

other methods to determine the degree of structural perturbation caused by mutation of the EF-hands. Inhibition of Ca^{2+} binding to the N-domain in CaM12 does not affect Ca^{2+} binding to the C-domain, however, inhibition of Ca^{2+} binding to the C-domain in CaM3 and CaM34 significantly increases the Ca^{2+} -binding affinity of the N-domain by decreasing the k_{off} for Ca^{2+} . 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 Ca^{2+} 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 (CaM₁₋₁₄₈), N-domain (CaM₁₋₈₀) and C-domain (CaM₇₆₋₁₄₈) 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 CaM₁₋₁₄₈, but affected both domains. At an 8:1 ratio of TFP:CaM, the effect reversed and the Ca^{2+} affinity of CaM increased.

¹H-¹⁵N-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-CaM₁₋₁₄₈ was determined to be 2:1, and 4:1 for (Ca²⁺)₄-CaM.

Crystallographic structures of TFP bound to (Ca²⁺)₄-CaM₁₋₁₄₈ 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 (Ca²⁺)₂-CaM₇₆₋₁₄₈-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-CaM₇₆₋₁₄₈ 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 ([Ca²⁺]_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 [Ca²⁺]_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 (IP₃R) 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 3 μM thapsigargin (TG), a SERCA inhibitor, or 60 μM 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 IP₃R 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.

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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