3523-Pos

Interplay between Calmodulin and the Ca^{2+} Channel Beta Subunit in Ca^{2+}-Dependent Inactivation of the L-Type Channel $Ca_v1.2$

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$Ca_v1.2$ is the most prominent L-type voltage dependent Ca^{2+} channel in heart and brain and has physiological importance in the cardiovascular and central nervous system due to its direct relation to cardiac muscle contractility, learning and memory. $Ca_v\beta$ subunits supports voltage gating of $Ca_v1.2$ calcium channel. Chronically increased $Ca_v1.2$ activity contributes to the etiology of heart disease and Alzheimer's disease. Ca^{2+} influx is tightly regulated through negative feedback via voltage-dependent inactivation (VDI) and Ca^{2+}-dependent inactivation (CDI) of $Ca_v1.2$. Our long term interest is to determine the molecular mechanisms that control VDI and CDI. At present we seek to understand the molecular details of how β2 subunit and the ubiquitous Ca^{2+}-binding protein calmodulin, regulate the function of voltage-gated Ca^{2+} channels. In the present study, we employed biochemical and fluorescence anisotropy techniques to identify the exact binding site for β2 subunit on the α1 subunit $Ca_v1.2$. Our findings indicate that the residues 1588-1675 of α1 subunit serves as a common binding region for β2 subunit and Calmodulin. Furthermore, we investigated the affinities of β2 subunit for different peptides within 1534-1697 fragments of $Ca_v1.2$ and observed an affinity in the nM range with peptides 1614-1635 and 1644-1670. This work may help us to form the basis for future work on the dysregulation of Cav1.2 under pathological conditions such as heart failure and ultimately also Alzheimer's disease.

3524-Pos

Role of AKAP150 in the Regulation of the Synaptic L-Type Chalcium Channel Cav1.2

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The A-kinase anchor protein 150 (AKAP150) is a major scaffolding protein for PKA localization in brain. It plays a critical role for PKA-mediated $Ca_v1.2$ regulation. The L-type calcium channel $Ca_v1.2$ contributes to Ca^{2+} influx into cardiac and smooth muscles, neurons, and endocrine cells. In brain, $Ca_v1.2$ is majorly localized in postsynaptic sites and important for synaptic function. Protein kinase A (PKA) upregulates $Ca_v1.2$ activity in response to adrenergic stimulation. The primary phosphorylation site by PKA is located on serine 1928 of the central, pore-forming $\alpha_1$1.2 subunit. We have shown previously that $Ca_v1.2$ forms a complex with the β_2-adrenergic receptor and PKA. We also found that AKAP150 associates with β_2AR in vivo and in vitro. To investigate the significance of AKAP 150 anchored PKA activity on $Ca_v1.2$ regulation, we analyzed the functional and physical association of β_2AR and $Ca_v1.2$ among wild type, AKAP150 KO, and AKAP D36 (lacking the PKA biding site) mice.

3525-Pos

The Second Loop of Orai Channels Fine-Tunes Ca^{2+} Feedback Regulation

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Interaction of STIM1, targeted to the endoplasmic reticulum and the Orai1 channel located in the plasma-membrane induce endogenous Ca^{2+} release activated Ca^{2+} (CRAC) currents. A key regulator and hallmark of CRAC currents is the Ca^{2+} dependent inactivation (CDI). Within the Orai family, Orai3 exhibits a striking CDI in comparison to Orai1. An attribute of Orai1 is its slower phase of reactivation. Several domains within the N-and C-terminus of Orai, Stim1 and the Ca^{2+} binding protein Calmodulin regulate CDI. Here we show, that a chimera of Orai3 with the second loop of Orai1 decreased CDI in comparison to wild-type Orai3 without inducing a reactivation phase. Among the Orai proteins, this 40 amino acid long stretch differs in a set of non-conserved residues. Side-directed mutagenesis in both Orai1 and Orai3 altered both CDI and reactivation, suggesting that several amino-acids within the loop contribute to the divergent feedback characteristics. These results suggest that the second loop of Orai channels is an essential site to fine-tune CDI in combination with further domains and proteins. Supported by FWF P21118, Hertha Firnberg grant T442 and DOCfFORTE of ÖAW.

3526-Pos

Positively Charged as Well as Hydrophobic Amino Acids in Orais' Conserved N-Terminal Domain Contribute to Orai Function

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The two proteins STIM1 and Orai1 represent the key components fully reconstituting the Ca2+ release-activated Ca2+ (CRAC) current. While the C-termini of both proteins play an essential role in their store-operated coupling, the role of Orai1 N-terminus in the STIM1/Orai1 signaling machinery remains so far elusive. Orai1 N-terminus contains proline- and arginine-rich regions at the very beginning which are lacking in Orai2 and Orai3. At the end of the N-terminal cytoplasmic stretch close to the first transmembrane segment all Orai proteins include a highly conserved amino acid domain that contains positively charged as well as hydrophobic amino acids. This stretch has also been suggested to interact with STIM1.

In attempt to elucidate the role of these conserved amino acids we mutated positively charged to either neutral or negatively charged amino acids. Single neutral mutations had no effect on store-operated currents, however, substantially reduced fast inactivation of Orai1 and Orai3, while double mutations strongly reduced current densities. Single negatively charged amino acids increased store-operated currents in correlation with abolished fast inactivation, while double negative mutations also decreased current density. Furthermore mutation of some hydrophobic amino acids within this conserved region resulted in 1.5-fold increased store-operated currents, while inactivation remained unaffected. In summary, we identified respective amino acids within Orais'conserved N-terminal region that play a modulatory role in Orai function. (supported by the Habilitation Scholarship JKU Linz, Austria and FWF P21118)

3527-Pos

Regulatory Role of N-terminal Orai Domains in Current Activation

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STIM1 and Orai1 represent the key components of the CRAC (Ca2+ release-activated Ca2+) channel signalling machinery. The Orai family comprises three members designated Orai1, Orai2 and Orai3. While the C-termini of all three Orai proteins are involved in the coupling of STIM1 to Orai, the exact role of their N-termini in the communication of STIM1 and Orai is still unclear. All Orai N-termini display a highly conserved cluster of positively charged and hydrophobic amino acids located close to the first transmembrane region. In contrast, only the Orai1 N-terminus but not that of Orai2 and Orai3 contains an arginine- and proline-rich region.

In this study we focused on the role of the N-termini of Orai 1/3 proteins for current activation. It has been shown that an Orai1 N-terminal deletion mutant lacking the first 73 amino acids is still sufficient for current activation. Accordingly, an Orai1 N-terminal deletion mutant Δ1-47 lacking the arginine- and proline-rich region displayed a similar STIM1-dependent store-operated activation but showed an altered reactivation profile compared to wild-type Orai1. Further, Orai1 and Orai3 mutants with corresponding N-terminal deletions exhibited distinct STIM1-dependent activation in response to store-depletion. Moreover, activation of Orai3 via STIM1 or 2-APB apparently involved separate N-terminal regions.

In summary, conserved regions within the N-termini of Orai 1/3 proteins play a distinct role in STIM1-mediated channel activation, and separate domains contribute to STIM1 or 2-APB induced Orai3 activation. (supported by a ÖAW scholarship and FWF P21118)

3528-Pos

Conformational Rearrangement within STIM1 C-terminus Crucial for Coupling to Orai1

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Ca^{2+} influx in non-excitable cells is mainly carried by store-operated channels (SOCs), where Orai1 (CRACM1) and STIM1 represent the two molecular key players in this process. STIM1 functions as an endoplasmic reticulum located Ca^{2+} sensor and transmits the signal of store depletion to the plasma membrane by coupling to Orai1, which in turn causes channel activation. STIM1 C-terminus itself can act as a surrogate of full length STIM1 and is sufficient for the activation of Orai1 currents. 2-aminoethoxydiphenylborate (2-APB) has been shown to induce enhanced association of STIM1 C-terminus with Orai1 suggesting a conformational change within the former that drives this association. Indeed we were able to monitor 2-APB induced STIM1 C-terminal conformational rearrangement by fluorescence microscopy. In the absence of 2-APB the change in conformation was only seen for STIM1 C-terminus coupled to Orai1 but not for that part remaining in the cytosol suggesting this conformational change crucial for Orai1 binding. Moreover, we were able to mimic this conformational rearrangement by introducing selective point mutations into

STIM1 C-terminus, which in line substantially increased binding to Orai1. In aggregate, our data support the theory of flexible regions within STIM C-terminus that undergo conformational rearrangement upon coupling to Orai1. (Supported by FWF-P21118)

3529-Pos

Cch1 Restores Intracellular Calcium in Fungal Cells during ER Stress

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Pathogens endure and proliferate during infection by exquisitely coping with the many stresses imposed by the host as a means to prevent pathogen survival. Recent evidence has shown that fungal pathogens and yeast respond to insults to the ER (endoplasmic reticulum) by initiating Ca^{2+} influx across their plasma membrane. Although the high-affinity Ca^{2+} channel, Cch1 and its subunit Mid1, have been suggested as the protein complex responsible for mediating Ca^{2+} influx, a direct demonstration of the gating mechanism of the Cch1 channel remains elusive. In this first mechanistic study of Cch1 channel activity we show that the Cch1 channel from the model human fungal pathogen, *Cryptococcus neoformans*, is directly activated by the depletion of intracellular Ca^{2+} stores. Electrophysiological analysis revealed that agents that enable ER Ca^{2+} store depletion promote the development of whole-cell inward Ca^{2+} currents through Cch1 that are effectively blocked by La^{3+} and dependent on the presence of Mid1. Cch1 is permeable to both Ca^{2+} and Ba^{2+} however, unexpectedly, in contrast to Ca^{2+} currents, Ba^{2+} currents are steeply voltage-dependent. Cch1 maintains a strong Ca^{2+} selectivity even in the presence of high concentrations of monovalent ions. Single channel analysis indicated that Cch1 channel conductance is small, similar to that reported for the Ca^{2+} current I_{CRAC}. This study demonstrates that Cch1 functions as a store-operated Ca^{2+}-selective channel that is gated by intracellular Ca^{2+} depletion. In ER stress conditions, Cch1 is poised to restore Ca^{2+} homeostasis and consequently fungal pathogens like *C. neoformans* require Cch1 activity for survival and colonization of the host.

3530-Pos

SERCA and IP_3R Expression and Function in Vascular Smooth Muscle is Altered Throughout Atherosclerotic Progression

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Peroxynitrite, the reaction product of superoxide and nitric oxide, forms in diseased vessels and has been shown to induce relaxation in vascular smooth muscle (SM). Here we demonstrate that relaxation of isolated aorta to peroxynitrite (30μM) is altered between healthy C57/BL-6 [25.2+/-6% (*n=10*)] and atherosclerotic $ApoE^{-/-}$ mice [59.3% and 22.4% after 2 (*n=13,p<0.05*) and 4 months (*n=13*) high fat diet (hfd)]. Inhibition of peroxynitrite relaxation by 3μM SERCA inhibitor thapsigargin (TG) or 60μM IP_3 receptor blocker 2-aminoethoxydiphenyl borate (2-APB) is also changed in atherosclerotic vessels [% reduction TG:30.1, 51.7 and 31.2 in C57 (*n=7*), 2mo (*n=13,p=0.05*) and 4mo (*n=11*)hfd $ApoE^{-/-}$; % reduction 2-APB:7.2, 60.3 and 26.7 in C57 (*n=9*), 2mo (*n=13,p<0.05*) and 4mo (*n=13*) hfd $ApoE^{-/-}$], potentially indicating altered SERCA and IP_3R Ca^{2+} handling mechanisms. Aorta expression levels of SERCA2b and IP_3R1 were found to be significantly down-regulated in atherosclerotic mice [56% SERCA (*n=8,p<0.05*) and 39% IP_3R (*n=9,p<0.05*)] confirming changes at the protein level. Further characterisation of functional changes was performed by estimation of SERCA activity. This was done by measuring calcium levels and rise rates following the addition of 10mM caffeine +/- 1μM TG. Increases in cytosolic calcium were found to be larger in $ApoE^{-/-}$ [ΔF/F0 caffeine:1.88+/-0.24, 1.96+/-0.22 and 2.62+/-0.24 in C57 (*n=6*), 2mo (*n=4*) and 4mo (*n=3,p<0.05)* hfd $ApoE^{-/-}$; ΔF/F0 TG:1.05+/-0.2, 1.01+/- 0.08, 1.41+/-0.2 in C57 (*n=6*), 2mo (*n=4*) and 4mo (*n=3*) hfd $ApoE^{-/-}$]. Calcium rise rates were also calculated [ΔF/ms:4.28· 2.95 and 5.82 x10^{-5} in C57 (*n=4*), 2mo (n=4) and4mo hfd $ApoE^{-/-}$ (*n=3*)] and indicate altered SERCA activity in atherosclerotic vessels. These data suggest that the changes observed in SM Ca^{2+} handling in atherosclerosis may be largely due to modulation of SERCA and IP_3R expression and function.

3531-Pos

Extracellular Ca2+ and Glutamate Modulating the Function of Metabotropic Glutamate Receptor 1 Alpha (mGluR1α)

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Metabotropic Glutamate Receptor subtype 1 alpha (mGluR1α) mediates the accumulation of IP3 and DAG, release of intracellular Ca^{2+} from ER, and activation of PKC and PKA, by interacting with extracellular glutamate and Ca^{2+}. While more than 12 X-ray Structures of different forms of the extracellular domain (ECD) has been determined, the detailed binding locations of Ca^{2+} still remain unidentified. In this study, we first report our prediction of several Ca^{2+} binding sites in the ECD of mGluR1a using our newly developed computational algorithms. Putative residues involved in the calcium binding were verified using a grafting approach. Their capability to bind Ca^{2+} and its trivalent analog, Tb^{3+} were determined using fluorescence energy transfer and NMR. The substitution of charged or polar ligand binding residues with Ile at Site 1 resulted in up to 99-fold decrease in the Tb^{3+} binding affinity. In transiently-transfected HEK cells, mutations on these proposed ligand binding residues in the predicted calcium binding site either resulted in increase or decrease of intracellular Ca^{2+} response toward changes in $[Ca^{2+}]_o$. When treated mGluR1α with glutamate and Ca^{2+} together, the intracellular Ca^{2+} response is significantly greater than individual effects by glutamate or Ca^{2+}, suggesting a cooperative effects by calcium binding and glutamate.

3532-Pos

Expression and Regulation of Shark NCX Gene in Transgenic Mouse Heart

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Inward currents generated by the mammalian cardiac Na^+-Ca^{2+}-exchanger (NCX1.1) have arrhythmogenic potential, especially in the failing heart where expression of NCX is up-regulated. We hypothesize that arrhythmogenesis might be alleviated if NCX were subject to the same cAMP mediated regulation (suppression of Ca^{2+}-influx, but enhancement of Ca^{2+}-efflux on NCX) as found in the native shark ventricle. To test this hypothesis, we created heterozygous transgenic mice that express the shark NCX protein with a myc-tag, under the control of the alpha-myosin heavy chain promoter (α-MHC). The construct was evaluated by expression and functionality prior to production of transgenic lines. The expression of the transgene was confirmed by immunocytochemistry staining using DAPI and myc-FITC antibody on transfected HL-1 cells. Using dual laser confocal microscopy, the pattern of staining was consistent with NCX expression at the protein level. To determine the functionality of the transgene, HL-1 cells were co-transfected with the shark NCX transgene and GFP. GFP positive cells were incubated with Fluo-4 AM and imaged confocally. These cells showed NCX activity in response to withdrawal and readmission of $[Na^+]_o$ consistent with electrophysiological data of native shark myocytes.
Echocardiography and ECG studies on transgenic mice showed no remarkable cardiac phenotype, but analysis of initial voltage-clamp and western blot studies verify robust exchanger currents and expression levels. Our findings show that shark NCX is functional in the transgenic mouse producing no discernable cardiac pathology, but it remains to be determined as yet whether shark NCX can confer anti-arrhythmic properties to the mammalian heart.

Exocytosis & Endocytosis II

3533-Pos

Chemomechanical Regulation of Snare Proteins Studied with Molecular Dynamics Simulations

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SNAP-25B is a neuronal protein required for neurotransmitter (NT) release and is the target of Botulinum Toxins A and E. It has two SNARE motifs that form a four helix bundle when combined with syntaxin1A and synaptobrevin. Formation of the three protein complex requires both SNARE motifs of SNAP-25B to align, stretching out a central linker. The N-terminal of the linker has four cysteines within eight amino acids. Palmitoylation of these cysteines are thought to target SNAP-25B to the membrane, however these cysteines are also an obvious target for oxidation, which has been shown to decrease SNARE complex formation and NT secretion. We hypothesize that since the linker is not much longer than the SNARE complex, formation of a disulfide bond between two cysteines could shorten it sufficiently to reduce secretion by limiting complex formation.
Molecular Dynamics simulations of the SNARE complex, including a modeled linker in the oxidized and reduced state, respectively, reveal drastic conformational differences and a reduction of helical content in SNAP-25B upon oxidation. Further, the conformations of three hydrophobic layers, crucial for the helix association are significantly different. We therefore suggest that oxidation of the cysteines leads to a dysfunctional SNARE complex, thus down-regulating NT release during oxidative stress.