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(YFP-RyR1$_{1:4300}$), which encodes the cytoplasmic domain of RyR1 ("foot") but lacks the C-terminal domains which form the ion pore for SR calcium release and which anchor RyR1 in the SR membrane. We found that YFP-RyR1$_{1:4300}$ targets to PM-SR junctions in dyspedic myotubes (null for endogenous RyR1) and transmits the retrograde signal to the DHPRs present in those myotubes. We have now begun to examine whether these interactions between the RyR1 foot and DHPR can occur in the absence of other proteins which are present in PM-SR junctions of muscle cells. Toward this end, we used tsA201 cells to co-express CFP-RyR1$_{1:4300}$, YFP-Ca$_V$1.1 (or Ca$_V$1.1/Ca$_V$1.2 chimeras) and the DHPR auxiliary subunit β1a. Most of the Ca$_V$ constructs appeared to be retained in a peri-nucuelar compartment (Ca$_V$1.1, and chimeras in which three Ca$_V$1.1 repeats were replaced by the corresponding Ca$_V$1.2 repeats). PM-targeting was observed for a construct composed of Ca$_V$1.2 with the cytoplasmic II-III loop replaced by that of Ca$_V$1.1. However, we have not yet observed co-localization of CFP-RyR1$_{1:4300}$ with any of the Ca$_V$ constructs. We are currently testing whether the presence of additional, junctional proteins will result in co-localization of RyR1$_{1:4300}$ and Ca$_V$. Supported by grants NIH AR055104 and MDA 277475 to K.G.B.

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Multiple Regions Inhibit Expression of Ca$_v$1.1 Ca^{2+} Channels in Non-Muscle Cells

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Compared to the cardiac Ca$_V$1.2, the skeletal Ca$_V$1.1 Ca^{2+} channel has been very difficult to express in non-muscle cells. To identify domains of Ca$_V$1.1 that inhibit such expression, we have co-transfected tsA201 cells with β_{1a} and chimeric constructs of Ca$_V$1.1 and Ca$_V$1.2 tagged with YFP. A tandem construct of Ca$_V$1.1 linked to Ca$_V$1.2 expressed robustly in tsA201 cells, suggesting that either Ca$_V$1.2 masks a sequence of Ca$_V$1.1 which causes ER retention in non-muscle cells, or that Ca$_V$1.1 lacks a signal important for ER export. Replacement of individual transmembrane repeats of Ca$_V$1.1 (in non-tandem constructs) with the corresponding repeat of Ca$_V$1.2 was ineffective at increasing functional expression as indicated by slowly activating, L-type Ca^{2+} current and/or membrane-bound charge movement. However, simultaneous replacement of all four Ca$_V$1.1 repeats with those of Ca$_V$1.2, while retaining Ca$_V$1.1 sequence for the cytoplasmic domains (construct ‘‘CSk9’’), resulted in increased expression, although not to the level of Ca$_V$1.2. By contrast, expression levels of CSk9 and Ca$_V$1.2 were equivalent in dysgenic myotubes. Thus, both the transmembrane and cytoplasmic domains appear to play a role in suppressing expression of Ca$_V$1.1 in non-muscle cells. To investigate which cytoplasmic domains might be particularly important at suppressing expression, we tested Ca$_V$1.2 constructs in which the I-II loop, II-III loop, I-II and II-III loops, or C-terminus were replaced with Ca$_V$1.1 sequence and found expression not to differ for any of these constructs from that of the entirely Ca$_V$1.2 construct. We are currently testing constructs of Ca$_V$1.2 with other combinations of cytoplasmic domains replaced by Ca$_V$1.1.

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Residues Critical for Voltage-Sensor Transitions Determining Gating Properties of Cav1.1

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Following membrane depolarization S4 segments of voltage-gated cation channels translocate through the membrane resulting in the opening of the channel pore. This mechanism has been extensively studied in potassium and sodium channels yet little is known about it in voltage-gated calcium channels. Here we used de-novo ROSETTA modelling and site-directed mutagenesis to study the transition of the IVth voltage-sensing-domain (VSD) of Cav1.1 calcium channel during gating. Previously we have shown that the embryonic Cav1.1e splice variant, lacking exon 29 in the IVS3-S4 linker, has an 8-fold higher current amplitude and 30mV left-shifted voltage-sensitivity compared to the adult Cav1.1a splice variant. Modeling 4 consecutive states of the VSD suggested that the number of H-bonds formed between the arginines of IVS4 and residues in IVS3 is higher in Cav1.1e than in Cav1.1a. Among them aspartate at position 1196 (D1196) shows the strongest difference between the two splice variants. Indeed, voltage clamp recordings show that neutralizing the negative charge in S3 (D1196N) has no effect on the voltage sensitivity of Cav1.1a but confers poor voltage sensitivity to Cav1.1e. Mutations of R1210, R1217, and R1220 of Cav1.1e are currently analyzed to identify the interaction partners of D1196 during gating. Furthermore, structure modeling revealed an extracellularly exposed hydrophobic cluster formed by two leucines in the middle of IVS3-S4 loop encoded by exon 29. Point mutations which eliminate this hydrophobic cluster partially recapitulate the effects of exon 29 deletion. Together these findings suggest a model according to which the extracellular loop S3-S4 controls the orientation of S3 and S4 relative to each other. This alters charge compensation of positive charges in S4 and dramatically reduces the voltage sensitivity of CaV1.1a calcium channels. Support: FWF W1101, P23479, LFU-P7400-027-011.

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Differential Stability of Ca$_V\beta_{2a}$ and Ca$_V\beta_3$ in a Ca$_V$1.2 Calcium Channel Complex

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In voltage-gated calcium channels, auxiliary Ca$_V\beta$ subunits function in membrane targeting of the channel and modulation of its gating properties. Structural studies have revealed that the high affinity Ca$_V\alpha_1/\beta$ interaction relies on a conserved Ca$_V\beta$-binding sequence in the I-II loop of the Ca$_V\alpha_1$ subunit, called the α-interaction domain (AID). We have previously shown that in skeletal muscle triads the β_{1a} subunit forms stable complexes with both Ca$_V$1.1 and Ca$_V$1.2, while β_{2a} forms dynamic complexes (Campiglio et al., JCS 2013). To further test the reversibility of the Ca$_V$1.2/β_{2a} interaction, here we injected an AID peptide into *Xenopus* oocytes expressing Ca$_V$1.2/β_{2a}/$\alpha_2\delta$-1 complexes and examined the effect of the competing peptide on current inactivation. Unexpectedly, the AID peptide had no effect on the currents of wildtype Ca$_V$1.2/β_{2a}/$\alpha_2\delta$-1 channel complexes. However, when a Ca$_V$1.2 AID residue known to decrease the interaction with β_{2a} by 1000-fold was mutated (Y437A), injection of AID peptide changed the inactivation properties of the channels similar to properties of channels expressed without a β subunit. These results suggest that under our experimental conditions binding of the wild-type Ca$_V$1.2/β_{2a} pair is too strong for peptide competition. In contrast, in wild-type Ca$_V$1.2 channel complexes containing the β_3 subunit injection of the AID peptide caused a strong effect on inactivation properties, indicating efficient competition. Consistent with a lower stability of Ca$_V$1.2/β_3 complexes compared to Ca$_V$1.2/β_{2a}, β_3 also showed a significantly reduced extent of co-clustering with Ca$_V$1.2 in the triads of dysgenic myotubes. Ongoing FRAP experiments will reveal whether diminished co-clustering of β_3 correlates with higher mobility and dynamic β subunit exchange in a native calcium channel signaling complex. Funding: FWF P23479 and W1101 (BEF), NIH R01 HL080050 (DLM).

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The Amino-Termini of RGK Proteins Dictate the mode of L-Type Ca^{2+} Channel Inhibition in Adult Skeletal Muscle

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Work in heterologous systems has revealed that RGK (Rad, Rem, Rem2, Gem/Kir) proteins inhibit L-type Ca^{2+} channels via three mechanisms: 1) reduction of membrane expression, 2) immobilization of the voltage-sensors, and 3) reduction of P_o without impaired voltage-sensor movement. However, the question of which of these modes is (are) critical for inhibition of L-type channels in their native environments persists. To address this conundrum in skeletal muscle, we overexpressed Rad and Rem in mouse *flexor digitorum brevis* (FDB) fibers via *in vivo* electroporation and examined the abilities of these two RGK isoforms to modulate the L- channel (Ca$_V$1.1). We found that Rad and Rem both potently inhibit excitation-contraction coupling and L-type current in FDB fibers. Charge movement was reduced (~35%) in fibers overexpressing Rad, but charge movement in Rem-expressing fibers was identical to charge movement observed in control fibers. This unexpected result indicated that Rem supports inhibition *solely* through a mechanism which allows for translocation of Ca$_V$1.1’s voltage-sensors whereas Rad utilizes at least one mode that limits voltage-sensor movement. Since Rad and Rem differ significantly only in their amino-termini, we constructed chimeras to probe the basis for the distinct specificities of Rad- and Rem-mediated inhibition. A chimera composed of the Rem amino-terminus and the Rad core/carboxyl-terminus inhibited L-current without reducing charge movement. Conversely, a chimera having the Rad amino-terminus

fused to Rem core/carboxyl-terminus inhibited L-current with a concurrent reduction in charge movement. Thus, our work demonstrates that Rad and Rem exert their inhibitory effects on L-type channels in differentiated muscle fibers via distinct mechanisms. We have identified the amino-termini of Rad and Rem as the structural elements which dictate these specific modes of inhibition of $Ca_V1.1$. Supported by AG038778 (RAB) and 2T32AG000279-11 (Schwartz).

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Pegylated-Cholesterol Decreases the Amplitude and Augments Time- and Voltage-Dependent Inactivation of L-Type CA^{2+} Current of A7R5 Cells from Rat Aorta

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Cholesterol (Chol) localizes at high density in lipid rafts and caveolae in the membrane and regulates ion channel functions. We studied Chol-dependent regulation of L-type Ca^{2+} channel current ($I_{Ca,L}$) by applying water- and lipid-soluble pegylated Chol. A7r5 cells, a cell line from fetal rat aorta, were incubated with PEG600:Chol (PC) for hours in microtubes. PC dose-dependently (0.1 - 10 mM) decreased the density of $I_{Ca,L}$ recorded by whole-cell clamp method with Ba^{2+} as a charge carrier to 51%, while similar pretreatment by methyl-beta-cyclodextrin (MbCD) dose-dependently (1 - 30 mM) increased it by 61%. Voltage-dependence of the activation was little affected by them. PC accelerated the time course of the current decay: the ratio of the amplitude at 500 ms to the peak amplitude (I_{500}/I_{peak}) at 0 mV was decreased from 0.41 in control to 0.26 by 1 mM PC, while the pretreatment by MbCD increased it to 0.63. The PC-pretreatment shifted f_{inf}-V curve to the left, shifting $V_{0.5}$ from control −29.5 mV to -39.2 mV at 1 mM. In contrast, MbCD shifted $V_{0.5}$ to the right to −25.5 mV at 30 mM. The PC-induced decrease of $I_{Ca,L}$ density was reversed to an increase by the addition of 30 mM-MbCD to the tube containing 1 mM-PC. However, it only moderately reversed the PC-induced decrease of I_{500}/I_{peak} and hyperpolarizing shift of f_{inf}-V. By inhibiting amplitude and augmenting inactivation, PC strongly inhibited window $I_{Ca,L}$ that is mainly responsible for depolarization-induced contraction in the arterial smooth muscles. PC-induced increase of membrane stiffness due to the increase of the total content of Chol and/or direct interaction of PC with signaling lipids and proteins including $Ca_V1.2$ could explain these changes of $I_{Ca,L}$.

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AKAP79/150-Anchored CaN and PKA Regulate Neuronal L-Type Calcium Channel Activity and NFAT Transcriptional Signaling

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In neurons, Ca^{2+} influx through L-type voltage-gated Ca^{2+} channels (LTCC) couples electrical activity to changes in transcription. LTCC activity is elevated by the cAMP-dependent protein kinase (PKA) and depressed by the Ca^{2+}-dependent phosphatase calcineurin (CaN), with both enzymes localized to the channel by A-kinase anchoring protein (AKAP) 79/150. AKAP79/150 anchoring of CaN also promotes LTCC activation of transcription through dephosphorylation of the nuclear factor of activated T-cells (NFAT). We report here that genetic disruption of PKA anchoring to AKAP79/150 also interferes with LTCC activation of CaN-NFAT signaling in neurons. Disruption of AKAP-PKA anchoring promoted redistribution of the kinase out of dendritic spines, profound decreases in LTCC phosphorylation and Ca^{2+} influx, and impaired NFAT movement to the nucleus and activation of transcription. Our findings support a model wherein basal activity of AKAP79/150-anchored PKA opposes CaN to preserve LTCC phosphorylation, thereby sustaining LTCC activation of CaN-NFAT signaling to the neuronal nucleus.

Voltage-gated K Channels I

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High Yield Expression of the Human *Ether-à-Go-Go* Related Gene (HERG) in *Saccharomyces Cerevisiae*

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The human *Ether-à-go-go* related gene (hERG) encodes a voltage-gated potassium channel and represents the molecular correlate of the IKr current, which is one of the potassium currents involved in the repolarizing of the cardiac action potential. Inhibition of hERG prolongs the QT interval in the electrocardiogram and enhances the risk for severe or even fatal arrhythmias. Detection of unintended interactions with hERG is therefore an important issue when compounds are approved for drug development. In the present study we have explored *Saccharomyces cerevisae* as a host for heterologous expression of the hERG channel. Yeast codon optimized hERG cDNA was used to generate expression plasmids producing the hERG channel C-terminally fused with either a His10 or a TEV-GFP-His8 tag. The latter was generated to ease quantification of the expression level, to allow live cell bioimaging, development of a purification protocol and assessment of the quality of the recombinant protein. Both gene fusions were expressed from a galactose inducible promoter located on a plasmid with a regulatable copy number in a yeast strain overexpressing the Gal4 transcriptional activator. 48 hours after induction recombinant hERG accumulated to approximately 1.6% of total membrane content when produced at 15°C in amino acid complemented media. A solubilization screen established Fos-Choline-12 as a superior detergent for hERG solubilization. Solubilization in Fos-Choline-12 supplemented with cholesterol hemisuccinate generated a monodisperse FSEC (fluorescent size-exclusion chromatography) profile and caused recombinant hERG to elute in its native tetrameric form. In-gel fluorescence of SDS-separated yeast membranes showed that recombinant hERG protein has the expected molecular weight. Complementation assays in *S. cerevisiae* revealed that the heterologuosly expressed hERG is able to rescue the high potassium requirement of a *trk1*Δ, *trk2*Δ yeast strain indicating that the recombinant hERG is functional.

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Expression and Purification of a Functional hERG Pore Domain for Biophysical and Electrophysiological Studies

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The long QT syndrome (LQTS) is a cardiac dysfunction that prolongs the heart repolarisation interval, leading to cardiac arrhythmia or failure. This pathology can be induced by off-target effects of drugs which block the human ether-a-go-go related gene (hERG) potassium channels located in the myocardium cell membranes. To reduce the risks of this acquired LQTS, regulatory authorities demand in vitro testing of all new drug entities for hERG-blocking potential. As the pore domain of the hERG channel is an important target of LQTS-prone drugs, the objective of our work was to express and purify this region (Asp540-Tyr673) in E. coli to allow biophysical and electrophysiological studies. The detergent sarkosyl was employed for the solubilisation and a His6 N-terminal tag was used to isolate the transmembrane pore domain of hERG with yields of approximately 0.5-1 mg per liter of LB media. Mass spectrometry and Western blot confirmed the identity of the protein and circular dichroism showed that the majority of the hERG pore domain segments adopt an α-helix structure as was expected from sequence homology with other K^+ channels. The functionality of the channel is proven by incorporating it into lipid bilayers formed using Montal Mueller method.

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Eag Domains Regulate LQT Mutant hERG Channels in Induced Pluripotent Stem Cell-Derived Cardiomyocytes

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The generation of human induced pluripotent stem cells (hiPSCs) by controlled delivery of reprogramming factors provides a novel path for a wide range of disease research, including type 2 long QT syndrome (LQT2). The human ether-a-go-go related gene (hERG) encodes the α-subunit of a voltage-gated potassium channel underlying IKr. hERG potassium channels contain nearly 300 different disease-causing mutations, which can lead to LQT2. Its N-terminal region contains an eag domain, which is important for modulating channel deactivation properties. R56Q is a LQT2-associated point mutation located in the eag domain, which is a defect known to increase the rate of deactivation profoundly. We previously showed that isolated eag (i-eag) domains rescued the dysfunction of hERG R56Q channels by replacing the covalently attached, but defective eag domains when the channels were expressed in Xenopus oocytes or HEK 293 cells. Our goal was to determine whether the rescue of