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Molecular determinants of Rem2 regulation of N-type calcium channels

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Abstract

Rem2 belongs to the RGK family of small GTPases whose members are known to interact with the voltage gated calcium channel β subunit, and to inhibit or abolish calcium currents. To identify the underlying functional domains of Rem2, we created several N- or C-terminally truncated Rem2 proteins and examined their abilities to interact with the Ca_v β subunit and to regulate the activities of $Ca_v2.2$ N-type calcium channels. Confocal imaging of Rem2 in tsA-201 cells revealed that it contains a membrane-targeting signal in its C-terminus, consistent with previous studies. Co-precipitation assays showed that Ca_v β_3 interaction depends on Rem2 residues 1–123. Only Rem2 proteins that targeted the cell membrane as well as bound the β subunit were able to reduce whole cell calcium currents.

Keywords: Calcium channel; GTPase; Rem2; Beta subunit; RGK protein

RGK proteins, consisting of Gem, Rad, Rem, and Rem2, are small GTPases of the Ras superfamily. They are expressed in a tissue- and region-specific manner, with Rem2 being expressed predominantly in brain [1]. They typically contain a Ras-like homology domain which is about 50% conserved among RGK family members and which is flanked by N- and C-terminal regions [2]. The N-terminal region is highly variable within the RGK family (7% conservation), whereas the C-terminus region shows a higher degree of conservation (40%, [2]). Co-expression of Gem, Rad and Rem with $Ca_v1.2$ (L-type) channels, and of Rem2 with $Ca_v2.2$ (N-type) channels in heterologous and native systems has been shown to abolish calcium current activity [3–8], presumably through their interactions with the calcium channel β subunit [3,5,9,10]. Recent work has demonstrated that all RGKs are trafficked to the plasma membrane by way of their C-termini which interact with phosphoinositol lipids [11–13].

In spite of homology, structural domains of RGK proteins seem to mediate different functions. Binding of Gem to the β subunit is reported to be GTP-dependent [5], while β-binding of Rem does not seem to require nucleotides [14]. Also, the central Ras-homology domain of Gem, lacking the N- and C-terminal extensions, is sufficient to pull down the Ca_v β_3 subunit [9]. In contrast, removing 32 residues of the Rem C-terminus reduced its ability to bind the Ca_v β_{2a} subunit as well as its effect on $Ca_v1.2$ currents [3], although a recent report shows Ca_v β_{1b}-binding remains intact in spite of this deletion [12]. Indeed, this suggests that RGK proteins may contain specialized sequences to bind the various β subunits.

Here, we have examined the Rem2 structural determinants that are responsible for its interaction with the calcium channel β_3 subunit and for regulating N-type calcium channel activity and membrane expression. We show that the N-terminal 123 residues of Rem2 are sufficient for β subunit binding, and that inhibition of calcium channel activity requires binding to the β subunit as well as the plasma membrane insertion domain of Rem2.

Materials and methods

Molecular biology. Rem2 cDNA was amplified by RT-PCR from mouse brain mRNA and cloned into the pCMV-HA (Clontech) expression vector, creating N-terminally HA-tagged Rem2. Premature stop codons were introduced into this vector using site-directed mutagenesis,

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each adding a silent restriction site for the purpose of screening. Mutated cDNAs were subcloned back into the HA-Rem2-pCMV vector after their sequences were verified. The shortest Rem2 truncation we generated (1–41 Rem2) never expressed in our system.

The C-terminally flag-tagged Ca_v β_3 subunit was created by inserting oligonucleotides containing the flag sequence immediately following the C-terminal Tyr residue in the β_3-pMT2 expression vector [15]. Wild-type calcium channel 2.2 constructs were provided by Dr. Terry Snutch.

Cell culture and transfection. The culturing and transfection of tsA-201 cells was conducted as described in detail by us previously [16]. Briefly, HEK tsA201 cells were transfected with calcium phosphate, using 15 μg DNA of each plasmid per 10 cm plate for co-precipitation, 1.5 μg per well ($Ca_v2.2$) and 0.5 μg per well (α_2–δ, β_3, and Rem2) of a 35 mm glass-bottom imaging dish (Mat-tek Corporation, Ashland, MA, USA), and 6 μg each construct per 10 cm plate containing coverslips for electrophysiology. Cells were washed 12–16 h later, and harvested for co-precipitation or fixed for imaging 48–60 h after transfection, or transferred to a 30 °C incubator for patch clamp analysis.

Co-immunoprecipitation. 10 cm plates were washed and pelleted, then resuspended in 200 μl lysis buffer (140 mM NaCl, 10 mM Tris, 0.5% NP-40) with protease inhibitor (Roche Complete) per plate. After a 30 μl cleared-lysate sample was removed, the remaining lysate was added to washed and prepared anti-flag M2 agarose beads, and rotated at 4 °C overnight. Bound complexes were then washed twice in 1 ml lysis buffer, eluted in 4× sample buffer (12% SDS, 6% β-ME, 30% glycerol, 150 mM Tris, pH 7.5, 0.05% Coomassie blue), separated on 16% Tricine SDS–PAGE, and transferred to 0.2 μm nitrocellulose membrane for Western blots. Membranes were probed with either anti-flag (1:1000, Sigma) or anti-HA (1:1000, Roche) antibodies to detect β subunits and Rem2 proteins, respectively.

Imaging. Cells on 35-mm plates were fixed in 4% paraformaldehyde/15% picric acid, washed, permeabilized in 0.5% Triton X-100, and probed with anti-HA antibody to detect HA-tagged Rem2 proteins. Coverslips were mounted over the glass portion of the dishes using Vectastain mounting media with DAPI (Vector Labs, Burlingame CA, USA) and imaged on a Zeiss LSM-510 inverted confocal microscope using a 63× oil immersion objective.

Electrophysiology. HEK tsA201 cells exogenously expressing $Ca_v2.2 + \alpha_2$–$\delta + \beta_3 \pm$ full-length or truncated Rem2 were subjected to whole cell patch clamp recordings as described previously [17]. Recordings were performed using an Axopatch 200B amplifier driven by pCLAMP software. Series resistance was compensated 85%, and recordings were conducted under the following ionic conditions: external solution (mM): 20 $BaCl_2$, 1 $MgCl_2$, 10 HEPES, 40 TEA–Cl, 65 CsCl, 10 glucose. Internal solution (mM): 108 Cs–methanesulfonate, 4 $MgCl_2$, 9 EGTA, 9 HEPES. The data were leak subtracted offline, and analyzed using Clampfit software. Peak current amplitudes were measured for each cell. Data were averaged across multiple transfections.

Statistical analysis. All error bars denote standard errors. Statistical analysis of peak calcium currents was performed using one-way ANOVAs with Bonferroni's post test for multiple comparisons versus a control group, using SigmaStat software.

Results

Subcellular localization of truncated Rem2 constructs

To examine structural determinants of Rem2 modulation of $Ca_v2.2$ calcium channels, we created a series of HA-tagged Rem2 expression constructs containing successive deletions at either the N-terminal or C-terminal end (Supp. Fig. 1). These constructs were transfected into in tsA-201 cells and their cellular localization analyzed by HA-immunofluorescence. Expression of full-length Rem2 and N-terminally truncated Rem2 proteins, all of which contained the PIP lipid-binding C-terminus [12,13], resulted in effective targeting to the plasma membrane, as evident from a rim-like fluorescence pattern seen in cells expressing these constructs (Fig. 1). In contrast, C-terminally truncated proteins tended to show a diffuse cytoplas-

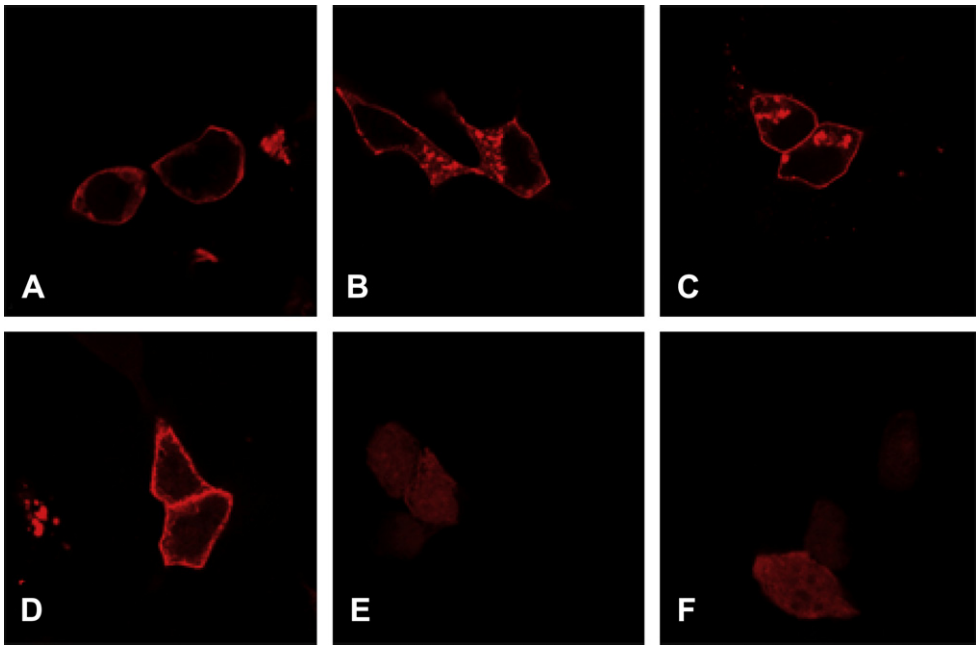

Fig. 1. Rem2 membrane-targeting. Representative confocal images of HA-tagged Rem2 proteins exogenously expressed in tsA-201 cells and probed with an HA antibody after permeabilization. Constructs imaged in panels (A) through (D) contain intact C-terminal sequences and are plasma membrane targeted. Constructs expressed in the cells shown in panels (E and F) lack the C-terminal region and are distributed diffusely in the cytoplasm. (A and D) Full-length Rem2 (1–341); (B) 179–341 Rem2; (C) 284–341 Rem2; (E) 1–282 Rem2; (F) 1–149 Rem2.