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

Martin Muik[1], Marc Fahrner[1], Isabella Derler[1], Rainer Schindl[1], Irene Frischauf[1], Judith Bergsmann[1], Reinhard Fritsch[1], Josef Madl[1], Klaus Groschner[2], Christoph Romanin[1].
[1]Institute for Biophysics, Linz, Austria, [2]Institute for Pharmacology and Toxicology, Graz, Austria.

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)

594-Pos Board B473

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

Isabella Derler[1], **Marc Fahrner**[1], Oliviero Carugo[2], Martin Muik[1], Judith Bergsmann[1], Rainer Schindl[1], Irene Frischauf[1], Said Eshaghi[3], Christoph Romanin[1].
[1]Institute for Biophysics, Linz, Austria, [2]Max Perutz Laboratories, Vienna, Austria, [3]Karolinska Institute, Stockholm, Sweden.

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

Laurel Hoffman, Hassane S. Mchaourab.
Vanderbilt University, Nashville, TN, USA.

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

Xu Wang, Quinn Kleerekoper, John Putkey.
Department of Biochemistry and Molecular Biology, University of Texas Medical School at Houston, Houston, TX, USA.

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.

597-Pos Board B476

Characterization of Calmodulin with Mutated Ca2+-Binding Sites

Liangwen Xiong, Quinn Kleerekoper, John Putkey.
The University of Texas, Houston, TX, USA.

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

other methods to determine the degree of structural perturbation caused by mutation of the EF-hands. Inhibition of Ca2+ binding to the N-domain in CaM12 does not affect Ca2+ binding to the C-domain, however, inhibition of Ca2+-binding to the C-domain in CaM3 and CaM34 significantly increases the Ca2+-binding affinity of the N-domain by decreasing the koff for Ca2+. This was associated with increased exposure of hydrophobic regions in the N-domain as detected by ANS fluorescence. Significantly, 1H-15N HSQC spectra collected in the absence of Ca2+ show large structural perturbations in the C-domain of CaM3, CaM4 and especially CaM34 relative to apo-CaM. This was observed as resonance broadening and a loss of dispersion. These data indicate that conversion of Asp 93 and 129 to Ala destabilizes the C-domain of apo-CaM.

598-Pos Board B477

Interactions of the Anti-Psychotic Drug Trifluoperazine with Calmodulin

Michael D. Feldkamp, Madeline A. Shea.
Univeristy of Iowa, Iowa City, IA, USA.

Calmodulin (CaM) is a Ca^{2+}-sensing protein essential to eukaryotic signal transduction pathways. It has two homologous domains (N and C), each binding two Ca^{2+} ions. The anti-psychotic drug trifluoperazine (TFP; Stelazine) is a CaM antagonist known to bind hydrophobic clefts of CaM that are exposed upon Ca^{2+} binding.

Equilibrium Ca^{2+} titrations monitored by changes in steady-state fluorescence of intrinsic Phe and Tyr residues were used to evaluate the effect of TFP on the Ca^{2+} affinity of full length CaM ($CaM_{1–148}$), N-domain ($CaM_{1–80}$) and C-domain ($CaM_{76–148}$) over a range of TFP:CaM ratios. Low levels of TFP (1:1, 2:1 ratios) decreased the Ca^{2+} affinity of CaM. TFP had the greatest effect on Ca^{2+} binding to sites III and IV, in the C-domain of $CaM_{1–148}$, but affected both domains. At an 8:1 ratio of TFP:CaM, the effect reversed and the Ca^{2+} affinity of CaM increased.

^{1}H-^{15}N-HSQC NMR showed that resonances assigned to apo and Ca^{2+}-saturated C-domain were the most perturbed during TFP titration, while a smaller subset of N-domain resonances were affected. The stoichiometry of TFP binding to apo-$CaM_{1–148}$ was determined to be 2:1, and 4:1 for $(Ca^{2+})_4$-CaM.

Crystallographic structures of TFP bound to $(Ca^{2+})_4$-$CaM_{1–148}$ indicate two possible orientations of TFP when bound in 1:1 vs 2:1 and 4:1 TFP:CaM ratios. A new structure of a $(Ca^{2+})_2$-$CaM_{76–148}$ -TFP complex showed the trifluoromethyl group of TFP in both positions seen previously; distinct conformation of Met 144 correlated with orientation of TFP. NMR of apo-$CaM_{76–148}$ will be used to determine whether apo CaM-TFP complex adopts the semi-open conformation of apo CaM bound to a myosin peptide.

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Integration of Extracellular and Intracellular Calcium Signals via Calcium-Sensing Receptor (CaSR)

Yun Huang[1], Yubin Zhou[1], Adriana Castiblanco[1], Hing-Cheung Wong[1], Yangyi Chen[1], Wei Yang[1], Edward M. Brown[2], Jenny J. Yang[1].
[1]Georgia State University, Atlanta, GA, USA, [2]Brigham and Women's Hospital, Boston, MA, USA.

Ca^{2+}, both as a first and a second messenger, is closely involved in the modulation and regulation of numerous important cellular events, such as cell proliferation, differentiation and cell death. Fine-tuned Ca^{2+} signaling is achieved by its reversible or irreversible binding to a repertoire of Ca^{2+} signaling molecules. Among them, the extracellular calcium sensing receptor (CaSR) senses Ca^{2+} concentration ($[Ca^{2+}]_o$) in the milieu outside of cells where Ca^{2+} serves as a first messenger. An array of naturally-occurring mutations in CaSR has been found in patients with inherited disorders of Ca^{2+} homeostasis, leading to abnormal intracellular responses toward $[Ca^{2+}]_o$.

In the present study, we have computationally predicted and experimentally characterized the metal-binding properties of five Ca^{2+}-binding pockets within the extracellular domain of CaSR. Two complementary methods of grafting approach and the subdomain approach were used to probe site specific and cooperative metal binding as well as metal induced conformational change. Based on our results, a model has been proposed to explain the distinct CaSR-mediated responses toward diseases related-abnormally ''high'' or ''low'' extracellular Ca^{2+} levels. We here further demonstrate that the cytosolic terminal is essential for proper intracellular Ca^{2+} response to external signals.

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Altered Calcium Handling Between Healthy And Atherosclerotic Vascular Smooth Muscle

Marie-Ann Ewart[1], Susan Currie[2], John G. McCarron[2], Simon Kennedy[1].
[1]University of Glasgow, Glasgow, United Kingdom, [2]University of Strathclyde, Glasgow, United Kingdom.

In order for smooth muscle (SM) contraction and relaxation to proceed efficiently, Ca^{2+} handling is under tight regulation. The cyclic strain associated with hypertension is thought to play an initiating role in atherosclerosis, suggesting dysregulation of SM Ca^{2+} handling may be a contributing factor. Peroxynitrite ($ONOO^-$), the reaction product of superoxide and nitric oxide, forms in diseased vessels and has been demonstrated to induce SM cell relaxation. In this study, we assessed function and expression levels of sarcoplasmic reticulum Ca^{2+} handling proteins; the inositol 1,4,5-trisphosphate receptor (IP_3R) and the Ca^{2+}ATPase (SERCA) in both healthy and atherosclerotic aorta. $ONOO^-$ dose-dependently relaxed U46619 pre-contracted aorta from both control and atherosclerotic $ApoE^{-/-}$ mice (2 months high fat diet) [51.2 ± 4.7% and 78.5 ± 4.3% maximal relaxation, respectively (3×10^{-5} $ONOO^-$)]. This relaxation was antagonised in both C57 and $ApoE^{-/-}$ by the addition of either 3μM thapsigargin (TG), a SERCA inhibitor, or 60μM 2-aminoethoxydiphenyl borate (2-APB), an IP3R blocker. In control aorta, relaxation was 4.3 ± 5% (TG), $p<0.001$; n=7 and 14.6 ± 6.2% (2-APB) $p=0.001$; n=9. In $ApoE^{-/-}$ aorta, % relaxation was 22.09 ± 3.1 (TG) $p<0.001$; n=8 and 7 ± 5.9% (2-APB) $p<0.001$; n=7. There was no significant difference between endothelial denuded or intact vessels. These data indicate an alteration in the effect of Ca^{2+} handling protein inhibitors between control and $ApoE^{-/-}$ mice. This has been further correlated to expression of both SERCA and IP_3R proteins. Studies with the potassium channel blocker tetraethylammonium (TEA) indicate plasma membrane hyperpolarisation is an effector of $ONOO^-$ induced relaxation [31.2% relaxation reduction with TEA in C57 aorta, $p=0.002$ vs 35.5% reduction in $ApoE^{-/-}$, $p=0.022$]. We provide additional evidence, through myography and biochemical analysis, of a time-dependent correlation between atherosclerotic development and SM Ca^{2+} handling machinery modulation.

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PEP-19 is an Intrinsically Disordered, Acidic/IQ Motif Regulator of Calmodulin Signaling

Quinn K. Kleerekoper, Liangwen Xiong, Xu Wang, **John A. Putkey**.
University of Texas Medical School, Houston, TX, USA.

PEP-19 is a small calmodulin (CaM) binding protein that inhibits apoptosis and protects cells against Ca^{2+}-toxicity. It binds to either apo or Ca^{2+}-CaM and greatly increases the k_{on} and k_{off} of Ca^{2+} binding but does not affect K_{Ca}. Here we investigate the molecular basis for modulation of Ca^{2+} binding to CaM by PEP-19. First, we identified an extended IQ motif that includes an N-terminal acidic sequence that is necessary for modulation of Ca^{2+} binding to CaM, and show the acidic/IQ motif is present in a variety of proteins from different species. Although PEP-19 binds to apo and Ca^{2+}-CaM with similar affinity, the k_{off} and k_{on} for binding to apo CaM are at least 50-fold slower than for Ca^{2+}-CaM, however, simulations show that these differences would not inhibit transfer of CaM from PEP-19 to a Ca^{2+}-dependent target protein during a Ca^{2+} pulse. Sequence analysis, CD and NMR show that PEP-19 is an intrinsically disordered protein, but with residual structure localized to its acidic/IQ motif. We also show that PEP-19 persists in a partially folded state when bound to either apo or Ca^{2+}-CaM, a feature of protein-protein interactions that has been called a fuzzy complex. These data show PEP-19 to be a representative of a class of acidic/IQ regulators of CaM signaling. They also support models in which intrinsic disorder confers plasticity that allows PEP-19 to bind to either apo or Ca^{2+} CaM, and that complex formation may be facilitated by conformational selection of residual structure in the acidic/IQ sequence. Moreover, conformational exchange of bound PEP-19 in a fuzzy complex with CaM could exert an allosteric effect that modulates or gates the k_{on} and k_{off} rates for binding Ca^{2+} to the C-domain of CaM.

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Preferential Binding and Orientation of Recoverin to Phospholipid Monolayers

Philippe Calvez, Julie Boucher, Philippe Desmeules, Christian Salesse.
Centre de recherche du CHUQ, Québec, QC, Canada.

Recoverin is a 201 amino acids calcium-myristoyl switch protein that is responsible for the regulation of the phosphorylation of the visual pigment rhodopsin. Calcium binding to myristoylated recoverin leads to a conformational change, which exposes its hydrophobic residues and its myristoyl moiety. We have previously demonstrated that the myristoyl group highly accelerates the membrane binding of recoverin in the presence of calcium. However, it is still unknown whether recoverin shows preferential membrane binding towards highly polyunsaturated phospholipids such as those found in photoreceptor membranes. In this study, we performed monolayer measurements to analyze the affinity of recoverin for different phospholipids that are representative of these membranes. We concluded that the affinity of recoverin increases with fatty acyl chain length and unsaturation of the phospholipids. In addition, we observed a preferential binding of recoverin for didocosahexaenoyl phosphatidylethanolamine