± 0.02, n = 55), which were expressed exclusively in the cytosol (p < 0.001) (Fig. 1, C and D). To understand this difference, we more closely examined the distribution of the GFP-Rem truncations by double-blind trial and noted that rather than a uniform membrane pattern of fluorescence, 73.96 ± 12.63% of cells expressing GFP-Rem-(1–276) displayed a punctate pattern of fluorescence at the cell bound-
ary (Fig. 1E), as compared with 15.15 ± 13.21% of cells expressing GFP-Rem-(1–265) (p < 0.01). Taken together, these data suggest that residues 270–282 within the Rem C terminus play a critical role in targeting Rem to the plasma membrane.

**Truncation of the Rem C Terminus Disrupts Phosphatidylinositol (PI) Lipid Binding**—Recent data suggest that many small GTPases bearing polybasic C termini are plasma membrane-localized and bind PI lipids, including the Gem and Rad GTPases (23). To examine whether the Rem C terminus also directs selective PI lipid binding, we performed an overlay assay utilizing 3× FLAG-tagged RemWT and Rem C-terminal truncations or empty 3× FLAG vector control, overexpressed in tsA201 cells, and PIP strips (Molecular Probes, Hybond membranes spotted with 15 different biologically active lipids). As shown in Fig. 2, RemWT and Rem-(1–282) displayed strong association with PtdIns(3)P, PtdIns(4)P, PtdIns(3,4)P2, PtdIns(3,5)P2, PtdIns(4,5)P2, PtdIns(3,4,5)P3, and phosphatidic acid, whereas greater C-terminal truncations resulted in substantially diminished lipid binding. These data correlate with

**FIGURE 1.** Deletion of the Rem C terminus prevents plasma membrane localization. A, diagram showing features of the Rem C terminus and the locations of Rem truncations. B, TsA201 cells were transfected with plasmids expressing RemWT and empty pCMVT7F2 vector, Ca1.2, and/or FLAG-Caβ2a. 72 h post-transfection, cells were examined by confocal microscopy. The localization of RemWT at the cell periphery is not significantly affected by co-expression of calcium channel components. C, TsA201 cells were transfected with plasmids expressing Rem truncations and empty pCMVT7F2 vector, Ca1.2, and/or FLAG-Caβ2a, as described in B. GFP-Rem-(1–265) and GFP-Rem-(1–270) show cytosolic localization, GFP-Rem-(1–282) displays very strong cell periphery enrichment, and GFP-Rem-(1–282) shows very strong cell periphery enrichment consistent with plasma membrane localization irrespective of Caβ2a or Ca1.2 co-transfection. D, confocal images were quantified by line scan from the cytosolic interior of the cell to the plasma membrane as described under “Experimental Procedures.” Intensity at the cell periphery was divided by the mean intensity over the total line scan to find cell peripheral enrichment. Line scan was performed four times for each cell examined, and the results were averaged. A significant difference (p < 0.05) between treatments is denoted by asterisks. E, selection of tsA201 cells from C and D. The arrows indicate patches of increased GFP-Rem-(1–276) expression at the cell boundary.

**FIGURE 2.** Rem membrane localization is positively correlated to PI lipid association. 3× FLAG-tagged Rem truncations or empty 3× FLAG vector (control) were overexpressed in tsA201 cells and cell lysates were exposed to PIP strips in an overlay assay. Spotted lipids are identified in the above figure as follows: 1, lysophosphatidic acid; 2, lysophosphatidylcholine; 3, phosphatidylinositol (PtdIns); 4, PtdIns(3)P; 5, PtdIns(4)P; 6, PtdIns(5)P; 7, phosphatidylethanolamine; 8, phosphatidylcholine; 9, sphingosine 1-phosphate; 10, PtdIns(3,4)P2; 11, PtdIns(3,5)P2; 12, PtdIns(4,5)P2; 13, PtdIns(3,4,5)P3; 14, phosphatidic acid; 15, phosphatidylserine; 16, blank. Association of Rem truncations with spotted lipids was observed using immunoblotting with biotinylated FLAG antibody. Although Rem-(1–282) and RemWT display robust association with phosphorylated PI lipids, further truncation of the Rem C terminus dramatically diminishes the interaction.
the observed reduction in plasma membrane association (Fig. 1, C and D) and suggest that membrane localization is mediated in part by association of the Rem C terminus with phosphatidylinositol lipids.

**Contribution of the C Terminus to Rem-mediated Ca²⁺ Channel Regulation**—The Rem truncation mutant Rem-(1–282) retains the ability to bind β2a and regulate Ca²⁺ channel activity, whereas Rem-(1–265) is incapable of HVA Ca²⁺ channel regulation and displays reduced β2a binding (9). However, since Rem-(1–265) is not plasma membrane-localized (Fig. 1, C and D), we next asked whether the intermediate Rem truncations were capable of binding β2a and regulating Ca²⁺ channel function. To this end, 3X HA-tagged versions of Rem-(1–265), Rem-(1–270), Rem-(1–276), and RemWT were analyzed for β2a binding (Fig. 3A). As reported previously, Rem-(1–265) displayed an almost complete loss of association with FLAG-β2a (9) as measured by co-immunoprecipitation, whereas binding of Rem-(1–270) to β2a was noticeably weaker than that between FLAG-β2a and RemWT or Rem-(1–276).

Interestingly, although co-expression of either RemWT or Rem-(1–282) has been shown to result in a complete blockade of ionic current expression (9), neither Rem-(1–270) nor Rem-(1–276) was capable of generating a complete channel block in the presence of 30 mM Ba²⁺ (Fig. 3, C and E). Whole cell currents elicited in the presence of GFP-Rem-(1–270) co-expression (−11.877 ± 4.128, n = 9) were statistically indistinguishable from control currents in HEK293 cells expressing Caᵥ1.2 + FLAG−β2a + GFP (−9.326 ± 1.914, n = 7) (Fig. 3E), suggesting that this truncation mutant has lost the ability to regulate Ca²⁺ channel activity. On the other hand, currents measured in the presence of Rem-(1–276) co-expression (−1.326 ± 0.627, n = 7) are 86% lower than control currents (p < 0.01) (Fig. 3E) but did not result in the complete block of current seen with RemWT. Since Rem-(1–276) displayed a slight but statistically significant increase in cell periphery localization when compared with Rem-(1–265) and Rem-(1–270) (Fig. 1D), it is possible that the difference in Ca²⁺ channel inhibition is due to a defect in membrane localization.

Recent studies have suggested that calmodulin association is critical for both Gem and Rad-dependent Ca²⁺ channel regulation (12, 14, 20), but the importance of calmodulin to Rem-mediated channel regulation is less clear (14). To explore this issue, we next examined the ability of the Rem truncations to regulate Caᵥ1.2-Caᵥβ2a channel complexes with 30 mM Ca²⁺ as charge carrier. Although GFP-Rem-(1–276) was not capable of completely inhibiting current expression in this system (−1.212 ± 0.609, n = 13), currents obtained for Caᵥ1.2 + FLAG−β2a + GFP-Rem-(1–276) were not significantly different from those seen in the presence of GFP-RemWT (−0.493 ± 0.258, n = 9), most likely due to the smaller currents expressed in this system (Fig. 3, D and F). As seen in Fig. 3B, in a calmodulin-Sepharose binding assay, only RemWT and Rem-(1–282) displayed Ca²⁺-dependent calmodulin binding. Since Rem-(1–276) is capable of partial channel regulation, these data suggest that calmodulin association is not required for Rem-mediated Ca²⁺ channel regulation.

**Caᵥβ Association Is Not Sufficient for Rem-mediated Ca²⁺ Channel Inhibition**—We next investigated whether the C terminus directly contributed to Caᵥβ2a association or if the effect was indirect, resulting from relocation of Rem to the cytosol. To this end, the ability of recombinant 3⁵S-labeled Caᵥβ2a to associate with recombinant GST-Rem-(1–265) was examined. As shown in Fig. 4A, in the absence of a cellular context, radiolabeled Caᵥβ2a displays binding to GST-Rem-(1–265). To extend this analysis, we next asked whether the Rem C terminus was necessary for in vivo association with a β subunit isoform (Caᵥβ1b), which, like Caᵥβ2a, is localized to the plasma membrane but is not palmitoylated and is thought to be targeted to the cell surface through its C terminus (3). Lysates from tsA201 cells co-expressing HA-tagged Rem-(1–265) or RemWT and empty vector (control) or FLAG-tagged Caᵥβ1b were subjected to anti-FLAG immunoprecipitation analysis, and bound HA-tagged proteins were visualized by SDS-PAGE and immunoblotting. HA-Rem-(1–265) and HA-RemWT proteins bind Caᵥβ1b with approximately equal efficiency (Fig. 4B), demonstrating that the Rem C terminus plays no direct role in Caᵥβ1b binding in vivo.

To determine whether Caᵥβ1b binding was alone sufficient to regulate channel function, we next examined the ability of both RemWT and Rem-(1–265) to regulate Caᵥ1.2-Caᵥβ1b channel current expression. Consistent with previous studies (16), tsA201 cells transiently co-transfected with GFP-tagged RemWT, Caᵥ1.2, and Caᵥβ1b resulted in a complete loss of detectable ionic current expression (0.407 ± 0.392, n = 8) (Fig. 4, C and D). In contrast, currents measured from cells co-expressing channel components along with Rem-(1–265) (−10.043 ± 2.837, n = 14) were significantly different (p < 0.01) and displayed no inhibition of Ca²⁺ channel activity (Fig. 4, C and D). Taken together, these data indicate that Caᵥβ subunit binding alone is not sufficient for Rem-mediated Ca²⁺ channel blockade and suggest that plasma membrane localization is a critical aspect of Rem-mediated channel regulation.

**The Isolated Rem C Terminus Does Not Regulate Channel Function**—To determine whether the isolated Rem C terminus was sufficient for Ca²⁺ channel regulation, tsA201 cells were co-transfected with Caᵥ1.2, Caᵥβ2a, and either empty RFP or RFP-Rem-(266–297), and currents were determined using the whole cell configuration of the patch clamp technique. Co-expression of RFP-Rem-(266–297) (−17.712 ± 5.069, n = 7) resulted in current not significantly different from that seen for channel components co-expressed with RFP (−22.275 ± 11.036, n = 6) (Fig. 5A; current at 5 nM displayed in Fig. 5B), indicating that the isolated Rem C terminus cannot regulate channel function. Confocal microscopy revealed that in contrast to full-length RemWT, Rem-(266–297) was found predominantly in punctate nuclear structures (Fig. 5C), suggesting that in the absence of the Rem GTP-binding core, the C terminus acts as a nuclear localization signal.

**Plasma Membrane Localization Is Critical for Rem-mediated Ca²⁺ Channel Inhibition**—To explore whether Rem-dependent Ca²⁺ channel regulation requires molecular contacts between the C terminus and known binding partners, such as calmodulin and 14-3-3, as suggested by recent studies (12–14), two chimeric proteins were created in which the C terminus of
a polybasic domain and does not bind calmodulin (24, 25). Confocal imaging of GFP-tagged versions of both Rem-(1–265)/K Ras4B-CAAX and Rem-(1–265)/HRas-CAAX displayed prominent localization to the cell periphery in a manner consistent with plasma membrane localization (Fig. 6B), and both proteins were found to co-immunoprecipitate with β2a (data not shown).

We postulated that plasma membrane targeting is required for Rem function. GFP-Rem-(1–265)/HRas-CAAX resulted in a strong reduction in detectable ionic current (−0.333 ± 0.422, n = 14) when co-expressed with Ca v,1.2 + β1b in tsA201 cells (Fig. 6C). Although GFP-Rem-(1–265)/K Ras4B-CAAX was also targeted to the plasma membrane, it was found to only partially inhibit Ca v,1.2 + β1b channel current expression (−1.827 ± 0.703, n = 13), reducing inward currents by 81.8% when compared with control GFP-Rem-(1–265) transfected tsA201 cells (Fig. 6C). Currents at 5 mV from channel complexes containing β1b co-expressed with GFP-Rem-(1–265)/K Ras4B-CAAX were significantly different from those measured in the presence of GFP-Rem-(1–265) (p < 0.01) but not significantly different from those channels co-expressed with GFP-Rem-(1–265)/HRas-CAAX (Fig. 6E). The relative potency of channel blockade was reversed when the fusion proteins were co-expressed with K-Ras4B and H-Ras were fused to Rem-(1–265) (Fig. 6A). The resulting proteins were designated Rem-(1–265)/K Ras4B-CAAX and Rem-(1–265)/HRas-CAAX. The K-Ras4B C terminus is a well characterized membrane-targeting domain that contains a C-terminal polybasic domain and a farnesylation motif and displays both PI lipid and calmodulin binding, maintaining many of the functional properties of the Rem C terminus (23–25). On the other hand, the H-Ras targeting domain lacks a polybasic domain and does not bind calmodulin (24, 25). Confocal imaging of GFP-tagged versions of both Rem-(1–265)/K Ras4B-CAAX and Rem-(1–265)/HRas-CAAX displayed prominent localization to the cell periphery in a manner consistent with plasma membrane localization (Fig. 6B), and both proteins were found to co-immunoprecipitate with β2a (data not shown).

We postulated that plasma membrane targeting is required for Rem function. GFP-Rem-(1–265)/HRas-CAAX resulted in a strong reduction in detectable ionic current (−0.333 ± 0.422, n = 14) when co-expressed with Ca v,1.2 + β1b in tsA201 cells (Fig. 6C). Although GFP-Rem-(1–265)/K Ras4B-CAAX was also targeted to the plasma membrane, it was found to only partially inhibit Ca v,1.2 + β1b channel current expression (−1.827 ± 0.703, n = 13), reducing inward currents by 81.8% when compared with control GFP-Rem-(1–265) transfected tsA201 cells (Fig. 6C). Currents at 5 mV from channel complexes containing β1b co-expressed with GFP-Rem-(1–265)/K Ras4B-CAAX were significantly different from those measured in the presence of GFP-Rem-(1–265) (p < 0.01) but not significantly different from those channels co-expressed with GFP-Rem-(1–265)/HRas-CAAX (Fig. 6E). The relative potency of channel blockade was reversed when the fusion proteins were co-expressed

FIGURE 4. Rem-(1–265) can bind Ca v,β1b but cannot regulate channel function. A, GST or GST-tagged Rem-(1–265) protein was incubated with 35S-labeled Ca v,β2a in the presence of glutathione-Sepharose. Bound proteins were eluted by the addition of free glutathione and resolved via SDS-PAGE, and Ca v,β2a association was observed via autoradiography. B, tsA201 cells were transfected with plasmids expressing Rem WT, Rem-(1–265), and either empty pCMV7T7F2 vector control or FLAG-Ca v,β1b. Co-immunoprecipitation was performed with FLAG antibody and interaction with Rem proteins examined by immunoblotting with biotinylated HA antibody. Rem-(1–265) and Rem WT were both capable of binding Ca v,β1b. C, tsA201 cells were transfected with plasmids expressing Ca v,1.2, FLAG-Ca v,β1b, and either GFP-Rem-(1–265) or empty pEGFP-C1 as control. Current through the Ca v,1.2-Ca v,β1b complex was examined using the whole cell patch clamp configuration. D, currents at 5 mV from C. A significant difference (p < 0.05) between treatments is denoted by asterisks.

K-Ras4B and H-Ras were fused to Rem-(1–265) (Fig. 6A). The resulting proteins were designated Rem-(1–265)/K Ras4B-CAAX and Rem-(1–265)/HRas-CAAX. The K-Ras4B C terminus is a well characterized membrane-targeting domain that contains a C-terminal polybasic domain and a farnesylation motif and displays both PI lipid and calmodulin binding, maintaining many of the functional properties of the Rem C terminus (23–25). On the other hand, the H-Ras targeting domain lacks

FIGURE 3. β2a association is not sufficient for Rem-mediated Ca 2+ channel regulation. A, HEK293 cells were transfected with 3× HA-Rem truncations and either empty pCMV7T7F2 (FLAG) vector or FLAG-Ca v,β2a. Co-immunoprecipitation was performed with FLAG antibody, and interaction with Rem was examined by immunoblotting with biotinylated anti-HA antibody. B, tsA201 cells were transfected with plasmids expressing GFP-Rem-(1–265), GFP-Rem-(1–270), GFP-Rem WT, or empty pEGFP-C1 as control. Lysates were pulled down onto calmodulin-Sepharose beads in the presence of 2 mM CaCl 2 or 2.5 mM EGTA, beads were boiled to release bound protein, and the ability of Rem truncations to associate with calmodulin was examined by immunoblotting with anti-HA antibody. C, HEK293 cells were transfected with plasmids expressing Ca v,1.2, FLAG-Ca v,β2a, and either GFP-Rem-(1–270), GFP-Rem-(1–265), GFP-Rem WT, or empty pEGFP-C1 as control. Current through Ca v,1.2-Ca v,β2a complex was examined using the whole cell patch clamp configuration in the presence of 30 mM Ba 2+. D, tsA201 cells were transfected with plasmids expressing Ca v,1.2, FLAG-Ca v,β2a, and either GFP-Rem-(1–270), GFP-Rem WT, or empty pEGFP-C1 as control. Current through the Ca v,1.2-Ca v,β2a complex was examined using the whole cell patch clamp configuration in the presence of 30 mM Ca 2+. E, currents at 5 mV from C. A significant difference (p < 0.05) between treatments is denoted by asterisks. F, currents at 5 mV from D. A significant difference (p < 0.05) between treatments is denoted by asterisks.
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Figure 5. The isolated Rem C terminus does not inhibit Ca\(^{2+}\) channel current. A, Tsa201 cells were co-transfected with Ca\(_{\text{v},1.2}\), β2a, and either RFP or RFP-Rem(266–297), and current was examined using the whole-cell patch clamp configuration. B, currents at 5 mV from A. There is no significant difference between the treatments. C, Tsa201 cells expressing either RFP-Rem\(^{\text{WT}}\) or RFP-Rem-(266–297) were analyzed 72 h after post-transfection by confocal microscopy.

DISCUSSION

To better characterize the mechanisms by which RGK proteins are regulated, we used confocal fluorescence microscopy to examine the role of the Rem C terminus in plasma membrane localization and found that residues 270–282 play a critical role in this process (Fig. 1). Recent work by Heo et al. (23) designed to examine the plasma membrane targeting mechanisms for a variety of small GTPases, including Rad and Gem, found that Ras family C-terminal domains containing polybasic motifs allow for direct association with both PI(4,5)P\(_2\) and PI(3,4,5)P\(_3\) lipids. The notion that a polybasic membrane targeting motif was required for Rem trafficking agrees with our localization data, since the loss of polybasic motifs in Rem-(1–265) and Rem-(1–270) prevented plasma membrane localization (Fig. 1, A, C, and D), whereas loss of one polybasic cluster in Rem-(1–276) led to a significant reduction in membrane localization (Fig. 1, A, C, D, and E). In further support of this model, Rem was found to selectively bind phosphoinositides (PIP\(_2\) and PIP\(_3\)) in an overlay assay using PIP strips, and truncation of the C terminus before position 282 resulted in a dramatic reduction in phosphatidylinositol lipid binding (Fig. 2). Taken together, these data suggest that the polybasic domains within the Rem C terminus provide plasma membrane targeting specificity by binding to negatively charged PIP\(_2\) and PIP\(_3\) lipids in the plasma membrane and that modulation of the membrane concentrations of these lipids may provide a molecular mechanism for regulating Rem signaling. Interestingly, previous studies have demonstrated potent up-regulation of N- and L-type Ca\(^{2+}\) channel function by PI(3,4,5)P\(_3\) lipids (26), and that PI 3-kinase activation increases L-type Ca\(^{2+}\) channel trafficking to the plasma membrane in a Ca\(_{\text{v},\beta2}\)-dependent fashion (27). It is possible, then, that the PI-mediated membrane association observed for Rem could serve as a negative feedback mechanism opposing an up-regulation of channel function following an increase in PI(3,4,5)P\(_3\) lipid concentration. Studies are ongoing to examine whether regulation of these lipid second messengers provides a novel mechanism for controlling Rem-dependent Ca\(^{2+}\) channel inhibition.

Since Rem directly binds to a variety of accessory Ca\(_{\text{v},\beta}\) subunits (9), and a number of studies suggest that this interaction is required for the regulation of functional Ca\(^{2+}\) channels at the plasma membrane (8, 9, 11, 16), we examined whether Rem localization would be altered by co-expression of either Ca\(_{\text{v},1.2}\) or Ca\(_{\text{v},\beta}\) subunits or in the presence of a functional Ca\(_{\text{v},1.2}\)-β2a Ca\(^{2+}\) channel. However, a similar fluorescence pattern was seen whether Ca\(_{\text{v},\alpha}\) and/or Ca\(_{\text{v},\beta}\) subunits were present or absent in tsA201 cells (Fig. 1B), indicating that plasma membrane trafficking of Rem is not dependent on Ca\(^{2+}\) channel subunit expression. Beguin et al. (12–14) report that wild-type RGK proteins display cytoplasmic, plasma membrane, and prominent nuclear localization when overexpressed in COS cells, a cellular distribution that is clearly different from that seen for GFP-Rem in tsA201 cells (Fig. 1). Whether these differences are cell line-specific or dependent on the level of Rem expression is unclear. Mutations within the C terminus of RGK proteins that disrupt calmodulin binding have also been reported to promote nuclear translocation (12–14). Our data show that Rem-(1–265), Rem-(1–270), and Rem-(1–276) fail to bind calmodulin resin (Fig. 3B) and are not trafficked to the nucleus (Fig. 1C). However, the isolated Rem C terminus expressed as an RFP fusion protein is localized to punctate structures within the nucleus, suggesting that the Rem C terminus contains a cryptic nuclear localization sequence (Fig. 5C). Although both Rem\(^{\text{WT}}\) and Rem-(1–282) displayed robust Ca\(^{2+}\)-dependent calmodulin binding and potent Ca\(^{2+}\) channel...
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blockade (Fig. 3), Rem-(1–276) was shown to partially inhibit Ca\(^{2+}\) channel function, yet this mutant is incapable of binding calmodulin resin (Fig. 3B). Although these data indicate that calmodulin binding is not required for Rem-mediated Ca\(^{2+}\) channel regulation, it might more subtly modulate Rem activity. Thus, it will be important in future studies to evaluate the effect of calmodulin and 14-3-3 binding or site-selective phosphorylation within the polybasic domain to modulate RGK protein plasma membrane targeting.

Previous studies have suggested an important role for the RGK C terminus in both \(\beta\) subunit binding and regulation of HVA channels (9, 15, 19), supporting the notion that \(\beta\) subunit binding was sufficient for RGK-mediated channel blockade. In this regard, the finding that truncation of the Rem C terminus before residue 276 resulted in a reduced ability to associate with Ca\(_{\alpha_\beta2}\) when assayed by co-immunoprecipitation (Fig. 3A) was expected. However, the finding that Rem-(1–276) associates with Ca\(_{\alpha_\beta2}\) just as well as Rem\(^{WT}\) but does not completely block L-type Ca\(^{2+}\) channel current expression in the presence of 30 mM Ba\(^{2+}\) was unexpected (Fig. 3C). Furthermore, although Rem-(1–270) had a reduced ability to co-immunoprecipitate Ca\(_{\alpha_\beta2}\), it was found to have no ability to inhibit Ca\(^{2+}\) channel activity (Fig. 3, C and E), suggesting that a \(\beta\)-binding threshold may exist for Rem-mediated Ca\(^{2+}\) channel regulation, consistent with a recent report demonstrating dose-dependent RGK-mediated channel modulation (17). Because the loss of Ca\(_{\alpha_\beta2}\) binding seen with progressive C-terminal deletions was mirrored by a reduction in plasma membrane trafficking (Figs. 1D and 3A), we examined whether this effect was specific for the palmitoylated Ca\(_{\alpha_\beta2}\) or whether Rem-(1–265) would demonstrate a reduction in binding to another membrane-localized Ca\(_{\alpha}\) subunit. In Fig. 4B we find that Rem-(1–265) binds the membrane-localized Ca\(_{\alpha}\) isoform, Ca\(_{\alpha_\beta1}\), just as well as Rem\(^{WT}\), suggesting that the loss of binding is specific for Ca\(_{\alpha_\beta2}\) and is not a consequence of reduced membrane localization for the Rem mutant. This notion is supported by

\[\text{in vitro} \text{ pull-down assays, which remove the complication of membrane localization from } \beta \text{ subunit interaction and demonstrate robust binding of Rem-(1–265) to Ca}_{\alpha_\beta2} \text{ (Fig. 4A).} \]

It is possible that the orientation in which the \(\beta\) subunit is anchored to the membrane affects the ability of Rem to bind, since it is known that \(\beta2a\) associates with the membrane through palmitoylation of its N terminus, whereas the C terminus of \(\beta1b\) is required for membrane association (3). Importantly, although the Ca\(_{1.2}\) subunit is inhibited by wild-type Rem (Fig. 4, C and D) (16), Rem-(1–265) was unable to inhibit ionic current expression (Fig. 4C). There are two major conclusions that can be drawn from these studies. First, since deletion of the majority of the C terminus does not disrupt Ca\(_{\alpha_\beta1b}\) association in vivo

\[\text{FIGURE 6. Membrane-targeted Rem-(1–265) inhibits } I_{\text{Ca}}. \text{ A, diagram showing construction of CAAX chimeric proteins and sequences of the K-Ras4B and H-Ras C-terminal and CAAX domains. B, TsA201 cells were transfected with plasmids expressing GFP-Rem-(1–265), GFP-Rem-(1–265)/Kras4B-CAAX, GFP-Rem-(1–265)/HRas-CAAX, or GFP-Rem\(^{WT}\). 72 h after transfection, cells were observed by confocal microscopy. Rem-(1–265) shows cytosolic localization, but fusion of either of the CAAX tags results in cell peripheral distribution stronger even than that of Rem\(^{WT}\) and consistent with plasma membrane localization. C, TsA201 cells were transfected with plasmids expressing Ca\(_{1.2}\), Ca\(_{\beta1b}\), and either GFP-Rem-(1–265), GFP-Rem-(1–265)/Kras4B-CAAX, or GFP-Rem-(1–265)/HRas-CAAX. Although GFP-Rem-(1–265)/HRas-CAAX can fully inhibit the activity of this channel complex, GFP-Rem-(1–265)/Kras4B-CAAX shows only partial inhibition. D, TsA201 cells were transfected with plasmids expressing Ca\(_{1.2}\), Ca\(_{\beta2a}\), and either GFP-Rem-(1–265)/KRas4B-CAAX, GFP-Rem-(1–265)/HRas-CAAX, or GFP as a control. Although GFP-Rem-(1–265)/KRas4B-CAAX can fully inhibit the activity of this channel complex, GFP-Rem-(1–265)/HRas-CAAX shows only partial inhibition. E, currents at 5 mV from C. A significant difference \((p < 0.05)\) between treatments is denoted by asterisks. F, currents at 5 mV from D. A significant difference \((p < 0.05)\) between treatments is denoted by asterisks.}\]
or Ca_\text{\textgamma} \beta_2 \text{a} \text{in vitro}, the \beta interaction domain is not located within the Rem C terminus. Instead, it appears to be located within the GTP-binding core of Rem and other RGK proteins (15, 28). Second, since Rem-(1–265) interacts with \beta_1 \beta but cannot regulate channel function (Fig. 4), Ca_\text{\textgamma} \beta binding alone is not sufficient for Rem-mediated Ca^{2+} channel regulation.

The observation that Ca_\text{\textgamma} \beta subunit binding, unlike plasma membrane association, is not dependent upon the Rem C terminus suggests that \beta binding and membrane localization are separable molecular events, and each may serve as an independent means of regulating Rem activity. To isolate the role of membrane trafficking from other functions of the C terminus, including PI lipid association (Fig. 2) and calmodulin binding (Fig. 3B) (14), we generated two chimeric Rem-(1–265) variants (Fig. 6) using the membrane targeting domains from K-Ras4B and H-Ras (25). Whereas the H-Ras CAAAX domain relies upon prenylation/palmitoylation to direct membrane localization (25), the K-Ras4B region has many properties in common with Rem, including both calmodulin association and a polybasic domain capable of PI lipid-mediated PM targeting (23–25). Importantly, both anchors reconstituted plasma membrane association and partially restored Ca^{2+} channel regulation (Fig. 6), in agreement with recent studies examining Rem2 function using a similar strategy (15). Therefore, directing plasma membrane association appears to be the primary function of the Rem C terminus. However, since the chimeric proteins display more pronounced membrane trafficking (Fig. 6B) but do not fully recapitulate Rem-mediated Ca^{2+} channel inhibition (Fig. 6, C–F), it is likely that previously described interacting partners of and/or modifications to the Rem C terminus (including PI lipids, calmodulin, and/or 14-3-3 association, or protein kinase A/protein kinase C-mediated phosphorylation), although not essential for channel regulation, may contribute to Rem signaling (12–14, 18, 20, 23, 29–33).

In summary, we have found that the Rem C terminus serves as an essential targeting signal, probably acting through binding of the positively charged polybasic region to negatively charged PIP_2 and PIP_3 lipids, to direct Rem plasma membrane association. Although membrane localization and Ca_\text{\textgamma} \beta subunit association are independent molecular events, we present strong evidence that both interactions play essential roles in Rem-mediated Ca^{2+} channel regulation. This new function for the conserved RGK C-terminal domain provides an opportunity for a variety of physiological pathways to influence RGK signaling. Clearly, additional studies will be needed to clarify the role of phosphatidylinositol lipid signaling and calmodulin/14-3-3 binding in both Rem trafficking and Ca^{2+} channel regulation.

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REFERENCES

1. Catterall, W. A. (2000) Annu. Rev. Cell Dev. Biol. 16, 521–555
2. Richards, M. W., Butcher, A. J., and Dolphin, A. C. (2004) Trends Pharmacol. Sci. 25, 626–632
3. Dolphin, A. C. (2003) J. Bioenerg. Biomembr. 35, 599–620
4. Finlin, B. S., and Andres, D. A. (1997) J. Biol. Chem. 272, 21982–21988
5. Finlin, B. S., Shao, H., Kadono-Okuda, K., Guo, N., and Andres, D. A. (2000) Biochem. J. 347, 223–231
6. Reynet, C., and Kahn, C. R. (1993) Science 262, 1441–1444
7. Maguire, I., Santoro, T., Jensen, P., Siebenlist, U., Yewdell, J., and Kelly, K. (1994) Science 265, 241–244
8. Beguin, P., Nagashima, K., Sonoi, T., Shibasaki, T., Takahashi, K., Kashiya, Y., Ozaki, N., Greer, K., Iwanaga, T., and Seino, S. (2001) Nature 411, 701–706
9. Finlin, B. S., Crump, S. M., Satin, J., and Andres, D. A. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 14469–14474
10. Finlin, B. S., Mosley, A. L., Crump, S. M., Correll, R. N., Oxzan, S., Satin, J., and Andres, D. A. (2005) J. Biol. Chem. 280, 41864–41871
11. Sasaki, T., Shibasaki, T., Beguin, P., Nagashima, K., Miyazaki, M., and Seino, S. (2005) J. Biol. Chem. 280, 9308–9312
12. Beguin, P., Mahalakhimi, R. N., Nagashima, K., Cher, D. H., Takahashi, A., Yamada, Y., Seino, Y., and Hunziker, W. (2005) J. Cell Sci. 118, 1923–1934
13. Beguin, P., Mahalakhimi, R. N., Nagashima, K., Cher, D. H., Kawamura, N., Yamada, Y., Seino, Y., and Hunziker, W. (2005) Biochem. J. 390, 67–75
14. Beguin, P., Mahalakhimi, R. N., Nagashima, K., Cher, D. H., Ikeda, H., Yamada, Y., Seino, Y., and Hunziker, W. (2006) J. Biol. Chem. 355, 34–46
15. Chen, H., Puhl, H. L., III, Niu, S. L., Mitchell, D. C., and Ikeda, S. R. (2005) J. Neurosci. 25, 9762–9772
16. Finlin, B. S., Crump, S. M., Pang, C., Crump, S. M., Satin, J., and Andres, D. A. (2006) J. Biol. Chem. 281, 23557–23566
17. Seo, L., and Pitt, G. S. (2006) J. Gen. Physiol. 128, 605–613
18. Crump, S. M., Correll, R. N., Schroder, E. A., Lester, W. C., Finlin, B. S., Andres, D. A., and Satin, J. (2006) Ann. J. Physiol. 291, H1959–H1971
19. Kelly, K. (2005) Trends Cell Biol. 15, 640–643
20. Ward, Y., Spinelli, B., Quon, M. J., Chen, H., Ikeda, S. R., and Kelly, K. (2004) Mol. Cell Biol. 24, 651–661
21. Andres, D. A., Crump, S. M., Correll, R. N., Satin, J., and Finlin, B. S. (2005) Methods Enzymol. 407, 484–498
22. Pan, J. Y., Fieles, W. E., White, A. M., Egerton, M. M., and Silberstein, D. S. (2000) J. Cell Biol. 149, 1107–1116
23. Heo, W. D., Inoue, T., Park, W. S., Kim, M. L., Park, B. O., Wandless, T. J., and Meyer, T. (2006) Science 314, 1458–1461
24. Villalonga, P., Lopez-Alcala, C., Bosch, M., Chioleches, A., Rocomora, N., Gil, J., Marais, R., Marshall, C. J., Bachs, O., and Agell, N. (2001) Mol. Cell Biol. 21, 7345–7354
25. Plovman, S. J., and Hancock, J. F. (2005) Biochim. Biophys. Acta 1746, 274–283
26. Blair, L. A., and Marshall, J. (1997) Neuron 19, 421–429
27. Viard, P., Butcher, A. J., Halet, G., Davies, A., Nurnberg, B., Heblich, F., and Dolphin, A. C. (2004) Nat. Neurosci. 7, 939–946
28. Opatowsky, Y., Sasson, Y., Shaked, I., Ward, Y., Chomsky-Hecht, O., Litvak, Y., Selinger, Z., Kelly, K., and Hirsch, J. A. (2006) FEBS Lett. 580, 5959–5964
29. Fischer, R., Wei, Y., Anaglia, J., and Berchtold, M. W. (1996) J. Biol. Chem. 271, 25067–25070
30. Zhu, J., Reynet, C., Caldwell, J. S., and Kahn, C. R. (1995) J. Biol. Chem. 270, 4805–4812
31. Moyers, J. S., Bilan, P. J., Zhu, J., and Kahn, C. R. (1997) J. Biol. Chem. 272, 11822–11839
32. Moyers, J. S., Zhu, J., and Kahn, C. R. (1998) Biochem. J. 333, 609–614
33. Finlin, B. S., and Andres, D. A. (1999) Arch. Biochem. Biophys. 368, 401–412