change in the pore size due to the intrinsic heterogeneity in the fluorescently labeled MscL pentamers where various stoichiometry of the donors and acceptors are present. Further, we have found that stacking the channel to a surface alters the function of them. To get around these two problems, we performed single-molecule FRET experiments using an Alternating Laser Excitation (ALEX) apparatus. By using alternating lasers of wavelengths 488 nm and 561 nm to excite directly the donors (Alexa488) and acceptors (Alexa568) present in single diffusing MscL molecules which are incorporated in 50 nm liposomes, both the distance-based FRET efficiency E and stoichiometry-based ratio S could be measured. Our single-molecule experiments show that the addition of an asymmetric lipid (LPC) to the liposomes opens the channels, consistent with the results from ensemble measurements.

Voltage-gated Ca Channels

**623-Pos Board B409**

A Short Basic Segment within a Non-Conserved Region of the Beta-Subunit Modulates the Rate of Inactivation of the R-Type Calcium Channel

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Besides opening and closing, high-voltage-activated (HVA) calcium Channels transit to an inactivated state from which they do not re-open unless the plasma membrane is repolarized. This process is critical for the temporal regulation of intracellular calcium signaling. CaV2.3, a particular class of HVA channels supporting R-type calcium currents, inactivates fully in few hundred milliseconds when expressed alone but when co-expressed with the palmitoylated form of the regulatory β-subunit (Cavβ2), this process is slowed several fold and is incomplete. The widely accepted view is that membrane-anchoring of the Cavβ2a immobilizes the channel inactivation machinery. Some evidence suggests that an additional structural determinant may be coded in the linker peptide that joins the two highly conserved domains composing Cavβ but whether these determinants target the subunit to the membrane has not been shown. Here we indentify a short positively charged segment lying at the boundary of the guanylate kinase domain of Cavβ2a that slows down channel inactivation without relocating the protein to the plasma membrane. Deletion analysis demonstrates that while neither the N-terminal nor the C-terminal variable region of the protein affects the regulatory effect of the basic segment the presence of the remaining residues within the linker does. These results demonstrate that membrane anchoring is not the only factor modulating inactivation rate Cav2.3 calcium channels and suggest the presence of intralinker interactions or posttranslational modifications that counteract the effect of this segment.

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The β1B Subunit Regulates the Activity of LVA Calcium Channels: Evidences for a Physical Interaction

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Voltage-gated Ca2+ (CaV) channels are classified as Low Voltage- (LVA; or CaV3) and High Voltage- (HVA) Activated channels. Both are formed by a main z1 subunit, which forms the conduction pore and senses the changes in membrane potential. It consists of four domains (I-IV), which are similar to one another and contain six transmembrane segments (S1-S6). Auxiliary subunits (β, α2δ y γ) have been described only for HVA channels; they regulate the main subunit expression level, voltage dependence and current kinetics. The loop between domain I and II of HVA z1 subunits contains a conserved sequence known as AID, where z1 and β subunits interaction take place. Despite such domain is not present in LVA z1 subunits, it has been shown that cells expressing heterologously LVA calcium channels significantly increase its current density in the presence of β subunits. Recently it has been suggested that a weak interaction between peptides of the I-II loop of CaV3.3 channels and the core of β1 subunit. Nevertheless this interaction has not been shown at the whole protein level. The main goal of this work was to investigate whether β subunits modulate LVA Ca2+ channels and to determine if these two proteins interact physically. By using the whole cell patch clamp technique, we found that β1b subunit increases the current density (63, 72 and 45 %) of HEK-293 cells expressing CaV3.1, CaV3.2 and CaV3.3, respectively. Then, with fusion fluorescent proteins and confocal microscopy, we detected changes in β1b cellular distribution in the presence of the CaV3.3 channel. Finally, by whole blot we identified the protein complex formed by CaV3.3 and β1b. Altogether, these data suggested that HVA β subunits modulate LVA Ca2+ channels probably by a physical interaction.

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Differential Requirement of GTP-Binding for Rem and Rad Inhibition of Cav1.2 Channels in Heart

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Ca2+ influx through L-type (Cav1.2) calcium channels in heart controls: action potential duration; muscle contraction; and gene expression. Rad/Rem/Rem2/Gem/Kir (RGK) GTPases are Ras-like monomeric G-proteins that bind auxiliary Cav channel beta subunits and potently inhibit Cav1-1/Ca2 channels. RGK proteins are expressed in heart, where their expression levels changes in disease. Whether GTP-binding is important for RGK-mediated inhibition of Ca2+ current (ICa,L) in heart is controversial. Here, we investigated whether GTP binding to Rem and Rad is important for their ability to inhibit Cav1,2 channels. Both wild-type (wt) Rem and Rad dramatically inhibited recombinant Cav1.2 channels reconstituted in HEK 293 cells. Putative GTP-binding-deficient mutants (RemT94N and RadS105N) similarly inhibited recombinant ICa,L. In heart cells (Department of Physiology expressing wt Rem or Rad (black lines) potently inhibited endogenous ICa,L (gray lines). Surprisingly, RemT94N (solid triangles) was functionally inert in heart, whereas RadS105N (solid squares) inhibited ICa,L to the same extent as wt Rad. The results contradict reports that RadS105N acts as a dominant negative in heart, and reveals a novel intrinsic difference between the two RGK proteins.

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Rare Missense Mutations in the Calcium Channel β2 Subunit Gene of Autistic Patients Reduce Time-Dependent Inactivation of Cav1.2

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Loss of function of voltage-dependent L-type calcium channels (VDCC) in patients with Timothy syndrome results in a multiorgan dysfunction including lethal arhythmias, immune deficiency, skeleton-dysplasia, syndactyly and autism. This single gene disorder serves as a model disease for autism spectrum disease (ASD), giving insights in a possible pathophysiology. A point mutation in the pore-forming calcium channel subunit Cav1.2 gene is involved in the development of the Timothy syndrome and leads to incomplete inactivation of the L-type calcium currents (Splawski et al., Cell 2004;119:19-31). Functionally similar biophysical effects can be induced by the influence of auxillary VDCC β subunits (Herzig et al., FASEB J.2007;21:1527-38; Jangsanthong et al., Pflugers Arch. 2010;459:399-411). Supported by findings in a meta-analysis of linkage data of ASD patients (Trikalinos et al., Mol Psychiatry. 2006;11:29-36), we are investigating a function-based candidate gene hypothesis linking the candidate gene Cav1.2 to the candidate gene hypothesis. By using the whole cell patch clamp technique, we found that β2 subunit mutations in ASD patients that result in a retardation of inactivation behavior, thus phenocopying the monogenic Timothy syndrome mutations of Cav1.2. β2 subunit mutations may influence neuronal function or development in some ASD patients.

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