The time- and calcium-dependent association of recombinant human copine I or annexin A1 with supported lipid bilayers composed of 25% brain PS and 75% DOPC was monitored by atomic force microscopy. Neither protein bound to featureless areas of the bilayer but both rapidly bound to small domains that appeared to be 0.5 to 0.8 nm lower than the rest of the bilayer. These domains may be enriched in PS and/or have a more disordered lipid structure. Copine I assembled into a reticular pattern made of 40nm linear elements that appeared to be one or two molecules high. In vivo such copine arrays might form a scaffold for the assembly of signalling proteins bound by copine I. Annexin A1 did not form ordered structures but appeared to promote the growth of the domains of lowered height to which it was bound. These enlarged domains created by annexin A1 provided binding sites for copine I when it was added subsequently. Therefore, in vivo, annexin A1 might recruit C2 domain-containing proteins like copine to membranes by modulating membrane structure.

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Depletion Of Intracellular Cholesterol Disrupts Carbachol But Not PTH-mediated Ca^{2+} Signals In HEK293 Cells

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In HEK cells stably expressing receptors for type 1 parathyroid hormone (PTH), PTH increases the sensitivity of IP_3 receptors (IP_3R) to IP_3 via a cAMP-dependent mechanism, thereby potentiating Ca^{2+} signals evoked by muscarinic M_3 receptors that stimulate IP_3 formation. The effect of PTH results from cAMP binding directly to a low-affinity site on either the IP_3R itself or a protein tightly associated with it. cAMP appears to pass directly from AC to IP_3R via an association we have termed an AC-IP_3R junction, formed selectively by AC6 and IP_3R2. Here we show disruption of cholesterol-rich lipid microdomains differentially disrupts M_3R signaling in HEK cells.

In the absence of extracellular Ca^{2+}, stimulation of HEK cells with a maximal concentration of carbachol (CCh, 1mM) caused an increase in $[Ca^{2+}]_i$ of 249 ± 33nM which returned to basal within 60–70s. Subsequent addition of PTH, in the continued presence of CCh, evoked further concentration-dependent (EC_{50} = 59 ± 15nM) increases in $[Ca^{2+}]_i$. Treatment with the cholesterol-depleting agent MβCD (2h; 22°C) caused an 86 ± 3% decrease in the response to CCh whilst having no significant effect on the response to PTH. Single-cell imaging revealed that treatment with MβCD caused a 30% decrease in the number of cells responding to CCh and a 45% decrease in amplitude of the Ca^{2+} signal in cells that did respond. Filipin staining of free cholesterol confirmed that MβCD caused depletion of cellular cholesterol.

Depletion of intracellular cholesterol with MβCD disrupts CCh but not PTH signalling in HEK293 cells. We hypothesise this may be due to the existence of either two different M_3R populations in the PM or differential distributions of IP_3R isoforms in the ER.

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Increased Store-Operated Ca^{2+} Entry in Skeletal Muscle with Knockdown of Calsequestrin

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Malignant hyperthermia (MH) is a life-threatening syndrome triggered by volatile anesthetics, in which uncontrolled elevation of myoplasmic Ca^{2+} leads to hypercontracture of skeletal muscle and elevation of body temperature. Our recent study showed that azumolene, an analog of dantrolene used to treat MH, inhibits a component of store-operated Ca^{2+} entry (SOCE) coupled to activation of the ryanodine receptor in skeletal muscle (*JBC* **281**: 33477, 2006). Given our previous observation that overexpression of calsequestrin-1 (CSQ1) suppressed SOCE in skeletal muscle (*JBC* **278**: 3286, 2003), here we tested the hypothesis that reduced CSQ1 expression would enhance an azumolene-sensitive SOCE in this tissue. A shRNA probe specific for CSQ1 (*JBC* **281**: 15772, 2006) was introduced into flexor digitorum brevis (FDB) muscles of living mice using electroporation. Individual transfected FDB muscle fibers labeled with a fluorescent marker were isolated for SOCE measurements using Mn-quenching of Fura-2 fluorescence. At room temperature (20–22°C), SOCE induced by caffeine/ryanodine was significantly enhanced in CSQ1-knockdown muscle fibers (in 10^{-4} ΔF_{360}/s, 9.36 ± 1.31) compared to those transfected with control (4.71 ± 1.29, $p<0.05$). Pre-incubation with azumolene (20 μM) completely inhibited the elevated SOCE detected in CSQ1-knockdown fibers (1.26 ± 0.38, $p<0.01$). To prevent muscle contraction, we used *N*-benzyl-*p*-toluene sulfonamide (BTS, 40 μM), a specific myosin II inhibitor. When temperature of the bathing solution was increased to 40°C, muscle fibers with knockdown of CSQ1 displayed a significant elevation in cytosolic Ca^{2+} over that seen in control fibers. Thus reduced CSQ1 expression is likely coupled to elevation of cytosolic Ca^{2+} due to increased SOCE function at higher temperatures. These results suggest that elevated SOCE activity in skeletal muscle may be linked to the pathophysiology of MH and the heat-sensitivity of MH-susceptible animals.

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Role of the Ryanodine Receptor/Calcium Release Channel in Beta-adrenergic Receptor Blocker Treatment of Heart Failure

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To explore the role of protein kinase A (PKA) phosphorylation of the cardiac ryanodine receptor (RyR2)/calcium release channel in the treatment of heart failure (HF) using beta-adrenergic receptor blockers (beta-blockers) we generated a knock-in mouse with aspartic acid replacing serine at residue 2808 in RyR2 (RyR2-S2808D). This mutation mimics constitutive PKA hyperphosphorylation of RyR2, a condition that occurs during HF. RyR2-S2808D+/+ mice developed an age-dependent cardiomyopathy characterized by moderate cardiac dysfunction and mild left ventricular dilatation indicating that PKA hyperphosphorylation of RyR2 alone can cause cardiac dysfunction. Following myocardial infarction (MI), RyR2-S2808D+/+ mice exhibited increased mortality compared to WT littermates. Treatment with the rycal S107, a 1,4-benzothiazepine derivative that inhibits PKA hyperphosphorylation-induced depletion of calstabin2 from the RyR2 complex, for 4 weeks significantly reduced HF progression in WT and RyR2-S2808D+/+ mice, confirming the important role of calstabin2 binding to RyR2 in preventing HF progression. In contrast, following MI, treatment with the beta-adrenergic receptor blocker (beta-blocker) metoprolol improved cardiac function in WT but not in RyR2-S2808D+/+ mice, indicating the important role of inhibition of PKA hyperphosphorylation of RyR2 as a key mechanism underlying the beneficial effects of beta-blockers in HF. Taken together, these data show that chronic RyR2 PKA hyperphosphorylation alone can cause a cardiomyopathy, preventing calstabin2 depletion from the RyR2 macromolecular complex can inhibit HF progression, and PKA phosphorylation of RyR2 is an important determinant of the therpaeutic efficacy of beta-blocker therapy of HF.

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Structural Basis for Calcium Sensing by GCaMP2

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Genetically encoded Ca2+ indicators are important tools that enable the measurement of Ca2+ dynamics in a physiologically relevant context. GCaMP2, one of the most robust indicators, is a circularly permutated EGFP (cpEGFP)/M13/Calmodulin (CaM) fusion protein, that has been successfully used for studying Ca2+ fluxes in vivo in the heart and vasculature of transgenic mice. Here we describe crystal structures of bright and dim states of GCaMP2 that reveal a sophisticated molecular mechanism for Ca2+ sensing. In the bright state, CaM stabilizes the fluorophore in an ionized state similar to that observed in EGFP. Mutational analysis confirmed critical interactions between the fluorophore and elements of the fused peptides. Solution scattering studies indicate that the Ca2+-free form of GCaMP2 is a compact, pre-docked state, suggesting a molecular basis for the relatively rapid signaling kinetics reported for this indicator. These studies provide a structural basis for the rational design of improved Ca2+-sensitive probes.

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Interference In Coiled-coil Mediated Coupling Between Stim1 And Orai Channels

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STIM1 and ORAI1, the two limiting components in the CRAC signalling cascade, have been reported to couple tightly upon store-depletion culminating in CRAC current activation. Based on the homology within the ORAI protein family, an analogous scenario might be assumed for ORAI2 as well as ORAI3 channels as both are activated in a similar store- and STIM1-dependent manner. A combined approach of electrophysiology and confocal Förster Resonance Energy Transfer (FRET) microscopy revealed a general mechanism in the communication of STIM1 with ORAI proteins that involved the predicted second coiled-coil motif in STIM1 C-terminus and the conserved putative coiled-coil domain in the respective ORAI C-terminus. Of the latter, a much higher coiled-coil probability is predicted for ORAI2 as well as ORAI3 than for ORAI1, compatible with our observation that a single point coiled-coil

mutation in ORAI1 C-terminus abrogated communication with STIM1 C-terminus, while an analogous mutation in ORAI2 and ORAI3 still allowed for their moderate activation. Conversely, destabilizing the second coiled-domain of STIM1 C-terminus by a single point mutation still enabled partial stimulation of ORAI2 and ORAI3 channels but not of ORAI1. A double mutation within the second coiled-coil motif of STIM1 C-terminus fully disrupted communication with all three ORAI channels. In aggregate, the impairment in the overall communication between STIM1 and ORAI channels upon mutual destabilization of putative coiled-coil domains in either C-terminus would be compatible with their heteromeric interaction.Supported by FWF P18169.

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An Orai1 Activating Minimal Fragment Of Stim1

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In immune cells generation of sustained Ca2+ levels is mediated by the Ca2+ release activated Ca2+ (CRAC) current. Molecular key players in this process comprise the stromal interaction molecule (STIM1) that functions as a Ca2+ sensor in the endoplasmic reticulum and ORAI1 located in the plasma membrane. Depletion of ER Ca2+ store leads to STIM1 multimerization into discrete punctae that co-cluster with ORAI1 thereby triggering coupling to and activation of ORAI1 channels. The C-terminus of STIM1 is sufficient for the activation of ORAI1 currents independent of store depletion. Here we unmasked an ORAI activating minimal fragment (OAMF) within STIM1 C-terminus that exhibited enhanced interaction with ORAI1 and resulted in threefold increased Ca2+ currents. STIM1-OAMF still showed the ability of a homomeric interaction similar to longer fragments as well as the full-length form of STIM1 C-terminus. In contrast, further deletion of a thirty amino acid region resulted in a substantial reduction of homomeric interaction concomitant to a loss of coupling to as well as activation of ORAI1. In aggregate, we have identified two key regions within STIM C-terminus that govern ORAI1 activation. (Supported by PhD-Program W1201 from the FWF)

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Increased Hydrophobicity At The N-terminus/membrane Interface Impairs Gating Of The Scid-related Orai1 Mutant

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Patients with severe combined immune deficiency (SCID) suffer from defective T cell Ca2+ signalling. At the molecular level a loss of Ca2+ entry has been linked to a single missense mutation R91W in the store-operated Ca2+ channel Orai1. Yet, the mechanistic impact of this mutation on Orai1 function remains unclear. Confocal FRET microscopy revealed that dynamic store-operated STIM1 coupling to Orai1 R91W was preserved similar to wild-type Orai1. Characterization of various point mutants at position 91 by whole-cell patch-clamp recordings revealed that neutral or even negatively charged amino acids did not impair Orai1 function. However, a substitution by hydrophobic leucine, valine or phenylalanine resulted in non-functional Orai1 channels. Bioinformatic analysis on secondary structure of the ASSR moiety (amino acid 88–91) that is located at the N-terminus/membrane interface suggested conformational constraints when R is substituted by these hydrophobic amino acids. Glycines substituting for the two serines in the ASSR moiety further promoted conformational flexibility and indeed increased channel activity. However, function of the Orai1 R91W mutant was not restored by these two additional glycine substitutions, pointing to a dominant role of tryptophan 91. Transmembrane probability plots revealed a substantial increase in probability for the first transmembrane segment in the case of all the hydrophobic, non-functional Orai1 R91X mutants in contrast to functional ones. We suggest that a substantial increase in the transmembrane probability of the first sequence of Orai proteins together with structural constraints at the N-terminus/membrane interface yields non-functional Orai1 channels. (supported by FWF 18169)

595-Pos Board B474

Structural dynamics of CaMKII activation

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The ubiquitously expressed calcium/calmodulin dependent protein kinase II (CaMKII) functions as a transducer of calcium (Ca^{2+}) signaling by responding to the amplitude, duration, and frequency of Ca^{2+} transients. During periods of elevated Ca^{2+}, CaMKII is activated by calcium-calmodulin (Ca^{2+}/CaM) binding. A subsequent autophosphorylation at Thr286 allows for Ca^{2+}-independent activity and endows this enzyme with a conformational memory of prior activation. CaMKII activity is regulated by a myriad of factors including CaM binding, autophosphorylation, and catalytic-regulatory domain interactions referred to as autoinhibition. While these variables have been linked to CaMKII function, the underlying structural and dynamic framework of activation and conformational memory is poorly understood. Here we utilize site-directed spin labeling and electron paramagnetic resonance (SDSL-EPR) to explore the conformational changes associated with CaMKII activation and conformational memory. EPR parameters were collected for the regulatory domain where CaM binding and autophosphorylation sites are located. Our results indicate the regulatory domain undergoes significant structural changes between several discrete conformations dependent on autophosphorylation and CaM binding. The CaM binding region is flexible in the apo state but has an induced rigidity in the presence of Ca^{2+}/CaM indicative of a binding event. Investigation of the regulatory domain outside the CaM binding region revealed an increase in protein backbone dynamics with a Thr286Glu autophosphorylation mimic and/or in the presence of Ca^{2+}/CaM. This data provides a structural and dynamic perspective consistent with the current biochemical activation model where CaM binding disrupts autoinhibition by disengaging regulatory and catalytic domains. We predict the enhanced flexibility facilitates Ca^{2+}/CaM binding and may play a role in Ca^{2+} independent activity. The adjacent regulatory loop showed similar flexibility suggesting this region functions as a hinge between regulatory and catalytic domains allowing for release and reinstatement of autoinhibition.

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Calcium binding and conformational properties of calmodulin complexed With PEP-19

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PEP-19 is an IQ calmodulin (CaM) binding motif that inhibits apoptosis and protects cells against Ca^{2+} toxicity. We showed that PEP-19 interacts predominantly with the C-domain of CaM, and that it greatly increases the k_{on} and k_{off} rates of Ca^{2+} binding, but has little effect on K_{Ca}. Here we used solution NMR to characterize the calcium binding and conformational properties of the Ca^{2+}-CaM-PEP-19 complex. Both $^3J_{HNHA}$ and ^1H, ^{15}N NOESY-HSQC experiments show the overall secondary structure of Ca^{2+}-CaM is not greatly affected upon binding PEP-19. ^{15}N backbone dynamics suggests that the Ca^{2+}-CaM-PEP-19 complex shows large-scale dynamics. Most residues in the C domain of CaM that experience significant chemical exchange on μs to ms timescale form a hydrophobic patch to interact with PEP-19.

We used a C-term fragment of CaM, CaM(76–148), which binds two Ca^{2+} ions, to determine the effect of PEP-19 on cooperative Ca^{2+} binding. Highly cooperative Ca^{2+} binding was seen in the absence of PEP-19, giving two sets of peaks in the ^1H-^{15}N HSQC spectra at substoichiometric levels of Ca^{2+}, corresponding to apo and 2-Ca^{2+} bound forms of CaM(76–148). However, in the presence of PEP-19, cooperativity was largely lost and most residues in CaM(76–148) showed line broadening, and spitting into multiple peaks at low Ca^{2+} levels. Amide markers in the Ca^{2+} binding loops showed sequential Ca^{2+}-binding first to site IV and then to site III. Furthermore, $^1H_\alpha$, $^{13}C_\alpha$ chemical shift perturbations indicate that the β-strand in Ca^{2+} binding loop III shifts toward the random coil direction in the presence of PEP-19. This indicates that loss of cooperativity and increased in k_{off} and k_{on} rates induced by PEP-19 is caused by destabilizing the antiparallel β-sheet formed between Ca^{2+} binding sites III and IV in the C-domain of CaM.

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Characterization of Calmodulin with Mutated Ca2+-Binding Sites

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Calmodulin (CaM) regulates cellular functions via its Ca2+ binding properties. The N- and C-domains of CaM, which are separated by a flexible tether, each bind two Ca2+ ions via EF-hand motifs. Mutation of position 1 in individual EF hands (the X coordination site) from Asp to Ala has been used to selectively inhibit Ca2+ binding to the N- and C-domains of CaM. We used this mutation strategy to investigate how the individual Ca2+ binding sites contribute to the association of PEP-19 with CaM. Four CaM mutants were made and designated CaM12, CaM3, CaM4 and CaM34 based on nomenclature established in the literature. Ideally, all mutant proteins should be structurally and functionally identical to native CaM in the absence of Ca2+, however, mutation of Ca2+ binding sites in the C-domain of CaM caused weak affinity and significantly different koff and kon rates for binding PEP-19. This led us to use NMR and