repulsion that adds to the other repulsive terms and overcomes the van der Waals attraction. An osmotic pressure of 10 atm is needed to reduce the DOPS interlamellar D-spacing to the fully hydrated DOPC spacing of 63.1 Å, while at 1 atm the D-spacing is about 30 Å larger for DOPS than for DOPC. From X-ray and volumetric measurements we calculate the area per DOPS molecule to be 65.1 ± 2.0 Å² compared to 72.2 Å² for DOPC. At the same D-spacing, bilayer fluctuations are smaller for DOPS than for DOPC, which might be related to the smaller area per lipid in the case of DOPS. From 31P NMR chemical shift anisotropy we infer a continuous change in headgroup orientation of DOPC as a function of water concentration that is not observed in DOPS. In addition, the quadrupole splittings of water between DOPS bilayers decay far less rapidly than in DOPC. This is either the result of a significantly larger hydration shell around the DOPS headgroup or additional orientational fluctuations in the DOPC headgroup region.

Symposium 15 - Awards

No Abstracts

Platform AP - Actomyosin Interactions

1589-Plat
Three-dimensional structure of the ADP and rigor states of β-cardiac S1 bound to F-actin
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Heart muscle contraction is powered by class II, cardiac myosin motors. Biochemical state transitions that occur during the myosin ATPase cycle are potentiated by actin-binding and are coupled to structural changes in the motor cleft and light chain binding domain (LCBD), resulting in the generation of a ~100 Å powerstroke. While the structural transitions in skeletal myosin II are complete after phosphate release, ADP release mediates an additional swing of the LCBD in other class II myosins such as smooth muscle myosin. We are investigating the structural characteristics of the LCBD of β-cardiac S1 bound to actin in the ADP and rigor states using cryo-electron microscopy and helical image analysis. β-cardiac S1 was prepared by papain digestion of purified porcine cardiac myosin. The S1 fragment decorates actin filaments in the presence (5 mM Mg-ADP) and absence of nucleotide. We observe an additional swing of the β-cardiac S1 LCBD upon ADP release that is similar in magnitude to that observed with the smooth muscle myosin S1 fragment. Our results support a strain-limited ADP release mechanism similar to that which has been proposed for smooth muscle myosin and provide further insight into the unique physiology of cardiac myosin.

1590-Plat
Myosin ATP binding to actin: effect on the measured working stroke
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In solution, actin not only binds to M.ADP.Pi, which accelerates ADP and Pi release, but also to M.ATP, which accelerates ATP release (Biochemistry, 17, 5423). This reaction has been investigated at the single molecule level by using ITP and related nucleotides for which the rate limiting step is hydrolysis. The duration of events is proportional to 1/[ITP] indicating that the observed state is AM and that the minimal sequence of reactions is A→M.ATP=AMP.I→AMP.ATP. No working stroke is associated with this sequence and thus repriming of the crossbridge cycle cannot take place between two AM.ATP states, as has been suggested (Science 227, 999), but must take place between states dissociated from actin. Low pH favors M.ATP over M.ADP.Pi and might be expected to increase the ratio of ATP release, relative to product release. Measurement of the working stroke at pH 6.5 gave a value of about 2 nm as opposed to the 6 nm observed at pH 7.5. The most likely explanation is not that the working stroke per se is a function of pH, but that the ratio of ATP release events (no working stroke) to product release events (working stroke) has increased. The best estimate of the true working stroke is probably about 10% greater than observed at pH 7.5 (~6.5 nm).

1591-Plat
Actomyosin motility on nanostructured resist polymers and silanes
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We have previously shown that heavy meromyosin may be adsorbed in a functional form to several resist polymers. Taking advantage of these findings we have used electron beam lithography to create tracks of the resist substance MRL6000.1XP for myosin adsorption. By this procedure we succeeded in constraining a significant number of actin filament images between 100 nm wide tracks without allowing the filaments to change sliding direction on the track. We have also tested several silanes with respect to their ability to bind heavy meromyosin in a functional form. The most interesting silane in this respect was chlorotrimethylsilane which supported high quality actin filament motility giving higher sliding velocities than observed on a conventional nitrocellulose surface (20 ± 2.8% higher velocity; mean ± SEM; n=4, p<0.01). Nanostructured silane surfaces have also been produced and the possibility to achieve actomyosin motility on such surfaces is explored. Our studies have, for the first time, described the use of nanofabrication techniques for constraining the motility of actin filaments to predefined tracks. This paves the way for nanotechnological applications and detailed studies of actomyosin function.

1592-Plat
Probes of the myosin cleft that sense actin binding
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The actin binding region of myosin comprises two sub-domains (upper and lower 50K) that are separated by a large cleft which is postulated to close on interaction with actin. In order to test this idea and resolve cleft dynamics, we have made mutants of the Dicyostelium myosin II motor domain. Single pairs of cysteine residues were incorporated across the cleft at positions 416/537, 400/537, 398/537 and 416/586 in a cysteine-deficient background. Cleft conformation was probed through sulfydryl oxidation, crosslinking with pPDM, labelling with fluorescence and epr probes. Here we focus on the 416/537 pair which has basal steady-state ATPase rates of 0.11 s⁻¹ and 0.03 s⁻¹ under reducing and oxidising conditions respectively, that increase to 0.9 s⁻¹ and 0.5 s⁻¹ in the presence of actin. Labelling the construct with N-(1-pyrene) iodacetamide results in a species with a marked pyrene eximer peak. The intensity of the pyrene eximer emission is reduced by 40% on binding actin, while ATP addition reverses this change. ATP binding to the construct in the absence of actin gives a 10% reduction. These data indicate that pyrene is a sensitive probe for resolving the dynamics of cleft movement. Supported by a Maggy Fellowship and the Wellcome Trust.
Differential smooth hydrolyse ATP, for the of Warshaw’s laboratory we head. We have each head as the smooth muscle S1 has demonstrated that double-headed myosin II produces approximately twice the unitary displacement as single-headed (Tyska et al., 1999). One possible explanation for the larger step is the summation of two small steps, one from each head. To confirm or refute this, we have expressed a heterodimeric smooth muscle HMM with one wild type head and the other containing the point mutation E470A. Single turnover assays showed the mutated head is compromised in its ability to hydrolyse ATP, effectively locking it in a weak binding state and incapable of producing motion in the in vitro motility assay. Therefore, heterodimer motion is only generated by the native head. Single molecule step size measurements using an optical trap reveal that the heterodimeric protein produces a 10nm step, comparable to the wild type homodimeric HMM, but distinct from the single-headed smooth muscle S1 (4nm). From these data, it is evident that one head alone is incapable of producing a complete displacement of 10nm, whereas a single head supplemented by a weak binding, Arthur is the minimal requirement for a full step. We propose that the weak association of the second head is enough to guide the lever arm rotation to the long axis of the actin, maximising the observed step size.

Motor domain strain and the origin of force in myosin
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We have shown that when myosin V is attached to actin by both its heads, the lead head is in the pre-power stroke conformation. We know this because the lever arm emerges from the rear edge of the motor domain, which shows that the converter sub-domain is in the pre-power position. This is the first demonstration of the pre-power conformation of any myosin on actin. The pre-power state of heads before attachment to actin has ADP and Pi bound, but a key issue is what is the nucleotide content of the lead head in myosin V? We have found that the rate of ADP dissociation from myosin V:ADP:Pi by actin is only slightly inhibited by two-headed binding. Therefore, in the rate limiting 1 μM ATP used in our microscopy, product release occurs without converter movement. The lead head lever arm is bent backwards and is either deformed throughout its length or at the plant point near the motor domain. This indicates that the lead head converter is under strain and is prevented from moving to its post-power position by being tethered through the trail head. Strain within the motor domain of the lead head without products is therefore likely to be the origin of myosin V force. This mechanism may be common to other myosins, for instance in isometrically contracting muscle.

Single molecule mechanical studies on the head and neck of myosin I & II
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We have measured the powerstroke, lifetime and flexural rigidity of single acto-myosin interactions using an optical tweezers transducer and direct observation of thermal fluctuations. We found that myo 1A (brush border myosin 1), myo 1B (150kDa, myr1) and myo 1C (110kDa, myr2) all exhibit a two-phase powerstroke. The timing of these phases is consistent with the rates of phosphate and ADP release from the actomyosin complexes. We are now testing if the phases correspond to different product release steps. Using S1 with a BDTC-tagged RLC coupled specifically to streptavidin (Iwane et al. 1997) we confirmed that in vitro sliding velocity was significantly increased over native S1, bound non-specifically to nitrocellulose. However, we found that the powerstrokes were similar (ca. 5nm). Observation of thermal vibration of actin about a single attachment point showed that torsional rigidity was lower for biotin-avidin coupled S1 and most complexes formed exhibited free diffusive motion about the attachment rather than spring-like behaviour (31 pN nm/ rad) typical of native S1 and BBM1 bound to nitrocellulose.

MYOSIN STRUCTURE AND FUNCTION AT LOW IONIC STRENGTH
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In vitro measurements of actin-myosin function often are made at low ionic strength (i.s.). We have investigated the effects of i.s. on electrical, hydrodynamic and functional properties of HMM and S1 in solution (20C). In 100 mM KOAc, the magnitude of the zeta potential (ζ) of HMM increases from 4.1 to 7.4 mV when Pi dissociates from the MgADP.Pi complex. Decreasing [KOAc] to 1 mM reduces ζ to -2.4 mV for the MgADP.Pi complex and increases ζ to -8.6 mV for the MgADP complex. S1 has similar changes in ζ. Transitions are below 50 mM. In 100 mM KOAc HMM hydrodynamic size (effective diameter, d(eff)) increases from 25.5 to 26.5 nm when Pi dissociates. Decreasing [KOAc] to 1 mM reduces d(eff) of the MgADP.Pi complex 24.1 nm, and increases the MgADP complex to 31.8 nm. Decreasing i.s. reduces: HMM and S1 MgATPase activities; their ratios of bound [MgADP.Pi] to [active site]; and their affinities for MgADP. The stability of HMM, estimated by activity loss when incubated at 25C, increases as i.s. is reduced. Clearly low i.s. changes motor domain structure and function. Our working hypothesis is that increased surface electrostatic interactions shift an intrinsic two state equilibrium of the motor domain. (NIH AR42895)

Recombination of Subunit Domains in Developmentally Regulated Transcriptomes of CACNA1G, the Human Cav3.1 Calcium Channel Gene
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The human gene CACNA1G was screened by cDNA exon scanning for sites of alternative mRNA splicing. Ten alternative splice sites within 8 exons, and two alternative 3' UTR sequences
yield a maximum of 2,048 possible transcripts and 1,024 ORFs. Full-length CACNA1G single-gene cDNA libraries prepared from fetal and adult human brain yielded 1,580 7 kbp cDNAs. Screening for alternative splicing, revealed 30 distinct ORFs. Both fetal (22) and adult (15) forms fell into coherent, developmentally regulated classes, based on structures and abundance. Monte Carlo and mutual information analyses of fetal and adult populations reveal patterns of variable splicing linkages that change and increase in enormously in complexity during brain development. Ensembles of 7-8 variable sites are selected in concert in adult transcripts. Patch-clamp analysis of selected adult and fetal variants reveal that splicing of individual domains, in general, elicit dramatically different effects depending on splicing configurations at multiple other sites. The concerted recombination of protein domains suggests that a flexible network of regulatory genes specifies changing calcium channel phenotypes during neural differentiation.

1598-Plat
Key determinant of CaM preassociation with L-type Ca channels
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L-type channel Ca\(^{2+}\)-dependent inactivation (CDI) involves multiple calmodulin (CaM) interactions: Ca\(^{2+}\)-free CaM (apoCaM) preassociates with channels; inflowing Ca\(^{2+}\) activates preassociated apoCaM; and Ca\(^{2+}\)-CaM binds to a channel IQ domain (IQ). The channel preassociation site and even the functional impact of CaM preassociation have been unclear. The IQ was initially proposed to be the preassociation site, but the hunt for sites shifted elsewhere when IQ did not bind apoCaM in vitro. We now report compelling evidence that the IQ is key to preassociation. Using a FRET 2- hybrid screen, we found robust binding between apoCaM and a 73-aa peptide containing IQ, with affinity comparable to that of apoCaM for intact channels. Alanine mutations in IQ reduced peptide affinity by ~20-fold. More telling were FRET images of intact channels and CaM in live cells: FRET efficiency in the membrane dropped from ~10% to 1% for IQ-mutant channels, showing disrupted preassociation in situ. Electrophysiologically, mutant channels lacked CDI, but had accelerated voltage-dependent inactivation (VDI) seen with Ba\(^{2+}\) currents. Overexpressing CaM markedly slowed VDI kinetics, providing functional evidence that increased CaM can repopulate weakened preassociation sites. Our findings clearly establish the IQ as crucial for the apoCaM/channel interactions underlying CDI.

1599-Plat
Direct Modulation of Ca,3.2 Calcium Channels by Calcium-Calmodulin Kinase II.
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LVA channels play a central role in control of functions as diverse as burst generation in thalamocortical neurons and steroid hormone secretion in adrenal glomerulosa cells. Using whole cell recording techniques in HEK293 cells and heterologous expression of Ca,3.1 or Ca,3.2 channels, we investigated the mechanism underlying the regulation of Ca,3.2 channels by CaM. CaMII activation induces a hyperpolarizing shift in the V_{1/2} of activation and a concordant increase in the steepness of the relative G\(_{V}\)-voltage relationship (\(\Delta V_{1/2} = -14.3 \pm 1.6\) mV; \(\Delta k = 4.5 \pm 1.4\) mV; p<0.05) of Ca,3.2 but not Ca,3,1 channels, despite a high degree of sequence homology. Analysis revealed several CaMII consensus motifs unique to Ca,3.2 intracellular domain-linkers. Exchange of these linkers resulted in chimeras with regulation lost on the Ca,3.2 or gained on the Ca,3.1 backbone. Rapid and stoichiometric phosphorylation of GST-domain-linker fusions by recombinant CaMII confirmed putative regulatory motifs. Mutagenesis of residues, singly or in combination, reduced \(^3^P\) incorporation and abolished regulation of Ca,3,2 channels by CaMII. We conclude that the dual changes in Ca,3,2 channel gating induced by CaMII are the consequence of a direct phosphorylation of intracellular linker-domains.

1600-Plat
Ca\(^{2+}\)/CaM-Dependent Protein Kinase II; A Tethered Frequency Decoder for Ca\(^{2+}\)-dependent facilitation of Cardiac Calcium Channels.
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Ca\(^{2+}\)-dependent facilitation (CDF) of L-type calcium channels (LTCs) is a frequency-dependent change in channel gating that underlies the positive ionotropy associated with an increase in heart rate. The multifunctional-CaMII is a good candidate for regulating CDF because its activity has been previously shown to be differentially regulated by the frequency of calcium oscillations. Here we demonstrate that CaMII phosphorylates the N and C-terminus of \(\alpha_{1C}\), the pore-forming subunit of the LTC, as well as \(\alpha_{1C}\) in vitro. Impairing the binding of CaMII on the C-terminus prevents CDF, indicating that this region is a critical binding site for CDF. Although Ca\(^{2+}\)/CaM and autophosphorylation of CaMII are required for initial binding, binding is maintained in the absence of calcium and following dephosphorylation. Anchored CaMII retains its dependence on Ca\(^{2+}\)/CaM, suggesting that the CaMII holoenzyme could form a dedicated calcium spike frequency detector by tethering to the LTC to locally regulate its gating properties.

1601-Plat
CHOLESTEROL MODULATES DIHYDROPYRIDINE RECEPTOR FUNCTION IN FETAL SKELETAL MUSCLE CELLS
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Caveolae and transverse tubules are membrane structures enriched in cholesterol and glycosphingolipids. They are thought to play an important role in calcium signaling and myogenesis. Our previous results indicated that treatment of skeletal muscle fibers with the cholesterol-depleting drug methyl-\(\beta\)-cyclodextrin (M\(_{100}\)) produced a significant decrease in the density of caveolae and morphological changes in the transverse tubular network. Here, we further investigated the effect of M\(_{100}\)CD in freshly isolated fetal skeletal muscle cells. M\(_{100}\)CD treatment decreased cell membrane capacitance indicating a loss of surface membrane and/or surface-connected membrane areas. L-type Ca\(^{2+}\) current amplitude was significantly reduced, and the voltage-dependence was shifted ~15 mV toward more positive potentials. Activation and inactivation kinetics were faster and sensitivity to Bay K 8644 was enhanced. In addition, intramembrane charge movement and calcium transients evoked by a depolarization were reduced, indicating a weakening of excitation-contraction coupling. In contrast, T-type Ca\(^{2+}\) current was not affected. These results suggest that membrane cholesterol modulates dihydropyridine receptor function.
Ca.1,4a1 subunits are exclusively expressed in the retina. Although full length cDNAs have been cloned their functional expression has not yet been reported and the classification as dihydroxypridine (DHP)-sensitive LTCCs is only hypothetical. We cloned a human Ca.1,4a1 cDNA and determined the biophysical and pharmacological properties of its Ba2+ (Iba) and Ca2+ (Ica) currents after expression (+ β3 or β2a + αδ1) in tsA-201 cells using the whole-cell patch-clamp technique. Iba showed strong DHP agonist dependence (-9-fold, 5 μM BayK8644). DHP antagonist sensitivity (isradipine) was ~15-fold lower than for Ca.1,2a. Iba was activated at negative voltages (-39.5 ± 1 mV; n = 21; 15 mM Ba2+), with faster activation time course and slower inactivation than Ca.1,3a1 (66.7 ± 4.3%; n = 7; 10-s pulse; +β3 + αδ1). Inactivation rate of Ica or Iba was similar indicating the absence of Ca2+-induced inactivation. Ca.1,4a1 exhibited voltage-dependent, G-protein independent facilitation by strong depolarizing pulses. Our experiments show that Ca.1,4a1 subunits can form L-type currents with biophysical and pharmacological properties similar to sustained Ca2+ influx coupled to neurotransmitter release in retinal neurons.

**Platform AR - Membrane Fusion and Protein-Lipid Interactions**

**1605-Plate**

Understanding structure-function correlations in cationic lipid-based gene delivery systems

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Cationic Liposome-DNA (CL-DNA) complexes are widely used in non-viral gene delivery, including clinical trials. However, transfection efficiencies of these complexes need improvement and their in-vivo lifetime is very short. Understanding the basic mechanism of gene delivery, the effect of lipid structure and of lipids such as cholesterol, is essential for the design of more efficient lipid vectors. We report the synthesis of new lipids designed to address current shortcomings of CL-DNA complexes. Cholesterol or two alkyl chains have been used as the lipophilic moiety, to which poly(ethylene glycol) (PEG) and multivalent cationic headgroups were attached. The length of the PEG and the headgroup charge were varied. PEG-chains should provide enhanced resistance to the immune system, while multivalent groups increase the membrane charge density of the lipid membrane. CL-DNA complexes of the new lipids and the neutral lipid DOPC have a lamellar structure and were investigated using x-ray diffraction and optical microscopy. Their transfection efficiencies were assessed using a luciferase assay. Funding provided by NIH R01-GM59288, NSF DMR-0203755

**1606-Plate**

Lipid Organization in Cisplatin Nanocapsules.

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Cisplatin is a commonly used anticancer drug. The clinical use of cisplatin is restricted by dose-limiting toxicities, which can be reduced by encapsulating the drug in liposomes. However, the low water solubility of cisplatin results in liposomal formulations with low drug-to-lipid ratios. Recently, we developed a new method to efficiently trap cisplatin in a lipid formulation, which involves hydration of a DOPC/DOPS lipid film with a concentrated solution of cisplatin and 10 freeze-thaw cycles. Examination by electron microscopy revealed the cisplatin nanocapsules, small precipitates of cisplatin coated by rod-like bilayer. The nanocapsules have in vitro cytotoxicity up to 1000-fold higher than the free drug. To investigate the lipid organization in the coat of the nanocapsules we used 31P NMR spectroscopy. The 31P NMR spectrum of a DOPC/DOPS dispersion consists of two resolved signals of DOPC and DOPS with line shapes characteristic of a liquid crystalline bilayer. In contrast, the spectrum of nanocapsules has a very different line shape, indicating the presence of two phospholipid phases differing in mobility. The role of the interactions between cisplatin and lipids in the formation and lipid organization of nanocapsules will be discussed.
1607-Plat
FUNCTIONAL AND SOLID STATE NMR STRUCTURAL STUDIES OF HIV-1 AND INFLUENZA FUSION PEPTIDES
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Structure/function studies were undertaken to characterize the HIV-1 and influenza fusion peptides in membranes. Solid state NMR spectra demonstrated that both peptides had significant antiparallel and helical character in lipid/cholesterol mixtures whose compositions are close to those found in the cells which the viruses infect. For HIV-1, the non-helical structure was found to contain both parallel and antiparallel arrangements of peptide strands. For the influenza peptide, the structure of the N-terminal region was found to be strongly lipid-dependent, with complete helical structure in POPC:POPG and non-helical structure in other lipid compositions. The peptide structure was independent of whether the peptide was monomeric or aggregated in aqueous solution prior to membrane binding, implying that the observed structure depends only specific peptide/membrane interactions. The influenza fusion peptide was found to induce significant intervesicular lipid mixing for all lipid compositions, with enhanced mixing at pH 5 relative to pH 7.4. The combination of structural and functional results suggests that both helical and non-helical peptide structures can play a role in membrane fusion. Progress will also be reported on solid state NMR structural measurements of the whole influenza fusion protein in membranes.

1608-Plat
The N- and C-terminal regions of gp41 ectodomain fuse membranes resembling the target and viral membrane, respectively
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Enveloped viruses employ fusion proteins to carry out the task of infecting target cells by inducing fusion between viral and target membranes. The ectodomain of the fusion protein TM subunit (gp41) is believed to be primarily responsible for fusion. The N-terminal fusion peptide (FP) of HIV inserts into and destabilizes the target membrane to initiate actual merging of the bilayers. A Trp-rich region, preceding the TM domain was shown to preferentially fuse bilayers mimicking the viral membrane. Since HIV buds from raft microdomains, the lipid composition of the viral and target membranes differs in that the virion is enriched in cholesterol and sphingomyelin. We show here that peptides from the C-terminal region comprising both the Trp-rich and C-terminal heptad repeat regions (56 aa) preferably fuse membranes modeling the viral envelope. In contrast, N-terminal fusogenic fragments comprising the FP and the N-terminal heptad repeat regions (70 aa) act at a markedly reduced level on vesicles modeling the virus compared to those modeling the target membrane. Our study implies the specific roles for the gp41 ectodomain regions in terms of virus evolution.

1609-Plat
Novel Mechanism of Membrane Fusion: Insights from Simulation and Theory
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We have performed both Monte Carlo (MC) simulations and self-consistent field theory (SCFT) calculations on the fusion of bilayers of amphiphilic molecules which are under stress. Initially a stalk forms, and profiles of it obtained from both MC and SCFT are in good agreement. Stalks greatly increase the rate of hole formation in each bilayer. Correlation functions from the MC simulations show that such holes form predominantly close to the stalk. We infer that their energy is greatly reduced by doing so, and calculate this reduction from SCFT. The stalk then surrounds the holes forming a fusion pore. Free energy barriers are estimated from a combination of MC and SCFT, and are of the same order of magnitude as estimated for the standard stalk mechanism; on the order of 200kT. Effects on this barrier of lipid architecture and mixtures of amphiphiles of different architectures are addressed. Our mechanism is consistent with the observed two time-scale fusion process. It predicts that fusion between model membranes should be leaky, and exhibit mixing of lipids between distal and proximal layers. These are in contrast to the predictions of the standard stalk mechanism, but are in agreement with experiment.

1610-Plat
pH Dependent Domain Formation in Phosphoinositide Model Membranes
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Phosphoinositides have been shown to mediate a large variety of important physiological functions by affecting the activity and/ or localization of membrane associated proteins. Growing evidence suggests that the temporal and spatial control of such membrane trafficking events requires selective phosphoinositide segregation into microdomains, which may either form only transiently or exist over longer time periods. Our research aims to study the mechanisms that lead to the formation of phosphoinositide enriched microdomains as well as to characterize such structures. We have investigated at different pH values various phosphoinositide/phosphatidylcholine model membrane systems by FTIR, NMR, DSC, fluorescence anisotropy and FRET measurements. By comparing several phosphoinositides, we have found that the segregating tendency of phosphoinositides is strongly dependent on not only the number but also on the position of the respective phosphomonoester groups at the inositol ring. Although 1H-NMR spectroscopy revealed substitution pattern dependent differences in the pKb values for phosphomonoester groups, these differences are too small to cause the observed alterations in the mutual interaction of phosphoinositides. Most likely these discrepancies can be attributed to orientational restrictions within the inositol headgroup region.

1611-Plat
Polyunsaturation Effects on Protein:Lipid Interactions: Molecular Dynamics Simulations of Rhodopsin in an Explicit DHA Bilayer
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Studies on model membranes have shown that lipid composition, especially the presence and degree of polyunsaturation, is important for full activity of rhodopsin and other G-protein coupled membrane receptors. To provide some insights into the effect of a polyunsaturated lipid (docosahexaenoic acid (DHA); 22:6n3) on rhodopsin motion we performed molecular dynamics computer simulations using the CHARMM program. Our system consists of more than 40,000 atoms and includes 99 DHA lipid molecules, 2 palmitate fatty acids covalently linked to rhodopsin, and full water hydration. In addition to describing the simulation system and results we will also include a description of the parameters developed for the DHA chain and the method used to construct the initial conformations of the system. Analysis presents the results of more than 10ns of simulation and concentrates on the effects of lipid on protein motion, on the energetics of protein-lipid interactions, and on the possible role of polyunsaturation in adjusting protein conformation and dynamics. These molecular insights suggest reasons for the unique role of polyunsaturated fatty acids in biological membranes.
1612-Plat
Characterization of Micro-domain Structure in ROS Disk
Model Membranes Using Differential Scanning Calorimetry
and Atomic Force Microscopy
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Membrane heterogeneity or microdomain structure in rod outer
segment (ROS) disk membranes was suggested nearly three
decades ago. A unique feature of disks is their high level of
docosahexaenoic acid (DHA, 22:6n-3) derived phospholipids,
which are essential for optimal visual function. Here, we have used
DSC and AFM to investigate the formation of microscopic domain
structure in mixed saturated and polyunsaturated PC bilayers, as
a first step in modeling ROS disk membranes. Spontaneous domain
formation was seen in lipid mixtures containing di16:0PC and
di22:6PC at various ratios both in DSC and in direct AFM
imaging. At room temperature, AFM images showed clear phase
separation into di16:0PC-rich domain and di22:6n3PC-rich domains
in mica supported bilayers. These domains differed in
height by ~1 nm, consistent with the height difference between
bilayers of di16:0PC and di22:6n3PC. Addition of cholesterol
resulted in a morphologically unique separation between these
domains, suggesting that cholesterol can play an important role by
altering preexisting microdomain structural. Our results reveal
domain structure in disk model membranes, which is driven by
phospholipid acyl chain composition rather than by sphingolipid-
cholesterol interaction.

Platform AS - Protein-Ligand Interactions

1613-Plat
Binding of Ochratoxin A to Human Plasma Proteins:
Implications in Toxicity Mechanisms
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Ochratoxin A (OTA), a mycotoxin produced by strains of
Aspergillus and Penicillium fungi, is a common contaminant
resulting from improperly stored food. Elimination of OTA has
proved futile; therefore research has centered on preventing its
toxicity. OTA is primarily nephrotoxic to mammals. Although
molecular mechanisms behind its toxicity are unclear, protein
binding seems essential to its biological activity. Previous research
indicates the presence of OTA in blood and sera of a majority of
human samples and binding to plasma proteins prolongs its half-
life within the body.

We have identified plasma proteins with high affinity for OTA
through optical spectroscopy, gel electrophoresis, and mass
spectrometry techniques. Several plasma proteins, including
human serum albumin, bind the toxin with binding constants.
Utilizing the photo-reactivity of OTA, we explored binding sites
by generating protein photo-adducts. In addition, OTA transport by
two organic anion transporters located in the liver and the kidney
was inhibited in the presence of plasma proteins.

1614-Plat
Resonance Energy Transfer Studies of Anthrax Lethal Toxin
Complex
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Formation of anthrax lethal toxin complexes involves binding of
lethal factor (LF) to proteolytically activated protective antigen
(PA). In solution, this complex is known to comprise a ring-
shaped heptamer of PA with up to 3 bound LF molecules. While
there are crystal structures of PA and LF individually, a complete
map of the PA:LF binding site is not available. We selected a pair of
non-oligomerizing PA mutants that complement to form dimers
containing a single LF site and added to this mixture the 30 kD
binding moiety of LF (LFα). The resulting ternary complex was
used as a platform for analyzing the structure of the LF site by
fluorescence resonance energy transfer. We introduced single
cysteine residues into solvent exposed regions of PA (K563C,
E465C, and D520C) and LFα (E126C, T199C, and N242C) by site
directed mutagenesis and chemically labeled them with the Alexa
Fluor 488 and Alexa Fluor 546 dyes. Steady state, polarization,
and time-resolved fluorescence measurements of energy transfer
efficiency were used to determine average distances between the
labeled sites in complex. On the basis of these results we propose
a model of the PA:LF solution structure.

1615-Plat
Comparative studies of different T cell receptors that bind
the same ligand in a topologically, thermodynamically, and
kinetically similar manner
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Here, the cellular immune response is dependent on the interaction of
the T cell receptor (TCR), a hypervariable molecule similar to an
antibody, with a peptide bound by a major histocompatibility
molecule (pMHC). Structures indicate that TCRs bind
peptide/MHC with a diagonal binding mode, with ~1/3 of the
recognized surface contributed by peptide. Intriguingly, the
binding mode is conserved regardless of the contacts made across
the interface. This is exemplified by the A6 and B7 TCRs, which
have different binding loops yet recognize the same peptide/MHC
(the Tax peptide bound by HLA-A2) in a very similar orientation.
Here we present kinetic and thermodynamic data for the binding of
A6 and B7 to Tax/HLA-A2. Despite different contacts within the
protein-protein interfaces, both receptors bind with affinities near 2
µM and off rates near 0.1 s-1. Both receptors are negatively
affected by substitution of R65 and K66 of HLA-A2 with Ala.
These amino acids are close to a region of negative charge in the
complexes with both A6 and B7. We hypothesize that R65 and
K66 play a key electrostatic role in the binding of different
receptors to this ligand.

1616-Plat
Alteration of Hemoglobin Cooperativity by Salt Bridge
Modification Is an Intra-Dimer Effect
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Cooperativity of human Hb (a dimer of α β dimers) is traditionally
modeled as a "two-state" system in which a low-affinity structure
(T) switches, upon ligation, to a high-affinity form (R), yielding a
net loss of H-bridges and salt bridges in the dimer-dimer interface.
Modifications that weaken these cross-dimer contacts destabilize
the T tetramer, yielding reduced cooperativity and enhanced heme
affinity. All studies, however, used symmetric, double
modifications, i.e., residue modified in both α- or both β- chains,
and yielded cross-dimer coupling models, ignoring possible intra-
dimeric coupling.

Here, using singly-modified tetramers only, the modified dimer
exhibits a perturbed cooperative free energy of ligation (ΔGc)
while the wild type dimer exhibits the ΔGc of wild type tetramer.
i.e., in spite of overall destabilization of the tetramer by
the modification. This asymmetric response by the half-tetramers
shows that loss of dimer-dimer contacts is not communicated
across the dimer-dimer interface, but is transmitted through the
dimer bearing the modified residue. These findings imply that the
classically inferred role of cross-dimer bonds in the Hb tetramer
332a
(e.g., salt bridge energies) are due, in large part, to coupling effects within the dimers rather than between them.
Funded by NSF and NIH.

1617-Plat
Ligand binding kinetics to the human neuroglobin and cytoglobin
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Neuroglobin and cytoglobin are newly discovered hemoglobins belonging to the vertebrate globin family; the first is found essentially in the nerve cells while the latter is expressed in all types of tissue. Neuroglobins are phylogenetically ancient while cytoglobin could arise from a common ancestor with myoglobin. However both exhibit comparable functional properties with regard to the O2 and CO binding. In particular the binding of an external ligand involves a competitive reaction with a distal histidine. By using time-resolved spectroscopy after flash photolysis and rapid mixing techniques we show that this competition for the heme decreases the intrinsic oxygen binding (for the penta-coordinated heme) giving a final O2 affinity between 1 to 10 torr depending on the state of the proteins. Furthermore the neuroglobin and more generally the globin known to be mainly hexa-coordinated in the absence of an external ligand show a weak temperature dependence of oxygen binding. Indeed the overall observed enthalpy for oxygen binding involves ligand replacement and corresponds to the difference in enthalpies for the two ligands as opposed to ligand binding to the heme in the absence of a competitive residue. Typical value for neuroglobin is 4 kcal mol-1 against 12 kcal mol-1 for horse myoglobin.

1618-Plat
How Do Leukocyte Adhesion Molecules Resist External Forces?
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The ligand-receptor bonds formed by leukocyte adhesion molecules and their ligands are known to be capable of resisting large external forces. Therefore, the force-dependent dissociation of these bonds is essential for leukocyte adhesion. Here we report on the atomic force microscopy (AFM) studies of four major human leukocyte adhesion complexes: LFA-1/ICAM-1, VLA-4/VCAM-1, P-selectin/ligand and E-selectin/ligand. Our data demonstrated that the dissociation of all these four complexes involve overcoming two major energy barriers before final separation. The height of the outer barrier determines the dissociation rates of these complexes under low or zero forces, whereas the inner barrier determines the dynamic strength of the complexes at high forces (~150pN for integrins and ~100pN for selectins). As a result of the steep inner activation barrier, these complexes are less sensitive to the change of high pulling forces. The inner barriers can be attributed to the electrostatic interactions mediated by a divalent cation at the binding pockets, since the presence of EDTA suppresses the inner barriers, eliminating the ability of these complexes to resist high forces. Based on these findings we proposed that the existence of two activation energy barriers is a common characteristic of leukocyte adhesion molecules.

1619-Plat
Ligand Binding Properties of cAMP Receptor Protein Having Amino Acid Substitutions at Positions 72 and 82.
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The cAMP Receptor protein (CRP) of E. coli binds cyclic nucleotides and DNA. When complexed with cAMP, CRP binds DNA and, at promoter sequences, seeds the formation of active transcription complexes composed of CRP, cAMP, DNA, and RNA polymerase. Three-dimensional structures of the CRP:cAMP complex exist; however, the details of CRP activation remain obscure. We have studied the effects of amino acid substitution at CRP positions 72 and 82 to evaluate the roles of these cAMP binding pocket residues in mediating cAMP activation of CRP. In the absence or presence of cAMP the secondary structure characteristics of E72Q, E72D and R82Q forms of CRP were similar to those of wild-type CRP. Cyclic AMP-binding studies showed that amino acid substitution at positions 72 and 82 reduced CRP affinity for cAMP by factors of 2- to 25-fold. DNA binding studies showed that the equilibrium binding constants of the mutant CRP:cAMP complexes measured for lacP were reduced to levels comparable to that of apo-WT CRP. In addition, the mutant complexes failed to footprint lacP in the presence of cAMP or in the presence of cAMP and RNAP. The results of this study demonstrate that E72 and R82 interactions with cAMP play an important role in both cAMP binding and in cAMP-mediated CRP activation.

1620-Plat
Salt effects on the lipid binding and stability of adipocyte lipid binding protein.
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Adipocyte lipid binding protein (ALBP) is a member of a family of small (14 kDa), ubiquitous, intracellular lipid binding proteins. Family members share nearly identical tertiary structures but have divergent primary structures and surface charge topologies. They bind fatty acids and other lipids in many mammalian tissues. ALBP, found in adipocytes and macrophages, has a highly polar surface charge topology, suggesting that electrostatics play a role in its function. Equilibrium binding titrations as a function of salt concentration reveal that salt weakens the binding of the fluorescent probe 1-anilinonaphthalene-8-sulfonate (ANS) to ALBP. Linkage analysis of this effect reveals that the binding of ANS to ALBP results in the release of one ion, and vice versa. Chemical denaturations reveal a concomitant stabilization of ALBP by ~3 kcal/mol upon increasing [KCl] from 0 to 500 mM. Calculation of the linkage between stability and binding indicates that ANS binds to the denatured state of ALBP with a 2G of ~2.5 kcal/mol at 50mM KCl. To further explore the role of surface charge on the salt-mediated function of ALBP, we have constructed a charge mutant of ALBP that neutralizes some of the positive potential near the binding portal. Binding kinetics data for the mutant and wildtype proteins will also be discussed.
1621-Plat
RyR2 mutations linked to catecholaminergic polymorphic ventricular tachycardia increase gating after PKA phosphorylation
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Catecholaminergic polymorphic ventricular tachycardia (CPVT) is a genetic disorder characterized by exercise-induced arrhythmias. Mutations in ryadonine receptor type 2 (RyR2), the cardiac Ca2+ release channel, have been linked to CPVT. Wild-type (WT) and CPVT-mutant RyR2 channels (CPVT-RyR2: P2328S, V4653F, Q4201R) were co-expressed with FKBP12.6 in HEK cells. Channel properties of nonstimulated and PKA-phosphorylated WT or CPVT-RyR2 were studied in lipid bilayers. Nonphosphorylated CPVT-RyR2 function was not different compared to WT. After PKA-phosphorylation, all three PKA-phosphorylated CPVT-RyR2 channels exhibited increased open probabilities, gating frequencies, and prolonged mean open times between 0 to 3 mM Mg2+, significantly increased open time constants (τ2), and long open events (40-100 ms) distinct from WT. CPVT-RyR2 have increased channel function after PKA-phosphorylation at inhibitory Mg2+ concentrations. These results imply PKA-dependent dysfunction of CPVT-mutant RyR2 Ca2+ release as a specific trigger mechanism for ventricular arrhythmias.

1622-Plat
Reduced binding of FKBP12.6 to mutant ryadonine receptors (RyR2) linked to exercise-induced sudden cardiac death
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Catecholaminergic polymorphic ventricular tachycardia (CPVT) is an autosomal-dominant disorder characterized by exercise-induced ventricular tachycardia and sudden cardiac death. Mutations in ryadonine receptor type 2 (RyR2), have been linked to CPVT. We examined RyR2 phosphorylation by PKA, and binding of the stabilizing subunit FKBP12.6 to RyR2 in a mouse exercise model. FKBP12.6 binding to recombinant wild type (WT) and four mutant RyR2s was assessed using a rapamycin competition assay. During mutant exercise, RyR2s became PKA phosphorylated (210% vs. control, and FKBP12.6 binding was decreased by 35%. Compared to recombinant WT RyR2, there was a ~10-fold decrease in the binding of FKBP12.6 to the S2246L, R2474S, and R4497C mutants, and a ~3-fold reduction in binding to the N410K mutant RyR2. These findings suggest that during exercise when the RyR2s become PKA phosphorylated, CPVT mutant channels are more likely to dissociate the stabilizing subunit FKBP12.6. This is likely to promote higher activity of mutant RyR2 channels during diastole causing Ca2+ leak out of the SR. These data provide a mechanism for delayed afterdepolarizations due to aberrant SR Ca2+ release that trigger fatal ventricular arrhythmias.

1623-Plat
[Na+] Imaging in Rat Ventricular Myocytes Using Two-Photon Microscopy of SBFI
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Intracellular [Na] ([Na+]) is important in the regulation of cardiac Ca and contractility, via Na/Ca and Na/H exchange. Functional data suggest that [Na+] might be non-uniformly distributed in myocytes not in steady-state, but little direct spatial information is available. Here we used two-photon confocal microscopy of SBFI to spatially resolve [Na+] in rat myocytes. SBFI was excited at 760 nm with a Ti:sapphire laser. In vivo calibration yielded an apparent Kd of 26.42±0.6 mM. Resting [Na+] (10±0.5 mM) here is similar to what we reported in whole-cell ratiometric measurements with SBFI (11.1±0.7 mM). We also reproduced our results with Na-pump inhibition ([Na+], rose by ~25 mM in 5 min) and reactivation (measuring Na-pump function). To assess [Na+], gradients in myocytes, we blocked the Na-pumps at one end of the cell (locally pipette-applied K free solution) with the rest of the cell in Na-free solution. This resulted in a marked increase in [Na+] at sites near the pipette (where the Na-pump was blocked). [Na+] rise was smaller at sites further away (upstream) from the pipette. A sustained [Na+] gradient of 12-14 mM could be induced in this way. We conclude that two-photon confocal microscopy of SBFI can be used to monitor spatial [Na+], distribution and that substantial [Na+] gradients can exist in cardiac myocytes.

1624-Plat
Cytosolic [Ca2+] Regulates Action Potential Duration Of Mouse Ventricular Myocytes
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Action potentials (APs) recorded with microelectrode in intact mouse hearts are significantly longer than whole-cell APs recorded in isolated ventricular myocytes. Since APs are commonly recorded in cells dialyzed with significant concentrations of Ca2+- buffers, the shorter APs may be a consequence of cytosolic Ca2+-buffering. To test this hypothesis, we recorded whole-cell APs from murine ventricular myocytes dialyzed with high (14 mM) EGTA, and from unbuffered myocytes using the perforated patch approach. At 1 Hz and 33 °C, APD90 of intact myocytes was 4-fold longer compared to APD90 of highly buffered myocytes (Mean: 127±16ms, n=16, vs 30±2ms, n=26, p<0.001). When APs and cytosolic [Ca2+] were measured simultaneously using the perforated patch technique in fura2-AM loaded myocytes, APD70 and APD90, but not APD30 and APD50 correlated significantly with peak cytosolic [Ca2+]. Suppression of the NaCa exchanger by rapid replacement of Na+ with Li+ significantly shortened APD90 and increased cytosolic [Ca2+], suggesting that the inward NaCa exchange current generated from Ca2+ extrusion contributes to the longer APD90 of unbuffered cells. We concluded that cytosolic [Ca2+] regulates the action potential duration of mouse ventricular myocytes, most likely via a NaCa exchanger-dependent mechanism.

1625-Plat
I Na,K and Classical Cardiac Na Channels are Encoded by Different Genes.
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To begin a molecular identification of channels generating the Na current component I Na,K we asked whether they are posttranslationally modified classical Na channels or a distinct isoform. We exposed rat ventricular cells in primary culture to an
antisense oligonucleotide (AON; 10μM; day 2 in culture) directed against H1 (the classical cardiac Na current (I\textsubscript{Na}) isoform). Mean maximum peak I\textsubscript{Na} densities (whole cell patch clamp) were – 2.50±0.31 (AON exposed; 18 cells), -8.23±0.60 (untreated controls; 18 cells) and -6.37±0.97 pA/pF (scrambled sequence exposed; 16 cells), for a reduction by the AON of 69.6% and 60.8% respectively. Both reductions were significant (P<0.001). Control group means were not significantly different from each other. Inhibition was specific for I\textsubscript{Na}. Mean maximum peak Ca current density was not significantly different for any treatment group. This confirms that H1 generates the classical cardiac I\textsubscript{Ca}.

This AON (same concentration, time of exposure and culture conditions) had no effect on I\textsubscript{CaTTX} (mean maximum peak I\textsubscript{CaTTX} current density of -4.72±0.15 (15 cells), -5.47±0.53 (13 cells) and -5.04±0.63 pA/pF (15 cells) for untreated control, scrambled sequence exposed and AON exposed respectively). None of these values were significantly different. I\textsubscript{CaTTX} and I\textsubscript{Na} are encoded by different genes.

1626-Plat
Effects of KCNQ2 mutation I57T on the HERG/KCNQ2 (I\textsubscript{Kr}) current.
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Co-association of KCNE2 with HERG was proposed to form the delayed rectifier potassium current I\textsubscript{Kr}.

Mutation I57T in KCNE2 was linked to Long QT Syndrome (Abbott et al. 1999) but remains to be characterized. We compared currents generated by co-expression of HERG with KCNE2 (WT) and I57T in Xenopus Oocytes and TSA201 cells. Electrophysiological analysis revealed that I57T increased the HERG/KCNEN1 maximal conductance by 31% with no significant effects on inward rectification or steady state activation. I57T significantly accelerated activation of the current and slowed the onset of inactivation. In oocytes, I57T current transiently increased up to 195 ± 20% of WT maximal amplitude at depolarized potentials. Acceleration of the activation kinetics at 37°C resulted in a 2.5 fold increase in I57T maximal current and a shortening of the time to peak to 20 ms at −10 mV. The combined kinetic and conductance effects of I57T at 37°C may lead to a 4-fold increase in I\textsubscript{Kr} amplitude during the first 50 ms of activation with modest changes on steady state currents and a decrease of HERG/KCN2 relative tail current. The effect of I57T on the balance of current at different time points during a cardiac action potential may modulate its duration by a mechanism more complex than a simple reduction in potassium currents during phase 3 repolarization.

1627-Plat
Gender differences in cardiac K currents in rabbit ventricular myocytes
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Females (F) have a longer corrected Q-T interval and action potential (AP) duration than males (M), but the mechanism is largely unknown. Delayed rectifier current (I\textsubscript{K}) has both slowly activating & rapidly activating components (I\textsubscript{Ks} & I\textsubscript{Kr}) that may contribute as major determinants of AP repolarization. We evaluated M vs F differences in I\textsubscript{Ks}, I\textsubscript{Kr} & I\textsubscript{K} density & kinetics in rabbit ventricular myocytes (whole-cell patch, 23°C). Total I\textsubscript{K} density was 37% lower in F vs M (at +20 mV, 0.45±0.05 vs 0.71±0.1 A/F, n=23,12, p<0.05). I\textsubscript{Ks} density (measured in presence of I\textsubscript{Kr} blocker dofetilde; 3 μM) was also 40% smaller in F vs M (at +20mV, 0.40±0.03 vs 0.66±0.08 A/F, n=12,12, p<0.01). However, I\textsubscript{Kr} density (dofetilde-sensitive difference current) did not differ (-0.1 A/F at +30mV). I\textsubscript{Kr} activation was shifted by ~7 mV in F vs M. Mean I\textsubscript{K} activation at +50 mV was slightly prolonged (1.5 vs 1.3 s) while deactivation was slower in F vs M at ~50 mV (τ = 620±48 ms vs 455±50 ms, n=11,12, p<0.05). I\textsubscript{Kr} activation & deactivation kinetics were similar. We conclude that F rabbits have lower I\textsubscript{Ks} density and Em-dependence of activation. This is sufficient to explain most of the longer APD in F vs M (LabHEART), and may cause gender-specific differences in arrhythmogenicity in pathophysioligic states & with pharmacologic interventions.

1628-Plat
Mechanism of automaticity induced by dominant-negative suppression of I\textsubscript{Kr} in guinea pig ventricular myocytes
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We have reported that simple adenoviral suppression of I\textsubscript{Kr} in adult guinea pig ventricular myocytes unleashes pacemaker activity \textit{in vitro} and \textit{in vivo}. However, theionic mechanisms responsible for this automaticity have not been defined. An adenovirus containing a dominant-negative Kir2.1 gene with pore-forming signature AAA for GYG (Kir2.1AAA) was injected into left ventricular (LV) cavity during clamping of the aortae. Freshly isolated LV myocytes were patch-clamped (37°C). I\textsubscript{Kr} was almost totally suppressed in Kir2.1AAA-transduced cells with spontaneous action potentials (SAP), while I\textsubscript{Kr} was maintained in controls with stable resting potentials. The pacemaker current I\textsubscript{Kr} was not recognized in both groups. Nickel (40 μM), nifedipine (10 μM), and E-4031 (5 μM), which selectively block I\textsubscript{CaL}, I\textsubscript{CaT}, and I\textsubscript{Kr}, respectively, terminated SAP in Kir2.1AAA cells. The I\textsubscript{Kr} blocker indapamide (1 mM), slowed SAP by increasing action potential (AP) duration. Block of I\textsubscript{Kr} caused (tetrodotoxin 10 μM) had no effect on SAP. Isoproterenol (1 μM) accelerated the pacing rate significantly. Analysis of current records obtained by the AP-clamp method using recorded SAP as the stimulus template confirmed that I\textsubscript{CaT}, I\textsubscript{CaL}, and I\textsubscript{Kr} were indispensable currents for SAP, and that I\textsubscript{Kr} was an important modulator of SAP duration.

### Protein Folding & Stability II

1629-Pos
Board # B1
Purification and Spectral Characterization of Domains 1 and 2 of Epithelial Cadherin.
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Cadherins are calcium-dependent cell adhesion proteins. They are a single polypeptide chain with between 3 and 7 independently folded extracellular domains. Calcium binds at the interface between these extracellular domains and changes their relative orientation. The protein is then activated to participate in parallel dimerization with an identical cadherin on the same cell surface. Cadherin dimers interact in an antiparallel fashion with cadherin dimers from a neighboring cell surface to form tetramers, thereby adhering two cells together. Our laboratory is interested in understanding the energetics of the calcium linkage in dimer and tetramer formation. Fortunately, we can study the stability and calcium binding properties of the extracellular domains separated from the rest of the molecule since they fold independently and are soluble. We report: (1) Creation of a family of clones that explore the importance of the choice of domain boundaries, (2) Purification of a two domain protein (extracellular domains 1 and 2) using anion exchange, size exclusion and affinity chromatography, (3) Preliminary calcium-dependent proteolytic footprinting studies with chymotrypsin, and (4) Fluorescence and CD spectra of the native protein in the presence and absence of calcium.