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Analysis of the Rem2 - Voltage Dependant Calcium Channel β Subunit Interaction and Rem2 Interaction with Phosphorylated Phosphatidylinositide Lipids

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Abstract

Voltage dependant calcium channels (VDCC) play a critical role in coupling electrical excitability to important physiological events such as secretion by neuronal and endocrine cells. Rem2, a GTPase restricted to neuroendocrine cell types, regulates VDCC activity by a mechanism that involves interaction with the VDCC β subunit ($Ca_V\beta$). $Ca_V\beta$ mapping studies reveal that Rem2 binds to the guanylate kinase domain (GK) of the $Ca_V\beta$ subunit that also contains the high affinity binding site for the pore forming and voltage sensing VDCC α subunit ($Ca_V\alpha$) interaction domain (AID). Moreover, fine mapping indicates that Rem2 binds to the GK domain in a region distinct from the AID interaction site, and competitive inhibition studies reveal that Rem2 does not disrupt $Ca_V\alpha$ - $Ca_V\beta$ binding. Instead, the $Ca_V\beta$ subunit appears to serve a scaffolding function, simultaneously binding both Rem2 and AID. Previous studies have found that in addition to $Ca_V\beta$ binding, Rem2 must be localized to the plasma membrane to inhibit VDCC function. Plasma membrane localization requires the C-terminus of Rem2 and binding studies indicate that this domain directs phosphorylated phosphatidylinositide (PIP) lipids association. Plasma membrane localization may provide a unique point of regulation since the ability of Rem2 to bind PIP lipids is inhibited by the phosphoserine dependant binding of 14-3-3 proteins. Thus, in addition to $Ca_V\beta$ binding, VDCC blockade by Rem2 is likely to be controlled by both the localized concentration of membrane PIP lipids and direct 14-3-3 binding to the Rem2 C-terminus.

Keywords

RGK; GTP; GTPase; Rem2; VDCC; $Ca_V\beta$; phosphatidylinositol

1. Introduction

Voltage dependant calcium channels (VDCC) regulate the entry of calcium into electrically excitable cells to control diverse physiological processes ranging from muscle contraction to secretion [1]. The channel consists of a pore-forming α_1 subunit ($Ca_V\alpha$), a cytosolic β

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($Ca_V\beta$) subunit, an α_2 subunit that is disulfide linked to the δ subunit, and in some channel types, a γ subunit [1]. The $Ca_V\beta$ subunit binds with high affinity to the intracellular loop connecting the homologous domains I and II of $Ca_V\alpha$ at a specific region called the α_1 interaction domain (AID) [2]. The $Ca_V\beta$ subunit is a critical regulatory subunit controlling both surface expression of the channel and the biophysical properties of the channel (reviewed in [3]). Different VDCC isoforms have been shown to be regulated by phosphorylation and heterotrimeric G-Proteins (reviewed in [1]). Recently, RGK (Rad, Rem, Rem2, and Gem/Kir) GTPases have been demonstrated to be potent inhibitors of channel activity [4-8] and a transgenic mouse expressing dominant negative Rad displays increased L-type calcium channel activity suggesting that RGK proteins play a physiologically relevant role in regulating L-type VDCC [9].

RGK GTPases [10-13] are a subfamily of the Ras GTPase superfamily of small GTP binding proteins. Rem2 displays a highly restricted tissue expression pattern whose mRNA is expressed in brain [13] and endocrine cells [6]. Rem2 mRNA is induced by glucose in the insulinoma cell line MIN6, and Rem2 has been reported to be capable of modulating L-type channel activity at the cell surface in pancreatic β cell lines [6]. Furthermore, Rem2 has been reported to regulate N-type channels at the cell surface in hippocampal neurons [7]. These data suggest that Rem2 may play important roles in regulating both insulin secretion in pancreatic β cells and neurotransmitter release. Intriguingly, Rem2 was recently identified in an siRNA screen for genes regulating synapse development [14] suggesting a physiological role for Rem2 in this process. Taken together, these studies suggest that Rem2 is an important regulator of VDCC in neuronal and endocrine cells.

Based on the current data, two biochemical mechanisms have been hypothesized to explain RGK regulation of VDCC, both involving the interaction of RGK GTPases with $Ca_V\beta$ subunits. Gem has been reported to inhibit the high affinity interaction between $Ca_V\alpha$ and $Ca_V\beta$ [4], and subsequent studies found that Gem and the $Ca_V\alpha$ AID compete for the same binding site on $Ca_V\beta$ [15]. The Gem mediated disruption of this interaction has been proposed to inhibit trafficking of $Ca_V\alpha$ to the cell surface [4]. Subsequent studies have reported that all four RGK GTPases can inhibit the trafficking of newly synthesized channel to the surface [16-19], suggesting that inhibition of $Ca_V\alpha$ surface expression is one potential mechanism of RGK-mediated VDCC inhibition. However, recent studies demonstrate that Rem, Rad, and Rem2 can inhibit VDCC function at the cell surface without altering $Ca_V\alpha$ surface expression [6,7, 9,20] and that Rem2 modulates the gating properties of newly synthesized channel at the surface in oocytes [21]. Furthermore, *in vitro* studies indicate that Rem does not inhibit the interaction between $Ca_V\alpha$ and $Ca_V\beta$ [22]. Crystallographic studies reveal that the AID binding pocket (ABP) resides entirely within the GK domain of the $Ca_V\beta$ subunit [23-25]. Rem has been shown to bind to the $Ca_V\beta$ subunit GK domain at a site distinct from the ABP and interact with the $Ca_V\beta$ - AID complex [20]. A subsequent study confirmed the presence of a Rem:$Ca_V\beta$:$Ca_V\alpha$ complex *in vivo* and found that Rad and Gem could enter into similar $Ca_V\beta$:$Ca_V\alpha$ complexes [19]. Thus, a second model in which $Ca_V\beta$ functions as a scaffold to bring the RGK protein to the $Ca_V\alpha$ subunit has been proposed [20]. The localization of the RGK protein to the plasma membrane is important feature of this model [7]. However, the precise mechanism used to inhibit VDCC activity after recruitment of the RGK GTPase to the $Ca_V\alpha$ subunit remains to be elucidated.

In order to gain a greater understanding of Rem2 mediated VDCC inhibition, it is necessary to biochemically characterize its binding to $Ca_V\beta$. Here, it is reported that Rem2 binds to the $Ca_V\beta$ subunit in a nucleotide independent manner *in vitro*. Rem2 binds to $Ca_V\beta$ within the same 130 amino acid domain required for Rem interaction [20], but is distinct from the ABP required for $Ca_V\alpha$ association. Rem2 does not inhibit the interaction between $Ca_V\beta$ and AID, instead Rem2 can form a Rem2:$Ca_V\beta$:AID complex. Additionally, the ability of Rem2 to

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inhibit channel activity depends upon its localization to the plasma membrane [7]. To begin to elucidate the mechanism utilized by the Rem2 polybasic C-terminus to localize Rem2 to the plasma membrane, the ability of Rem2 to bind phosphatidylinositide -monophosphate, -bisphosphate, -and trisphosphate (PIP1, PIP2 and PIP3) lipids was examined. Rem2 was demonstrated to be capable of binding PIP1, PIP2 and PIP3 lipids and this association is shown to be negatively regulated by 14-3-3 protein binding. Thus, these studies begin to provide details of the mechanism that Rem2 uses to inhibit channel function and suggest that both PI lipid phosphorylation and 14-3-3 protein association may figure prominently in this process.

2. Materials and Methods

2.1. Recombinant Rem2 protein production

Full-length mouse Rem2 was produced in baculovirus as follows. Full-length mouse Rem2 was cloned in pFastBacHT (Gibco) to generate Rem2 with an amino terminal hexa-histidine affinity tag (His_6-Rem2). Plaque purified baculovirus was produced according to the manufacturer's instructions. Sf-9 cells (4L) were infected with baculovirus at an MOI of 1.0 for 48 h in spinner flasks at 27° C. The cells were harvested in buffer A (40 mM imidazole, 50 mM Tris pH8.0, 500 mM NaCl, 5 mM β-ME, 1 mM $MgCl_2$) supplemented with 0.3% Triton X-100. The cells were sonicated with a Branson Sonifier and the lysate centrifuged at 100,000 × g for 30 minutes. The supernatant was applied to an Ni^{2+}-NTA sepharose (GE Life Sciences) column (8 mL) and the column was washed with 5 column volumes of buffer A supplemented with 0.3% Triton X-100 and then 5 column volumes of buffer A. His_6-Rem2 was eluted from the column with a linear gradient of buffer A to buffer B (buffer A with 500 mM imidazole) over 5 column volumes. Fractions containing His_6-Rem2 were pooled and dialyzed versus buffer A. The His_6 tag was enzymatically cleaved with His_6-TEV protease by adding 1 mg protease per 20 mg protein followed by incubation at 16° C for 1 h. Contaminating proteins and His_6-TEV protease were removed by incubation with 2 mL Ni^{2+}NTA sepharose for 20 min with end over end rotation at 4° C. Rem2 was purified from the Rem2-14-3-3 complex by dialyzing the protein versus buffer MQ A (20 mM Tris pH 7.5, 5 mM β-ME, 1 mM $MgCl_2$). The dialyzed protein was applied to a Mono Q 5/5 column (GE) which was then washed with 5 column volumes of buffer MQ A. Proteins were eluted using a linear gradient (10 column volumes) of buffer MQ A to buffer MQ B (MQ A + 1M NaCl). Fractions containing Rem2 and Rem2 -14-3-3 were pooled and dialyzed versus (50 mM Tris pH 7.5, 100 mM NaCl, 5 mM β-ME, 1 mM $MgCl_2$, 10% glycerol). The Rem2-14-3-3 complex was further purified by applying it to a Superdex 200 column equilibrated with dialysis buffer. Protein concentrations were determined using the Bradford assay using BSA as a standard. GST-rat Rem2 and GST-AID were purified as described [6,13]. The proteins that co-purified with Rem2 were confirmed to be 14-3-3 proteins by immunobloting with SC Pan 14-3-3 [26].

2.2. *In vitro* and *in vivo* binding experiments

In vitro binding studies were carried out as described [20] except that DTT was added to a concentration of 10 mM for the binding buffer in reactions in which $Ca_V\beta_{2a}{}^{1\text{-}604}$ was used. Recombinant full-length Rem2 was used to assess its ability to inhibit the GST-AID - $Ca_V\beta$ interaction. GST-Rem2 (N69 to C310) [13] was used for mapping and nucleotide binding studies. $Ca_V\beta_{2a}{}^{1\text{-}355}$ was used to test the nucleotide dependence of the Rem2- $Ca_V\beta$ interaction and to test the ability of Rem2 to inhibit the interaction between GST-Rem2 and $Ca_V\beta$ because it is made better than $Ca_V\beta_{2a}{}^{wt}$ in the *in vitro* transcription and translation system and therefore displays better binding to Rem2 than full-length Rem2 thus giving an increased dynamic range in the assay. *In vivo* binding studies to test for GST-AID-$Ca_V\beta_{2a}$-Rem2 complex formation were done by transfecting tsA201 cells with the indicated plasmids or empty vector controls. Cell lysates were prepared and subjected to GST pull down assays as described [20]. The membranes were blotted as indicated.

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2.3. Electrophysiology

TsA201 cells were transfected using the calcium phosphate method and whole-cell patch clamp experiments were performed as previously described [27]. Pipette solutions (in mM) consisted of 150 CsCl, 1 $MgCl_2$, 5 Mg-ATP, 3 EGTA, 5 Hepes (pH 7.36). The bath solution (in mM) consisted of 112.5 CsCl, 30 $BaCl_2$, 1 $MgCl_2$, 10 tetraethylammonium chloride, 5 glucose, 5 Hepes (pH 7.4). Traces were analyzed using Origin statistical software. Values are reported as normalized mean at 5 mV ± SE.

2.4. Anti-Rem2 Rabbit affinity purified antibody production

Rem2 was produced as a thioredoxin fusion protein and purified over Ni^{2+}-NTA sepharose. The immunogen was injected into rabbits, sera collected and affinity purified against THX-Rem2 coupled to Affigel. The antibody was eluted with 100 mM glycine pH 2.5 and immediately dialyzed versus PBS with 10% glycerol.

2.5. Phosphorylated PI lipid interaction assay

PIP strips or PIP arrays were from Invitrogen. The membranes were blocked with TBST (10 mM Tris pH 8.0, 150 mM NaCl, 0.1% Tween 20,) plus 3% fatty acid free BSA (Sigma). For experiments in which purified recombinant proteins were used, the membranes were incubated with the indicated concentration of purified Rem2 or Rem2-14-3-3 complex for 1 h. The membranes were washed three times with TBST and incubated with affinity purified anti-Rem2 antibody diluted 1:1000 in TBST plus 3% fatty acid free BSA. The membranes were washed and incubated with Zymax FITC conjugated goat anti rabbit antibody (Zymed) diluted 1:2000 in TBST plus 3% fatty acid free BSA. The membranes were washed three times with TBST and imaged with a Typhoon imaging system (GE Life Sciences). For enzymatic dephosphorylation of the Rem2-14-3-3 complex, the complex was incubated without (control) or with 12.5 units of protein phosphatase I (PP1) (NEB) or 12.5 units PP1 with 10 μg protein phosphatase inhibitor 2 (I-2) in 200 μL PP1 buffer (NEB) at 30° C for 1 h. For analysis of cell lysates, tsA201 cells were transiently transfected with pCDNA3.1 (+) zeo (empty vector control) or HA-mRem2 pCDNA or HA-$mRem2^{1-310}$ pCDNA. Forty hours post transfection, the cells were sonicated in lysis buffer (20 mM Tris pH 7.5, 250 NaCl, 1% Triton X-100, 10 mM $MgCl_2$, 0.5 mM DTT and 1X Calbiochem protease inhibitor cocktail). The lysate was centrifuged at 100,000 g for 10 min and the supernatant isolated. Two mg of the supernatant was added to 10 mL blocking buffer supplemented with 1 mM $MgCl_2$ and this was incubated overnight at 4° C with PIP strips that had been blocked in the same buffer. The membranes were then processed as described above except that HA antibody (12CA5) was used to detect HA-mRem2 or HA-$mRem2^{1-310}$. Fifty μg of lysate was immunoblotted with HA antibody to confirm the expression of both proteins.

3. Results

3.1. The Rem2-$Ca_V\beta$ interaction is nucleotide independant

While the Rem2 GTPase has been demonstrated to potently inhibit L-type calcium channel activity, the mechanism remains to be established. RGK proteins bind to the $Ca_V\beta$ subunit to inhibit channel activity, however this binding has been reported to be nucleotide dependent for Gem [4] and nucleotide independent for Rem [20]. To examine the nucleotide dependence of $Ca_V\beta$ binding, GST-Rem2 was loaded with GDP or GTPγS and incubated with *in vitro* transcribed and translated [^{35}S]$Ca_V\beta$ subunit. As seen in Fig. 1A, $Ca_V\beta$ subunit binding was nucleotide independent. To extend this analysis, the Rem2 binding surface on $Ca_V\beta$ was mapped by incubation of GST-Rem2 with a series of [^{35}S]$Ca_V\beta$ subunit truncation mutants containing either amino or carboxyl termini deletions (Fig. 1B). GST served as a negative control and GST-AID was used to define the AID binding pocket (ABP) and as a control to

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demonstrate the proper folding of wild type $Ca_V\beta$. As shown in Fig. 1B, Rem2 was capable of binding to $Ca_V\beta$ mutants ($Ca_V\beta^{1\text{-}355}$, $Ca_V\beta^{226\text{-}355}$, $Ca_V\beta^{261\text{-}355}$) that had lost the ABP domain and thus the ability to bind GST-AID. These data suggest that Rem2 interacts with a 130 amino acid stretch (261-355) within the GK domain that is indicated at the bottom of the schematic (Fig. 1B). Importantly, this binding domain is distinct from the ABP.

3.2. Rem2 does not inhibit the AID- $Ca_V\beta$ interaction

Gem has been demonstrated to inhibit the high affinity interaction between the $Ca_V\beta$ subunit and AID peptide *in vivo* and *in vitro* [4,15] while Rem does not inhibit this interaction [20]. Therefore, it was next tested whether Rem2 would inhibit the interaction between GST-AID and $Ca_V\beta$. Although the binding studies presented above suggest that Rem2 does not directly compete with $Ca_V\alpha$ for a common binding site on $Ca_V\beta$, Rem2 might cause a conformational change in $Ca_V\beta$ that would alter its affinity for $Ca_V\alpha$ AID. Since full-length Rem2 appears to be necessary for channel inhibition (Fig. 4 and [7]), a method for producing this protein was necessary to test its ability to inhibit the $Ca_V\alpha$ - $Ca_V\beta$ interaction *in vitro*. In our hands, full-length Rem2 cannot be made in bacteria [13]. Therefore, Rem2 was produced using baculovirus infection of Sf-9 insect cells as a His_6-tagged protein to facilitate purification and the His_6 tag was subsequently removed with His_6-TEV protease. The cleaved protein was resolved by MonoQ anion exchange chromatography and recombinant Rem2 migrated at approximately 46 kDa (Fig. 2A, left, lanes 1-4). In order to confirm that the purified protein was Rem2, an affinity purified antibody was produced and its specificity characterized (Fig. 2D). The purified Rem2 protein was shown to react with this antibody using immunoblot analysis (Fig 2A, right). Inhibition binding assays were then performed by incubating 10 ng GST-AID with the indicated concentrations (up to 10 μg) of full-length Rem2 and $[^{35}S]Ca_V\beta_{2a}$. As shown in Fig. 2B, even a large excess of Rem2 was unable to inhibit the high affinity AID- $Ca_V\beta$ interaction. However, unfused Rem2 in the range of 1 to 10 μg was shown to inhibit the interaction between GST-Rem2 (10 μg) and $Ca_V\beta^{1–355}$(Fig. 2C) indicating that the recombinant proteins were active. In addition it was also determined whether Rem2 expressed *in vivo* could inhibit the AID- $Ca_V\beta$ interaction. As shown in Fig. 3A, $Ca_V\beta$ associated with GST-AID in the presence of Rem2. Therefore, these studies suggest that inhibition of the $Ca_V\alpha$-$Ca_V\beta$ interaction is not the mechanism of Rem2 modulation of VDCC activity.

3.3. Rem2 can form a complex with AID that is mediated by $Ca_V\beta$

Since the binding sites on $Ca_V\beta$ are different for Rem2 and AID, it was next determined whether Rem2 could form a complex with GST-AID and $Ca_V\beta$. TsA201 cells were transfected with the indicated plasmids and cell lysates subjected to pull down assays using recombinant GST or GST-AID. As shown in Fig. 3B, Rem2 does not directly associate with GST-AID. However, when $Ca_V\beta$ was cotransfected with Rem2, Rem2 could be isolated by GST-AID pull-down indicating a trimeric complex was formed between GST-AID, $Ca_V\beta$, and Rem2. These results suggest that Rem2 may regulate channel at the cell surface by forming a complex with the channel in a $Ca_V\beta$ subunit dependent manner.

3.4. The C-terminus of Rem2 is required for L-type channel regulation

The C-terminus of Rem has been shown to be necessary for L-type channel regulation [5]. The C-terminus of Rem2 is required for its localization to the plasma membrane [7,13] and plasma membrane targeting of Rem2 is essential for N-type calcium channel regulation [7]. Therefore, it was next examined whether the C-terminus of Rem2 was necessary for the inhibition of L-type calcium channel activity. As shown in Fig. 4A, when tsA201 cells are transfected with GFP, $Ca_V\beta$, and $Ca_V1.2$, these cells display robust L-type calcium channel currents (−10.18 ± 2.75 pA/pF; n=11) and as expected, expression of GFP-$Rem2^{1\text{-}310}$ failed to inhibit current expression (−28.59 ± 7.9 pA/pF; n=11). GFP-Rem2 was used as a positive control in this study

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and produced dramatic inhibition of current (−0.46 ± 0.38 pA/pF; n=8), as expected [6]. As seen in the box and whiskers plot of the data at 5 mV (Fig. 4B), a sub-population of cells transfected with $Rem2^{1-310}$, $Ca_V\beta$, and $Ca_V1.2$ developed higher currents than seen in the GFP transfected controls. The reason for the generation of this subpopulation is unknown, but when taken together, these data indicate that the Rem2 C-terminus is necessary for VDCC inhibition.

3.5. Rem2 interacts with PIP lipids

The finding that an intact C-terminus was necessary for Rem2 mediated VDCC regulation motivated studies to understand how Rem2 is anchored to the plasma membrane. The majority of membrane anchored Ras-related GTPases contain either prenyl- or acyl- group modifications [28]. Rem2 has a polybasic C-terminus that does not contain a lipid modification site [7,13]. Recently, GTPases that have polybasic domains have been shown to bind PIP1, PIP2 and PIP3 lipids [22]. As seen in Fig. 5A, full-length recombinant Rem2 was found to interact with PIP1, PIP2, and PIP3 lipids when incubated with Hybond membranes spotted with 15 biologically active lipids (PIP strips). The study was repeated with PIP arrays in which the PIP1, PIP2 and PIP3 lipids were titrated (Fig. 5B) and Rem2 displayed a preference for PI lipids phosphorylated at the 4 position, with PIP3(3,4,5) displaying the best binding. To localize the PIP lipid binding site on Rem2, the polybasic C-terminus was removed from the protein ($Rem2^{1-310}$). As shown in Fig. 5C, $Rem2^{1-310}$ could not bind to PIP lipids despite the fact that it was expressed at an equivalent level to Rem2 (Fig. 5D).

3.6. 14-3-3 proteins regulate the Rem2 PIP interaction

Since 14-3-3 proteins associate with the Rem2 C-terminus near the PIP lipid interaction site and 14-3-3 binding has been proposed to regulate the subcellular distribution of Rem2 [17], the ability of the Rem2 -14-3-3 complex to interact with PIP lipids was examined. It was observed in the initial purification of Rem2 that the later fractions from the MonoQ column contained two proteins of approximately 30 kDa (Fig. 2A, right lanes 5-7) that did not react with polyclonal Rem2 antibody (data not shown). Since full-length Rem2 has recently been shown to interact with 14-3-3 proteins [17], it was reasoned that these proteins could be 14-3-3 proteins endogenous to Sf-9 cells. In order to purify the Rem2-14-3-3 complex from unbound Rem2, the fractions labeled II from the Mono Q column (Fig. 2A) were pooled and applied to a Superdex 200 column (Fig. 6A). Fractions free of uncomplexed Rem2 (Fig 6A, labeled III) were collected and immunoblot analysis was used to determine that the copurifying proteins were 14-3-3 proteins (Fig. 6C). As shown in Fig. 6D, the Rem2-14-3-3 complex failed to interact with PIP lipids, although Coomassie Blue staining demonstrated equal amounts of Rem2 were present in each reaction (Fig. 6B). Since the binding of 14-3-3 proteins to the Rem GTPase can be disrupted by treatment with the serine/threonine phosphatase PP1 [26], the Rem2 - 14-3-3 complex was treated with either PP1 or PP1 together with a phosphatase inhibitor (I-2). As shown in Fig. 6D, PP1 pretreatment restores PIP lipid interaction indicating that the Rem2 in the Rem2-14-3-3 complex is functional but blocked for PIP lipid interaction by 14-3-3 proteins. Taken together, these studies suggest that Rem2 - PIP lipid association *in vivo* may be subject to complex regulation since the interaction could be controlled by either PI lipid phosphorylation or Rem2-14-3-3 association.

4. Discussion

Rem2 is expressed in endocrine cells as well as neuronal cells and is likely to play an important role in regulating VDCC in these cell types by a mechanism that involves its interaction with the $Ca_V\beta$ subunit [6,7,14]. It is therefore important to understand the biochemical mechanism by which Rem2 regulates VDCC function. There are two major findings presented in this study. First, the mechanism Rem2 uses to inhibit channel does not involve direct blockade of the $Ca_V\alpha$ – $Ca_V\beta$ interaction. Instead, it likely involves Rem2 binding to the $Ca_V\beta$ subunit while

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$Ca_V\beta$ is bound to the $Ca_V\alpha$ subunit AID. Second, the C-terminus of Rem2 binds phosphorylated PI lipids, and as described recently for other polybasic C-termini containing GTPases [22], this may aid in the recruitment of Rem2 to the plasma membrane. Moreover, it is shown that 14-3-3 binding inhibits PIP lipid association suggesting that the plasma membrane localization of Rem2 is regulated both by the concentration of phosphorylated PI lipids and the 14-3-3 binding status of Rem2.

The calcium channel $Ca_V\beta$ subunit has a conserved domain structure consisting of an N-terminal SH3 domain and a C-terminal GK domain and each domain uniquely contributes to modulate VDCC [3,29-31]. The GK domain has been shown to bind with high affinity to the AID and regulates trafficking of the channel to the plasma membrane [3] while the SH3 domain regulates the biophysical properties of the mature channel at the plasma membrane [29-31]. The finding that Rem2 binds multiple $Ca_V\beta$ isoforms [6] suggested that it would bind to a conserved $Ca_V\beta$ subunit domain(s). Using a series of truncation mutants in $Ca_V\beta_{2a}$, it was demonstrated that Rem2 interacts with the GK, but not SH3 domain *in vitro* (Fig. 1B). Furthermore, these deletion mutants define a 130 amino acid sub-region within $Ca_V\beta_{2a}$ as the Rem2 binding site. This domain is not part of the ABP (Fig. 1B) as it lacks critical alpha helices that form the ABP [25], but is identical to the domain found in recent studies to direct Rem association [20]. In support of this analysis, a recent mutagenesis study identified two amino acids within in this same region of $Ca_V\beta3$ as important for RGK interaction [19].

An outstanding issue in the field is whether the interaction of RGK proteins with $Ca_V\beta$ is nucleotide dependent. Initial work suggested that Gem preferentially bound $Ca_V\beta$ subunits *in vitro* when GTP-bound [4]. However, recent *in vitro* binding studies indicate that Rem interaction with the $Ca_V\beta$ subunit is not influenced by the nucleotide state of Rem [20]. Indeed, the G2 effector domains of the RGK proteins are not conserved [13], suggesting that $Ca_V\beta$ binding may involve residues outside of the effector loop, the domain known to serve as the primary binding site for effector protein binding within the Ras GTP-binding protein family [28]. In this study, Rem2 was found to bind $Ca_V\beta$ in a nucleotide independent manner (Fig. 1A). Recently, it has been suggested that R236 of mRem2, which is located in the nucleotide binding core of the protein, is a critical residue for $Ca_V\beta$ binding [19]. This arginine residue is conserved in all RGK GTPases; however, it is far from the G2 effector domain and may explain the nucleotide independence of the interaction. Consistent with the observation that the Rem2-$Ca_V\beta$ interaction is nucleotide independent, a recent study presented two lines of evidence that N-type VDCC modulation is not dependant on the nucleotide status of Rem2 [7]. Finally, another study has recently found that $Ca_V\beta$ binding is not sufficient to mediate inhibition of VDCC activity by Rem [32]. Therefore, it is possible that another RGK-protein interaction is GTP dependent allowing for nucleotide dependant regulation of VDCC activity.

The Gem GTPase has been proposed to inhibit the high affinity (low nM [3]) interaction of $Ca_V\alpha$ and $Ca_V\beta$ subunits [4,15]. However, a recent study found that Rem does not inhibit this interaction *in vitro*; instead Rem can form a trimeric complex consisting of $Ca_V\beta$ and AID [20]. Moreover, a subsequent *in vivo* study suggested that Gem can form a higher order complex consisting of $Ca_V\beta$ and $Ca_V\alpha$ [19]. It was therefore important to determine whether Rem2 inhibits the $Ca_V\alpha$–$Ca_V\beta$ interaction. Several observations suggest that Rem2 would not be able to inhibit a high affinity interaction between AID and $Ca_V\beta$. First, while Rem2 interacts with the $Ca_V\beta$ subunit, the *in vitro* interaction is not as efficient as the AID-$Ca_V\beta^{1–604}$ interaction (Fig. 1B). Second, coimmunoprecipitation of Rem2 by $Ca_V\beta$ demonstrates that the interaction is inefficient since the majority of Rem2 remains in the unbound fraction [6], similar to earlier studies with Rem [5], suggesting that the *in vivo* interaction is not high affinity. Finally, deletion mapping (Fig. 1B) indicates that the binding site for Rem2 is distinct from the conserved ABP domain in $Ca_V\beta$, since $Ca_V\beta$ mutants ($Ca_V\beta^{1\text{-}355}$, $Ca_V\beta^{226\text{-}355}$, $Ca_V\beta^{261\text{-}355}$) are found that interact with Rem2 but do not associate with GST-AID (Fig. 1B). When inhibition assays were

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performed, it was observed that even at a large molar excess, full-length Rem2 does not inhibit either the AID–$Ca_V\beta$ interaction *in vitro* (Fig. 2B) or *in vivo* (Fig. 3A). Taken together, these data indicate that inhibition of the $Ca_V\alpha$–$Ca_V\beta$ interaction is not the mechanism Rem2 uses to regulate VDCC activity.

Studies examining the interaction of Rem and $Ca_V\beta$ were the first to suggest that the $Ca_V\beta$ subunit can function as a scaffold to recruit Rem to the $Ca_V\alpha$ subunit [20]. It was subsequently shown that Gem, Rem, and Rad can each associate with a $Ca_V\alpha$-$Ca_V\alpha$ complex [19]. The experiment in Fig. 3B indicates that a similar complex can form between Rem2- $Ca_V\beta$ and AID. Thus, the ability of Rem2 to participate in a higher order $Ca_V\beta$-$Ca_V\alpha$ complex may be critical to its ability to regulate VDCC. However, the molecular mechanism used by Rem2 to inhibit channel activity remains to be determined. Importantly, it has been shown that Rem2 localization to the plasma membrane is critical for its ability to inhibit VDCC function (Fig. 5 and [7]) and that Rem2 overexpression in several cell systems does not reduce the surface density of VDCC [6,7]. Restricting Rem2 to diffusion within the plasma membrane could be an important factor in driving Rem2:$Ca_V\beta$: AID association since the Rem2-$Ca_V\beta$ interaction does not appear to be high affinity (Fig. 1). It will be of future interest to determine the dissociation constant for Rem2 and the $Ca_V\beta$ subunit.

Plasma membrane binding of Rem2 relies upon its polybasic C-terminus [7,13] and is critical to its ability to inhibit VDCC ([7]and Fig. 4). PIP lipids have recently been shown to be capable of recruiting Ras-related GTPases with polybasic C- termini to the plasma membrane [22], and it is demonstrated that the Rem2 C-terminus directs PIP binding (Fig. 5C). These lipid binding studies support a role for PIP lipids as possible Rem2 binding targets at the plasma membrane and suggest a preference for PIP phosphorylated in the 4 position with the strongest binding to PIP3(3,4,5) (Fig. 5B). These data also suggest that this lipid interaction may provide an important regulatory mechanism for controlling both the subcellular localization of Rem2 and possibly other RGK GTPases [22]. Moreover, the PIP interaction with the Rem2 C-terminus may be subject to regulation since the interaction is blocked by Rem2 association with 14-3-3 proteins (Fig. 6D). Importantly, it is shown that this is a reversible process since disruption of the Rem2-14-3-3 complex by phosphatase treatment restores Rem2 - PIP lipid binding (Fig. 6D). Taken together, these studies suggest that regulation of PI kinases/phosphatases as well as modulation of the Rem2-14-3-3 binding could play important roles in regulating the subcellular localization of Rem2, which in turn would provide a physiological mechanism for controlling Rem2 mediated VDCC inhibition. Studies are underway to define the requirement for PIP binding for Rem2 mediated VDCC inhibition and to identify the kinase/phosphatase (s) that regulate Rem2-14-3-3 association.

5. Conclusions

While it is clear that all members of the RGK GTPase family function to potently inhibit VDCC function, a unifying molecular mechanism remains unresolved. Toward this goal, we demonstrate that Rem2 does not inhibit AID-$Ca_V\beta$ association, indicating that a simple $Ca_V\beta$ sequestration model does not explain Rem2-mediated VDCC blockade. Furthermore, we find that $Ca_V\beta$ can simultaneously associate with both Rem2 and $Ca_V\alpha_1$-AID, and that the Rem2-$Ca_V\beta$ interaction is nucleotide-independent. These data suggest a model in which the $Ca_V\beta$ serves a constitutive scaffolding role, promoting formation of a Rem2-$Ca_V\beta$-AID complex to inhibit plasma membrane localized $Ca_V\alpha1$. Regulation of this novel control mechanism appears to rely, at least in part, upon the Rem2 C-terminus. C-terminal mediated plasma membrane trafficking of Rem2 is required for VDCC regulation, and is likely mediated by interactions of the polybasic C-terminus with PIP2 and PIP3 lipids. Moreover, we find that phosphoserine dependant 14-3-3 protein binding negatively regulates Rem2-PIP lipid association, suggesting an unexpected role for kinase signaling in Rem2 mediated VDCC

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inhibition. Clearly, additional studies will be necessary to clarify the cellular signaling pathways that control Rem2 regulation of VDCC activity.

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Abbreviations used

RGK, Rem, Rem2, Rad, and Gem/Kir Ras Related GTPases
VDCC, Voltage Dependant Calcium Channel
$Ca_V\beta$, VDCC beta subunit
$Ca_V\alpha$, VDCC alpha subunit
PI, phosphatidylinositol
PIP, phosphorylated phosphatidylinositol
PIP1, phosphatidylinositol-monophosphate
PIP2, phosphatidylinositol-bisphosphate
PIP3, phosphatidylinositol-trisphosphate
AID, VDCC alpha subunit Interaction Domain
ABP, VDCC alpha subunit Interaction Domain Binding Pocket
GK, Guanylate Kinase
SH3, Src Homology 3 domain

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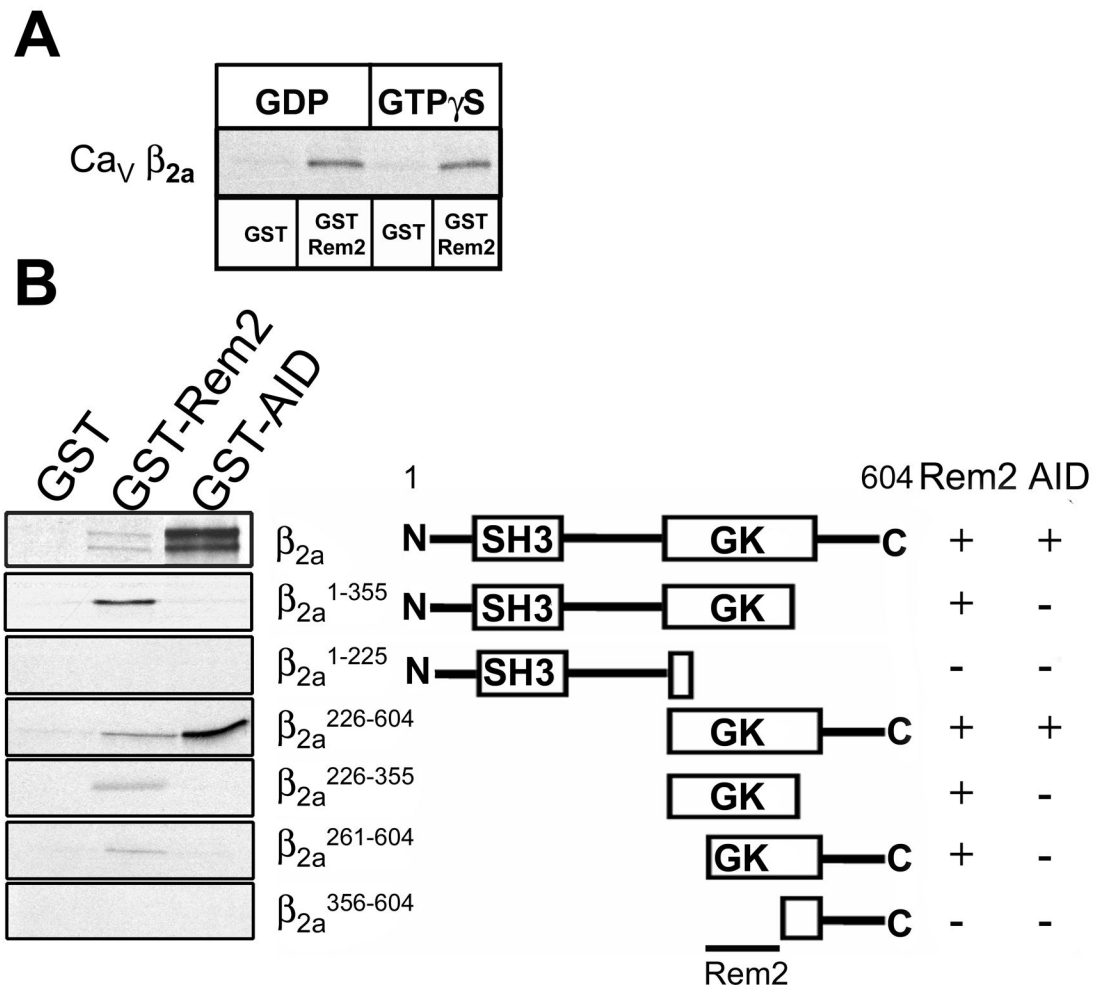

Fig. 1. Rem2 binds to Ca$_V$β subunits in a nucleotide independent manner and binds to the Ca$_V$β subunit at a site that is distinct from the AID binding pocket

A) Recombinant GST or GST-Rem2 was loaded with the indicated guanine nucleotide and then incubated with *in vitro* transcribed and translated [^{35}S]Ca$_V$β subunit for 3 h. The reactions were then processed as described in the Materials and Methods section and the bound fractions analyzed on a 10% SDS-PAGE gel that was dried and exposed to film. B) Left, GST, GST-Rem2, or GST-AID was incubated with the indicated [^{35}S]Ca$_V$β subunit truncated at either the N- or C-terminus for 3h. The reactions were processed as described in the Materials and Methods section. The bound fraction was resolved on a 10% SDS-PAGE gel that was dried and exposed to film. Right, a schematic of the Ca$_V$β$_{2a}$ subunit deletions used. The Rem2 interaction domain is underlined along with the SH3 and GK domains indicated as boxes. Interaction of the indicated fragment with Rem2 or AID is indicated with a (+) sign. The data are representative of at least three independent experiments.

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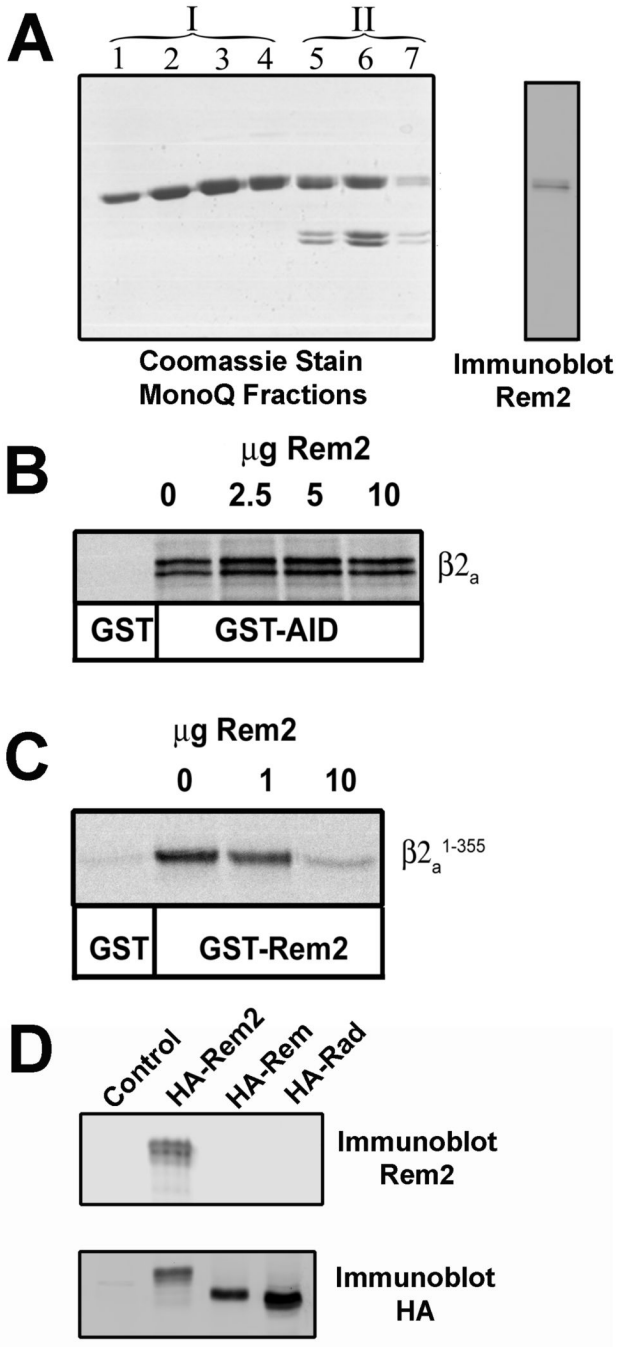

Fig. 2. Rem2 does not inhibit AID- $Ca_V\beta$ interaction

A) Left, recombinant baculovirus expressing His_6-Rem2 was used to infect Sf-9 cells and His_6-Rem2 was purified using Ni^{2+}-NTA sepharose as described in the Materials and Methods section. The His_6 tag was enzymatically cleaved using His_6-TEV protease, and contaminating proteins extracted using Ni^{2+}-NTA sepharose. Rem2 was then applied to a Mono Q column and column fractions (lanes 1-8) were run on a 10% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel that was analyzed by Coomassie Blue staining. This chromatographic step yielded purified Rem2 (I). Right, the identity of purified Rem2 was confirmed by immunoblot analysis of 20 ng of the pooled fractions labeled I with affinity purified Rem2 polyclonal antibody. B) GST or GST-AID (10 ng) was incubated with [^{35}S]

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$Ca_V\beta_{2a}$ and the indicated amount of recombinant full-length mRem2 for 3 h. The reactions were processed as described in the Materials and Methods section and the bound fraction run on a 10% SDS-PAGE gel that was then dried and exposed to film. C) GST or GST-Rem2 (10 μg) was incubated with $[^{35}S]Ca_V\beta_{2a}^{1\text{-}355}$ and the indicated amount of recombinant full-length mRem2. The reactions were processed and the bound fraction analyzed as described in "B." The data are representative of at least three independent experiments. D) Top, the specificity of the affinity purified antibody was confirmed by analyzing its reactivity to TSA201 cell lysates transiently transfected with empty vector (control), HA-Rem2, HA-Rem, or HA Rad as indicated. Bottom, the expression of the HA affinity tagged proteins was confirmed by immunoblot analysis with HA antibody.

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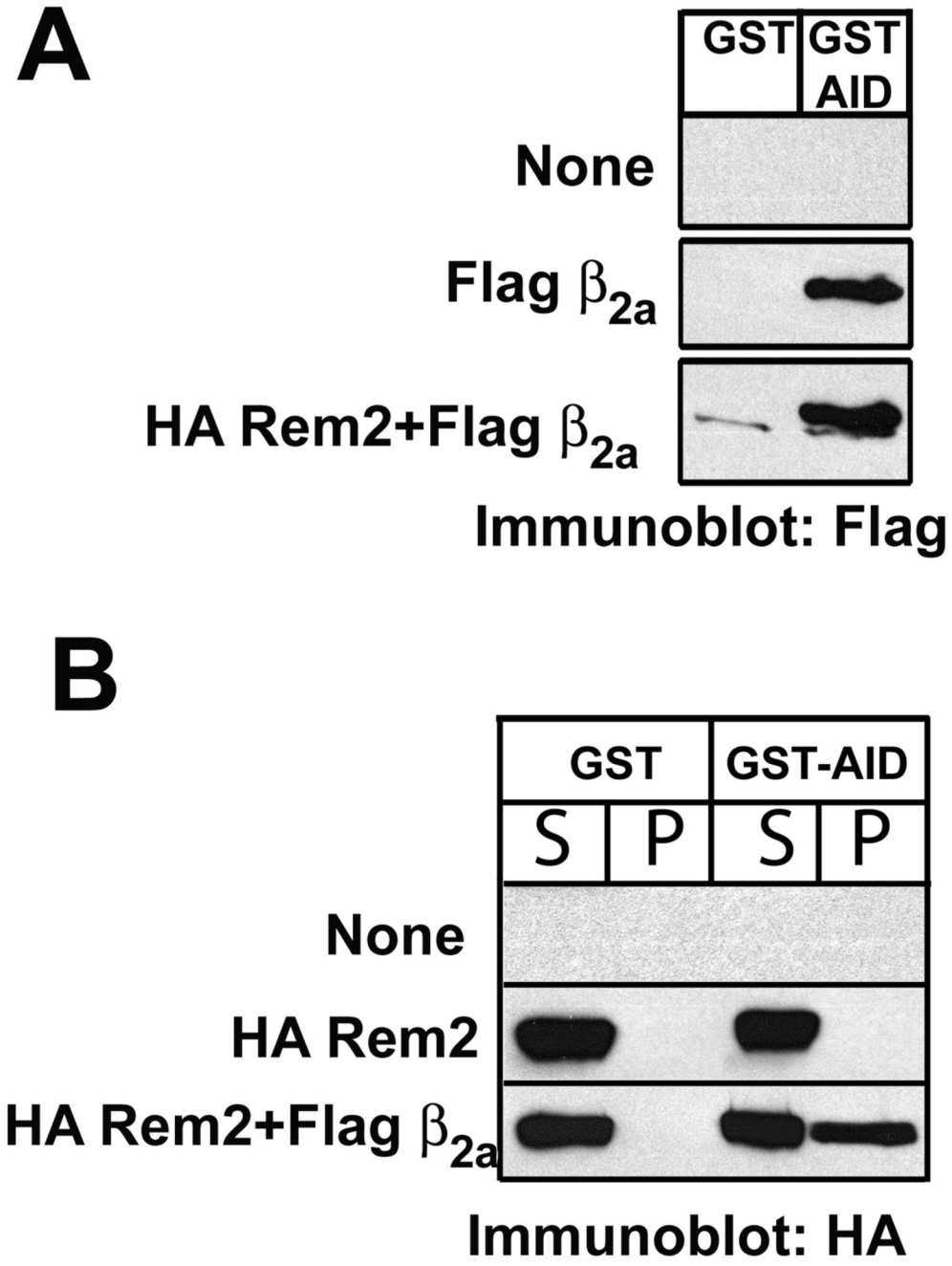

Fig. 3. Rem2 does not inhibit the GST-AID interaction with *in vivo* expressed $Ca_V\beta_{2a}$ and Rem2 can enter a complex with $Ca_V\beta$-AID

A) TsA201 cells were transfected with the indicated plasmids. Cell lysates were prepared and subjected to GST pull-down with GST or GST-AID. The reactions were processed as described in the Materials and Methods section and the ability of Flag β_{2a} to interact with GST-AID assessed by immunoblotting with FLAG antibody. B) TsA201 cells were transfected with the indicated plasmids and lysates prepared and subjected to GST pull-down as described in (A). The eluted proteins were then run on 10% SDS-PAGE gels, transferred to nitrocellulose and the ability of HA-Rem2 to enter a GST-AID complex analyzed by immunoblotting with HA

antibody. S, supernatant (unbound fraction), P, pellet (bound fraction). The data are representative of three independent experiments.

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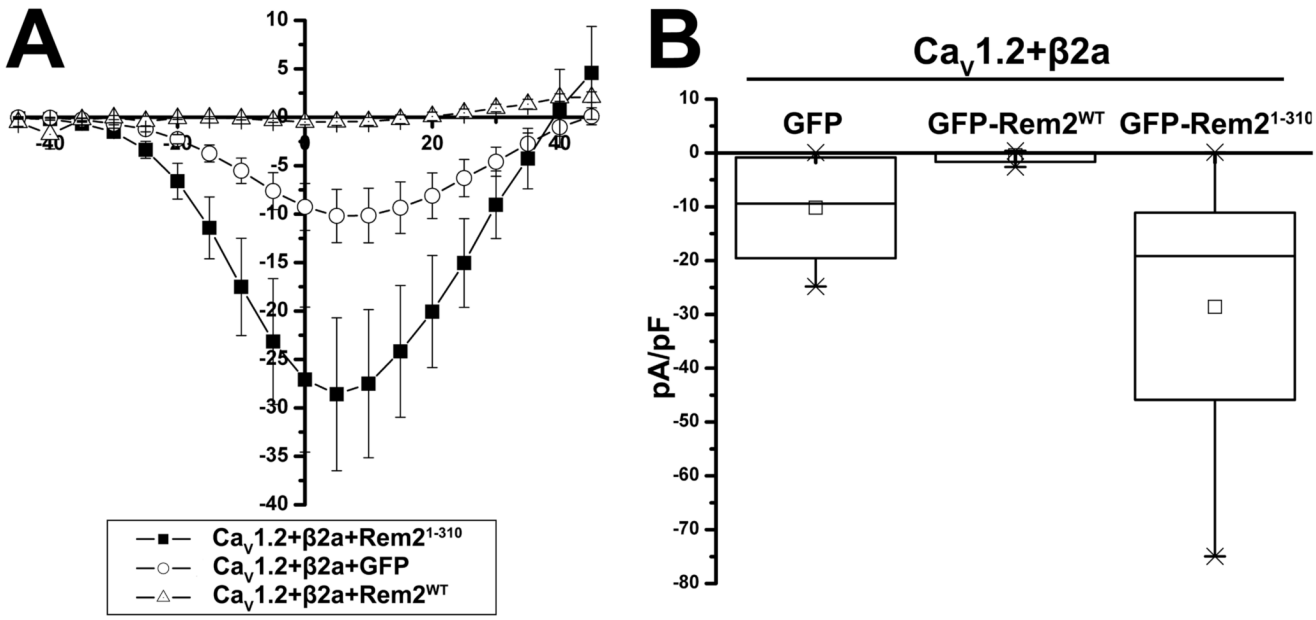

Fig. 4. Rem2 requires its C-Terminus to inhibit L-type VDCC activity

A) Current-voltage relationships for tsA201 cells transiently transfected with $Ca_V1.2$, $Ca_V\beta_{2a,}$ and GFP (open circle; n=11); $Ca_V1.2$, $Ca_V\beta_{2a,}$ and GFP-Rem2 (open triangle; n=8); and $Ca_V1.2$, $Ca_V\beta_{2a,}$ and GFP-Rem2$^{1-310}$ (filled square; n=11) are shown. The error bars represent standard error of the mean. B) Box plot of the data from Fig 5A. Box includes first quartile, median and third quartile. Open square represents the mean of the data set and "whiskers" extend to the highest and lowest values.

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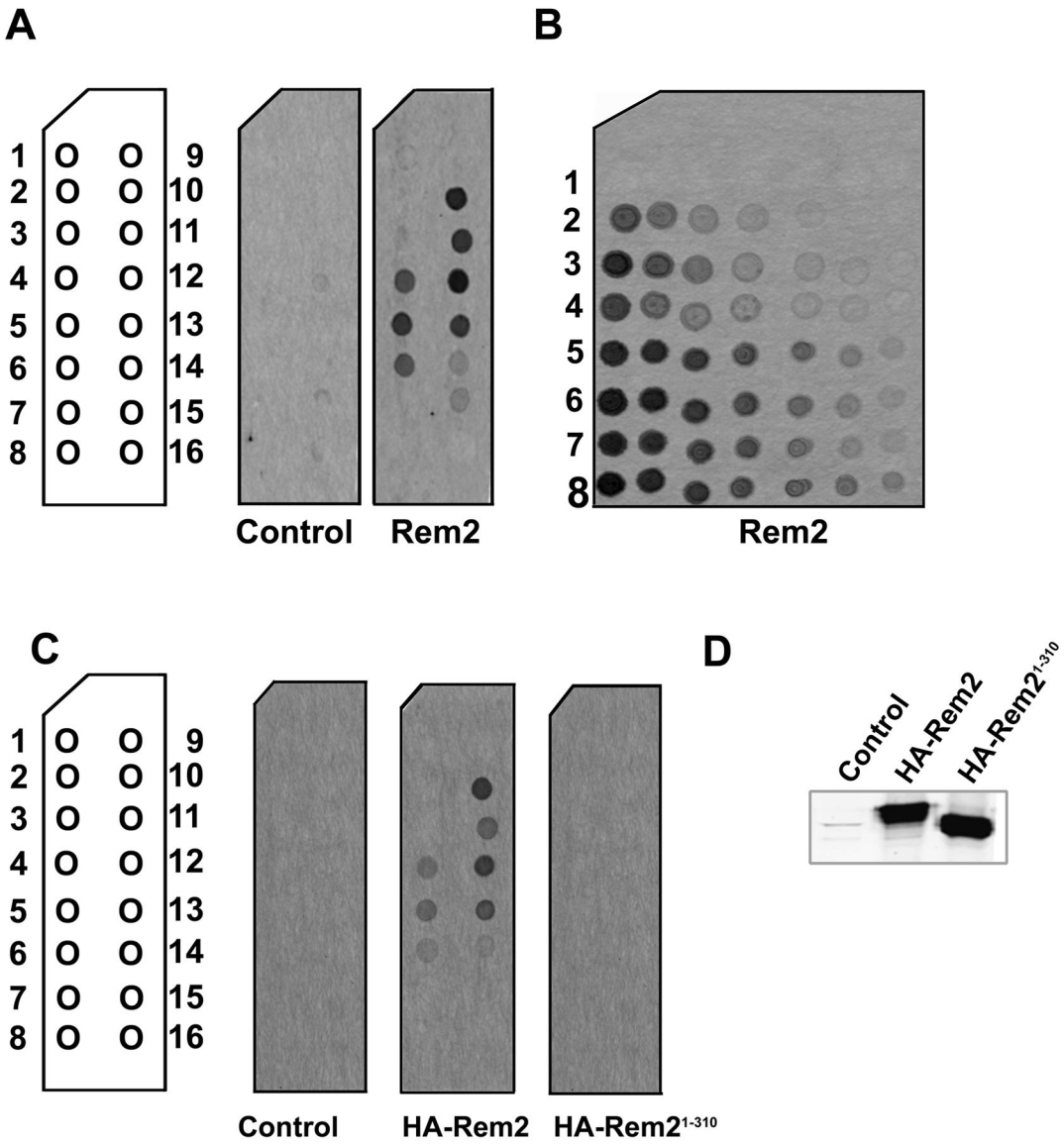

Fig. 5. Rem2 Interacts with Phosphorylated Phosphatidylinositide Lipids and the Rem2 C Terminus is Critical for Binding

A) Left, a schematic of lipids spotted on the PIP strip is shown. The lipids are: 1. Lysophosphatidic acid, 2. Lysophosphocholine, 3. Phosphatidylinositol (PI), 4. PI(3)P, 5. PI (4)P, 6. PI(5)P, 7. Phosphatidylethanolamine, 8. Phosphatidylcholine, 9. Sphingosine-1-phosphate, 10. PI(3,4)P_2, 11. PI(3,5)P_2, 12. PI(4,5)P_2, 13. PI(3,4,5)P_3, 14. Phosphatidic acid, 15. Phosphatidylserine, 16. Blank. Right, Rem2 (0.5 μg/mL) or no protein (control) were incubated with blocked PIP strip membranes as described in the Materials and Methods Section. The membranes were then washed and immunoblotted with Rem2 antibody as described in the Materials and Methods section to detect Rem2-lipid interaction. B) Rem2 (0.5 μg/mL) was incubated with a PIP array which is spotted with decreasing amounts of lipid. The left column has 100 pmol lipid spotted and each column to the right is spotted with half that amount. The lipids used are: 1) Phosphatidylinositol (PI), 2) PI(3)P, 3) PI(4)P, 4) PI(5)P , 5) PI(3,4)P_2, 6) PI(3,5)P_2, 7) PI(4,5)P_2, 8) PI(3,4,5)P_3. C) TsA201 cells were transiently transfected with the indicated plasmids. The cells were lysed and the 100,000 g supernatant

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was incubated with blocked PIP strips as described in the Materials and Methods section. The PIP strips were washed and immunoblotted with HA antibody to detect HA-Rem2-lipid interactions. The key to the lipids is the same as in (A). D) The tsA201 lysates from panel (C) were run on 10% SDS-PAGE gels, transferred to nitrocellulose, and immunoblotted with HA antibody. The data are representative of two independent experiments.

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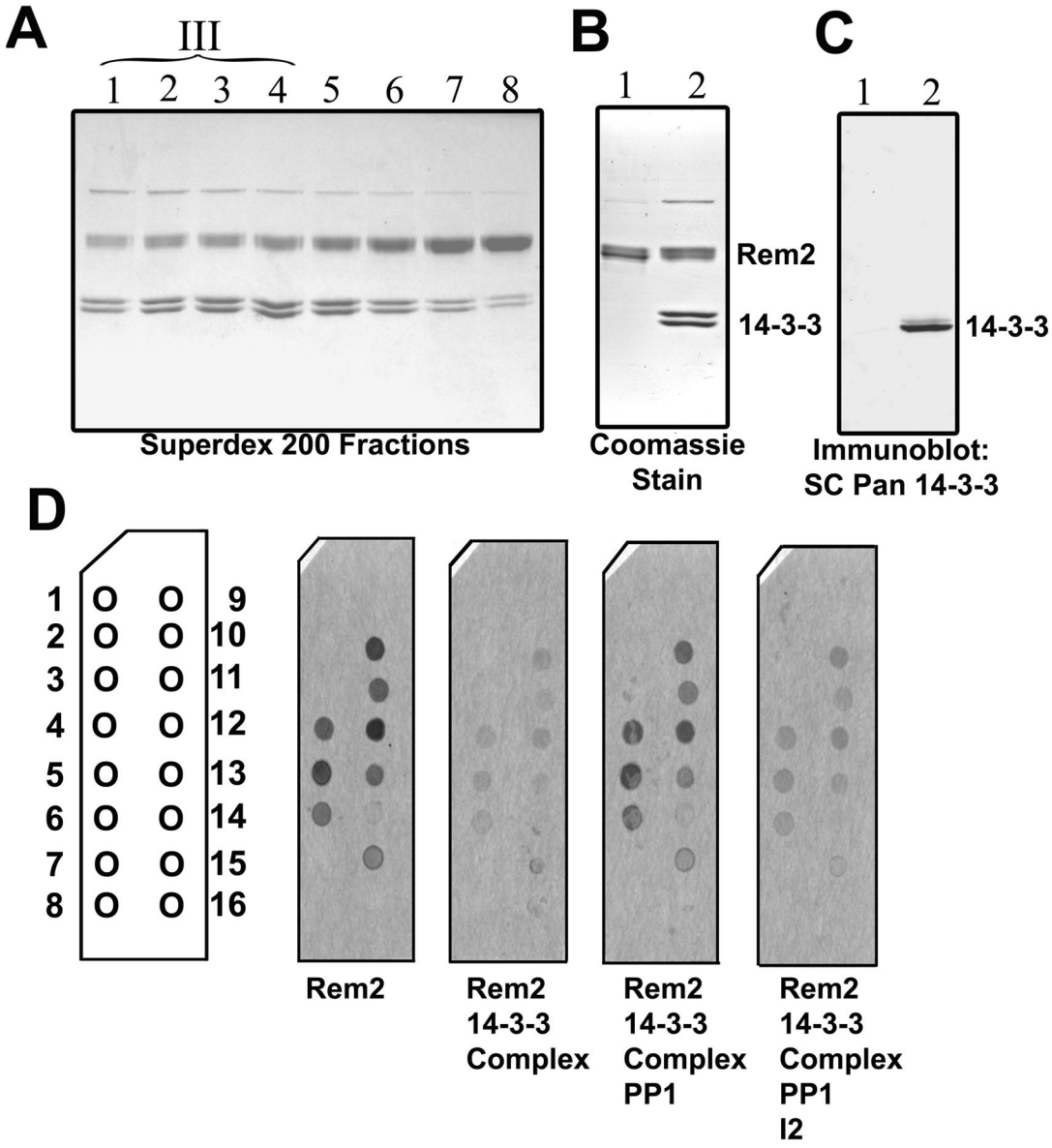

Fig. 6. 14-3-3 protein regulates the association of Rem2 with phosphorylated PI lipids

A) The Rem2-14-3-3 complex (III) was further purified using size exclusion chromatography and the indicated fractions (lanes 1-8) analyzed after being run on a 10% SDS-PAGE gel by Coomassie Blue staining. Equal molar amounts of Rem2 and the Rem2-14-3-3 complex were resolved by SDS-PAGE and then subjected to analysis by either Coomassie Blue Staining (B) or by immunoblot analysis with pan-reactive SC 629 rabbit anti 14-3-3 antibody (C). D) Left, the key to the lipids spotted is the same as in Fig. 5A. Right panels, the Rem2-14-3-3 complex was treated without (control), or with PP1, or PP1 with I-2 phosphatase inhibitor as described in the Materials and Methods section. An equimolar amount of Rem2 or the Rem2-14-3-3 complex treated with PP1 and I2 as indicated were then incubated with blocked PIP strips. The PIP strips were then washed and immunoblotted with Rem2 antibody to detect Rem2-lipid interactions as described in the Materials and Methods section. The data are representative of two independent experiments.