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Using Exofacially Tagged Functional Cav2.2 to Investigate the Modulation of Pore Subunit Trafficking by Auxiliary Calcium Channel Subunits

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N type Calcium Channels are voltage gated channels composed of a pore forming α1B (Cav2.2) subunit and 2 auxiliary subunits α2δ and β. N type calcium channels regulate calcium influx and exocytosis in presynaptic terminals of neurons. Tight control of Cav2.2 trafficking is important as small changes in channel density at the cell surface can result in large changes in calcium ion influx and therefore neurotransmitter release. The auxiliary subunits α2δ and β modulate the biophysical properties and surface channel density of Cav2.2 through mechanisms that are not fully defined. To investigate the contribution of these subunits to Cav2.2 trafficking we have created a fully functional exofacially tagged Cav2.2. Using this tool we are able to quantify the contribution of each subunit on Cav2.2 trafficking to the surface of N2a cells (mouse neuroblastoma derived cell line). Our results support previous electrophysiological data indicating the absolute requirement for the β subunit and additional enhancement by the α2δ subunit. Our data also suggest an intracellular site of interaction between Cav2.2 and α2δ. Epitope obstruction of α2δ bound to Cav2.2 may indicate the region of α2δ involved in interaction with Cav2.2.

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Cardiomyocyte Endpoints Distinguish Non-Canonical Regulation of the RGK Gtpases Rad and Rem

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Rad/Rem/Rem2/Gem/Kir (RGK) proteins are Ras-like GTPases with diverse (and expanding) functions including: regulating cytoskeleton dynamics, cell proliferation, synaptogenesis, and inhibiting voltage-dependent calcium (Ca_V) channels. Whether RGKs are canonical GTP-regulated ON-OFF switches similar to Ras is ambiguous. Here, we used a range of functional measurements in cardiomyocytes ($Ca_V1.2$ currents, Ca^{2+} transients, $Ca_V\beta$ binding) as biosensors to probe nucleotide regulation of Rad and Rem. We utilized as tools RadS105N and RemT94N, two mutants analogous to RasS17N which is a Ras variant that displays a strongly decreased GTP binding affinity. As a reference for expected canonical behavior, RasS17N loses effector binding, but acts as a dominant negative *in situ* due to increased avidity for guanine nucleotide exchange factor. Adenovirus mediated expression of either wild type (wt) Rad or RadS105N in cardiomyocytes dramatically blocked endogenous $I_{Ca,L}$, contradicting reports that RadS105N acts as a dominant negative in heart. Both Rad and RadS105N diminished cardiac EC coupling as reported by the amplitude of rhod-2-reported Ca^{2+} transients. However, RadS105N increased the propensity for cardiac arrhythmias compared to wt Rad, hinting at a functional difference. By contrast with Rad/RadS105N, RemT94N was significantly less effective than wt Rem in inhibiting either $I_{Ca,L}$ or Ca^{2+} transients, revealing a functional dichotomy between the two RGKs. FRET analyses of binding interactions between auxiliary $Ca_V\beta$s and RGKs revealed a reduced binding affinity for RadS105N and RemT94N relative to their wt counterparts. Finally, proteomic analyses of Rem and RemT94N interactomes in cardiomyocytes displayed subtle differences in binding partners. The results indicate that while Rad and Rem are non-canonical GTPases, their GTP-binding status can be sensed to yield a spectrum of functions in an isoform-dependent manner.

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Differential $Ca_V2.1$ and $Ca_V2.3$ Channel Inhibition by Baclofen and α-Conotoxin Vc1.1 via $GABA_B$ Receptor Activation

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In the nervous system, $Ca_v2.1$ (P/Q-type), $Ca_v2.2$ (N-type) and $Ca_v2.3$ (R-type) calcium channels contribute to synaptic transmission and are modulated via G protein-coupled receptor pathways. The analgesic α-conotoxin Vc1.1 has been identified as a selective inhibitor of $Ca_v2.2$ channels acting via $GABA_B$ receptors. We investigated baclofen and Vc1.1 modulation of human $Ca_v2.1$ or $Ca_v2.3$ channels via human $GABA_B$ receptor activation in HEK cells. Baclofen (50 μM) inhibited $Ca_v2.1$ and $Ca_v2.3$ channel Ba^{2+} currents by ~ 40%, whereas Vc1.1 did not affect $Ca_v2.1$, but potently inhibited $Ca_v2.3$, with a half-maximal inhibitory concentration of ~ 300 pM. Depolarizing paired-pulses revealed that ~ 75% of the baclofen inhibition of $Ca_v2.1$ was voltage-dependent, and could be relieved by strong depolarization. In contrast, baclofen or Vc1.1 inhibition of $Ca_v2.3$ channels was solely mediated via voltage-independent pathways that could be disrupted by pertussis toxin, GDP-β-S or the $GABA_B$ receptor antagonist, CGP55845. Over-expression of c-Src kinase significantly increased inhibition of $Ca_v2.3$ by Vc1.1. Conversely, co-expression of a double mutant c-Src or pre-treatment with a phosphorylated pp60c-Src peptide abolished the effect of Vc1.1. Site-directed mutational analyses of $Ca_v2.3$ demonstrated that tyrosines 1761 and 1765 within exon 37 are critical for mediating the inhibition by Vc1.1 and are involved in baclofen inhibition of these channels. Remarkably, point mutations introducing specific c-Src phosphorylation sites into human $Ca_v2.1$ channels conveyed Vc1.1 sensitivity. Our findings demonstrate that Vc1.1 inhibition of $Ca_v2.3$ is due to specific c-Src phosphorylation sites in the C-teminus, which defines $Ca_v2.3$ channels as potential targets for analgesic α-conotoxins.

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Calorimetric and NMR Characterization of a Rem2 G-Domain Interaction with the Sh3-GK Core of the Ca^{2+} Channel Beta4 Subunit

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The RGK (Rad, Rem1, Rem2 and Gem/Kir) subfamily of small GTPases has potent inhibitory effects on high voltage-activated Ca^{2+} channels. The inhibition has been shown to occur through a mechanism that is dependent on the Ca^{2+} channel beta subunit. In this study, we characterized an interaction between the G domain of Rem2 and the beta4 guanylate kinase (GK) domain using NMR and isothermal titration calorimetry (ITC) techniques. We determined that the interaction is endothermic, has a stoichiometry of 1, and a K_d 156 uM, which is three orders of magnitude weaker than the beta4 interaction with the pore-forming alpha1 subunit. NMR chemical shift perturbation analysis reveals that Rem2 interacts exclusively with the GK domain of the SH3-GK core by way of residues located in the former GMP binding region of the inactive kinase. The site is composed of residues just proximal to 3_{10} helix sequences 2 and 3, and residues in alpha helix 6. Thus, the RGK binding site is clearly distinct from the binding site for the alpha1 subunit AID peptide. Site-directed mutagenesis of surface residues combined with ITC indicate that multiple GK sequences contribute to Rem2 binding collectively. These results support the idea that Ca^{2+} channel inhibition by Rem2 is unlikely to be the result of direct competition with alpha1 binding to the beta subunit. Instead, it argues in favor of a mechanism in which beta subunit-anchored Rem2 executes its inhibitory effect through other contacts with alpha1 subunits. This study demonstrates that NMR combined with ITC can provide detailed information on important weak protein-protein interactions involved in Ca^{2+} channel regulation.

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A Timothy Syndrome Causing Mutation Perturbs Voltage Sensor Operation in Human $Ca_V1.2$ Channels

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The pore-forming α_{1C} subunit of the voltage-gated L-type calcium channel ($Ca_V1.2$) consists of four concatenated Repeats (I-IV), each contributing a voltage-sensing domain (VSD, transmembrane helices S1-S4) and a quarter of the central ion-conductive pore domain (S5-S6). In Timothy Syndrome (TS), a rare and severe multi-organ disorder, specific alterations in $Ca_V1.2$ voltage-dependent properties lead to autism, immunodeficiency, QT prolongation (LQT8) and lethal arrhythmias. The most frequent TS-causing mutation (G406R) is located at the intracellular flank of S6 in Repeat I and promotes Ca^{2+} influx by (1) shifting $Ca_V1.2$ voltage- dependent activation towards more negative potentials and (2) impairing voltage-dependent inactivation. To gain insight on the molecular basis underlying the TS-causing voltage-sensitivity anomalies, we studied the structural rearrangements of VSDs I and III in human $Ca_V1.2$ channels carrying the G406R mutation, using the voltage-clamp fluorometry technique. Briefly, this involves conjugating a small, environment-sensitive fluorophore at a specific VSD that reports local conformational rearrangements as fluorescence changes. Under voltage-clamp, we simultaneously acquired ionic