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.

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Interactions of the Anti-Psychotic Drug Trifluoperazine with Calmodulin

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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)

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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

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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

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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.

602-Pos Board B481

Preferential Binding and Orientation of Recoverin to Phospholipid Monolayers

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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

with a maximum insertion pressure of 30 mN/m, which is in the range of the lateral pressure postulated for biological membranes. Moreover, results show that the size and the charge of the polar head group of phospholipids are also implicated in recoverin adsorption in monolayers. Furthermore, polarization-modulated infrared reflection absorption spectroscopy (PM-IRRAS) has been used to determine the secondary structure and orientation of recoverin in monolayers. PM-IRRA spectra indicated the prevalence of α-helices in the secondary structure of recoverin, which is consistent with its known structure. In contrast, non-myristoylated recoverin is quickly denatured after its adsorption in monolayers. Finally, the amide I/amide II ratio allowed to determine that the α-helices of myristoylated recoverin are oriented perpendicular to the plane of the monolayer.

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Characterization Of Ca^{2+} Photo-release From DM-nitrophen Using Photothermal Beam Deflection

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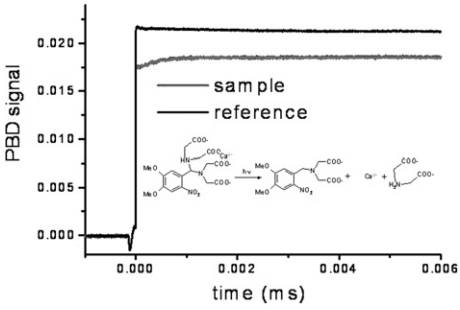
DM-nitrophen is a popular caged calcium compound that allows for a rapid increase in calcium concentration from hundred nanomolar to tens to hundred micromolar level on the microsecond timescale. To fully understand the mechanism of calcium photo-release from DM-nitrophen, we have used photothermal beam deflection technique to investigate time-profiles of volume and enthalpy changes associated with DM-nitrophen photodissociation. Our data show that the photodissociation of calcium loaded DM-nitrophen occurs as a two-step process. The first step takes place within ~ 10 μs upon photolysis and is associated with a volume decrease of -7 mL mol^{-1} and enthalpy change of 66 kcal mol^{-1}. On the longer timescale ($\tau = 200$ μs), the second event with a positive volume change of 7 mL mol^{-1} and enthalpy change of 8 kcal mol^{-1} was detected. These data are in agreement with the previous fluorescence studies showing that calcium release from DM-nitrophen occurs as a two step process.



604-Pos Board B483

Altered Structure Of The Cerebellar Granule Cell Layer Of Mice Lacking Calretinin

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Calcium binding proteins, such as calretinin, are abundantly expressed in distinctive patterns in the central nervous system, but their physiological functions remain poorly understood. Calretinin is highly expressed in cerebellar granule cells and calretinin deficient mice exhibit alterations in motor coordination. Using confocal microscopy, we demonstrate that the cerebellar cortex of calretinin deficient mice exhibit a significantly decreased granule cells density. Interestingly, it has been shown that the migration of granule cells is tightly associated with intracellular calcium oscillations . Therefore, we hypothesize that an alteration of these calcium oscillations in calretinin deficient mice could be involved in the observed morphological alterations. To test this assumption, we are currently developping two strategies. First, using confocal microscopy and cerebellar microexplant culture, we are studying calcium oscillations occuring during granule cell migration in the wild type control and calretinin knock-out mice. This allow us to characterize the impact of variations in calcium buffering capacity over neuronal development and on the generation of the calcium oscillations observed during the granule cell migration. On the other hand, we are developping a theoretical model to study the impact of calcium buffering modifications on the dynamics underlying the observed calcium oscillations . This dedicated computational model will shed light on the possible mechanism responsible for the modulation, by calretinin, of calcium oscillations during the granule cell migration.

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Characterization Of Zebrafish (Danio Rerio) NCX4: A Novel Na/Ca Exchanger With Distinct Electrophysiological Properties

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Members of the Na+-Ca2+ exchanger (NCX) family are important regulators of cytosolic Ca2+ in myriad tissues and are highly conserved across a wide range of species. Three distinct NCX genes and numerous splice variants exist in mammals, many of which have been characterized in a variety of heterologous expression systems. Recently, however, we discovered a fourth NCX gene (NCX4) which is found exclusively in teleost, amphibian and reptilian genomes. The zebrafish (Danio rerio) NCX4a encodes for a protein of 939 amino acids and shows a high degree of identity with known Na+-Ca2+ exchangers. Although knock down of NCX4a activity in zebrafish embryos has been shown to alter left-right patterning, it has not been demonstrated that NCX4a functions as a Na+/Ca2+ exchanger. In this study, we: 1) demonstrate for the first time that this gene encodes for a novel NCX; 2) characterize the tissue distribution of zebrafish NCX4a and 3) evaluate its kinetic and transport properties. While ubiquitously expressed, the highest levels of NCX4a expression occur in the brain and eyes. NCX4a exhibits modest levels of Na+-dependent inactivation and requires much higher levels of regulatory Ca2+ to activate outward exchange currents. NCX4a also exhibited extremely fast recovery from Na+-dependent inactivation of outward currents, faster than any previously characterized wild-type exchanger. While this result suggests that the I1 inactive state of NCX4a is far less stable than in other NCX family members. We have demonstrated that a new putative member of the NCX gene family NCX4a encodes for a Na+/Ca2+ exchanger with unique properties including an extremely rapid recovery from Na+-dependent inactivation. These data will be useful in understanding the role that NCX4a plays in embryological development as well as in the adult where it is expressed ubiquitously.

Calcium Signaling Pathways

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Modeling [Na+] in PM-SR Nanodomains of Vascular Smooth Muscle Cells

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We present a stochastic computational model aimed at elucidating the mechanism of site-specific signalling between a source and a target ionic transporter, both of which are localized on the plasma membrane (PM) and are part of a nanodomain: nanometer-scale subplasmalemmal signalling compartments comprising the PM, the sarcoplasmic reticulum (SR), Ca2+ and Na+ transporters (channels, exchangers and pumps), and the intervening cytosol. In this chain of events, the physical and functional link between non-selective cation channels (NSCC) and Na+/Ca2+ exchangers (NCX) needs to be elucidated in view of two interesting recent findings: the identification of the TRPC6 as the NSCC in VSM cells and the observation of localized Na+ transients following purinergic stimulation of these cells. Having previously helped clarify the signalling step between NCX and SERCA behind sarcoplasmic reticulum (SR) Ca2+ refilling, this quantitative approach now allows us to make inroads into this important signalling step. We have implemented a random walk (RW) Monte Carlo (MC) model with simulations mimicking a Na+ diffusion process originating at the NSCC within PM-SR junctions. Physical features of the system (junctional dimensions, diffusivity of Na+ in cytosol, channel capacity, etc.) were obtained in our laboratory and from the existing literature. The model calculates the average [Na+] in the junction and also produces iso-concentration profiles for [Na+] as a function of distance from the Na+ source. It also analyzes the influence of the junctional geometry on the signaling ability of the nanodomain. Our results emphasize the necessity of a strategic juxtaposition of the relevant signalling channels and organelles to form nanodomains that permit adequate [Na+] build-up to provoke NCX reversal and Ca2+ influx via NCX eventually to refill the SR during asynchronous Ca2+ waves.

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A 3d Pseudo-stochastic Model Of Intercellular Calcium Signaling In Smooth Muscle

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We present a general 3D pseudo-stochastic model of intercellular calcium signaling (ICS) in smooth muscle, composed of independently tunable units aggregated through a flexible convolution procedure. ICS ensures propagation and synchronicity of myocytes' contraction within muscular syncytia from