averaging and image analysis confirms the octameric nature of the hetero-oligomer and reveals an unexpected linear arrangement (rod) of the subunits. The position and identity of each subunit in the rod is determined using immuno-EM with antibodies directed against each septin and MBP or GFP proteins fusionned with septin subunits.. Shs1, the non-essential yeast septins is now being added so that our model can be made more complete and even closer to the *in vivo* situation. Alternatively we are also studying the structure and behavior of partial complexes and complexes containing mutant proteins, this in the aim of judging the relevance of different septins and septin domains to the architecture of the assembly unit and its propensity to self-assemble. The kinetic of assembly of the filaments is also studied by scattering methods in various ionic and nucleotide conditions.

1758-Plat Desmosome Structure in Intact Cells by Electron Cryotomography

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Demosomes are intercellular junctions that mediate cell-cell interactions and provide mechanical strength to cellular tissues. They are composed of transmembrane glycoproteins (cadherins) that directly bind one another to establish the intercellular bond, and a heterogeneous protein plaque in the cytoplasm that couples the cadherins to the intermediate filament network. Electron tomography provides a method for generating 3D structures of these complex molecular assemblies, from which we are trying to deduce the molecular interactions that characterize the desmosome. A previous electron tomographic study of freeze-substituted, plastic sections offered the first 3D insight into the organization of the extracellular cadherin domains. These results suggested flexibility in the binding interface between individual cadherins, which led to the formation of pleimorphic groups within the extracellular space. This result contrasted with projection images of frozen-hydrated vitreous sections, which revealed straight, rod-like densities with an apparent 5 nm periodicity. To better define their native structure, we have developed a new technique for preparing desmosomes in the frozen-hydrated state that avoids the technical challenges of cryoultramicrotomy. In particular, we have flash-frozen intact keratinocytes which were cultured on electron microscopic grids. This approach involves minimal manipulation of the samples and provides images of the entire desmosome rather than thin sections. Although cell bodies are too thick for electron microscopy, desmosomes initially form between thin filopodia that extend between the cell borders. Thus, we have been able to use a 300 kV electron microscope equipped with an in-column energy filter to acquire suitable images. Projection images of these desmosomes reveal features comparable to those previously observed in frozen-hydrated sections. We are currently recording tomographic reconstructions to elucidate the 3D organization of these densities and ultimately the molecular interactions of the constituent proteins.

1759-Plat Study of Magainin-induced Pores in Phospholipid Vesicles

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Magainin is a peptide antibiotic that interacts directly with the lipid membrane in the target cell. The prevalent mode of action for Magainin and related peptides is formation of large nonselective pores in the membrane leading to cell lysis. A number of methods, like Circular Dichroism, solid state NMR, Raman Scattering, Neutron Scattering, etc., have been used to infer this mode of action. However, none of those methods has directly observed the structure of an individual pore, and some puzzling inconsistencies exist in the current models. Here we present a new method for studying peptide/lipid interactions, which employs Cryo-EM to image Magainin-induced pores in phospholipid vesicles. Since the size of the putative pores is quite small for direct visualization under the dose limitations that exist for Cryo-EM, we are also analyzing the Cryo-EM data in a scattering context. That is, performing 'virtual' electron scattering experiments through analysis of the power spectra of selected vesicles. Unlike X-ray and Neutron Scattering experiments, this technique permits selective masking of the regions of the specimen for which the power spectrum is computed. This permits, for example, isolation of the in-plane power spectrum from the transverse power spectrum. While we eventually intend to directly image individual pores, as a first step, this permits observation of the effects of Magainin in bulk specimen, and relates Cryo-EM results to previous X-ray and Neutron Scattering experiments.

Platform AJ: Mitochondrial Channels & Calcium Signaling

1760-Plat Cytoskeleton Regulates Mitochondria Respiration Through A Tubulin-VDAC Direct Interaction

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Mitochondria have long been known to localize within the tubulin-microtubule network in heart and many other cells (Appaix et al., 2003). It is also well-known that in permeabilized cardiac cells the apparent Km for exogenous ADP in the control of mitochondrial respiration is significantly higher than in isolated mitochondria. It has been suggested that the low permeability of the mitochondria outer membrane (MOM) for ATP and ADP in cells is due to interaction of mitochondria with some cytoplasmic proteins (Saks et al., 2003). Here, for the first time, we demonstrate that tubulin is the factor which controls MOM permeability by regulating VDAC, the major channel of MOM. By direct measurements we show that nanomolar concentrations of mammalian tubulin induce highly voltage-sensitive reversible closure of VDAC channels reconstituted into planar phospholipid membranes. Analysis of VDAC single channel fluctuations in the presence of tubulin shows that channel closure occurs at very low potentials (as low as 10 mV)

compared to VDAC gating in control. The tubulin-VDAC interaction requires the presence of negatively charged C-terminal tails of tubulin. Tubulin with proteolytically removed C-terminus does not induce VDAC closure. We propose a model of tubulin-VDAC interaction in which the tubulin C-terminus penetrates into the channel lumen, interacting with VDAC with high specificity and blocking channel conductance. The experiments with isolated heart mitochondria strongly confirm our findings. Apparent Km for exogenous ADP increases 10 times after addition of 1–10 μM of tubulin to isolated heart mitochondria. We conclude that tubulin strongly limits ADP entry to mitochondria across its outer membrane. Our results suggest a new general mechanism of regulation of mitochondrial outer membrane permeability under normal and apoptotic conditions.

1761-Plat Pharmacology of The Cytochrome c Release Channel, MAC

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The Mitochondrial Apoptosis-induced Channel (MAC) forms early in apoptosis to allow cytochrome c release from mitochondria. Once in the cytosol, cytochrome c triggers the activation of caspases and endonucleases, culminating in cell death. Formation of MAC is the commitment step of apoptosis, since it occurs prior to cell damage. Also over-expression of its main components, Bax and/or Bak, is sufficient to induce cell death via release of cytochrome c. For these reasons, MAC is a potential therapeutic target for the regulation of cell death. Previously, compounds were screened and found to block the release of cytochrome c from liposomes induced by activated recombinant Bax. Patch clamp techniques were then used to evaluate the efficacy of these compounds to directly inhibit MAC activity in proteoliposomes containing mitochondrial outer membranes of apoptotic FL5.12 cells. Nanomolar concentrations of several compounds blocked MAC activity; the IC_{50}'s ranged from 25 to 700 nM. MAC blockade was specific in mitochondria since these compounds had no effect on the channel activity of the protein translocase, TOM. Importantly, while MAC activity was blocked by nM drug levels, the lethal dose of these compounds for neonate cardiomyocytes was typically >5 μM. Future studies will determine the effect of these MAC inhibitors on the progression of apoptosis.

1762-Plat Function-linked Changes in the Topology of the Mitochondrial Inner Membrane: Responses to Oxidative Stress

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Electron tomography has dramatically improved our understanding of the topological complexity of the mitochondrial inner membrane. Cristae are not random infoldings of the inner membrane but rather invaginated microcompartments exhibiting considerable diversity. As a rule, the changes observed in mitochondrial inner-membrane topology cannot be explained as passive osmotic adjustments of a flexible membrane. Rather they are remodelings that correlate with specific functional outcomes, involving alterations to membrane curvature and connectivity. The inferred topologic control of the inner membrane requires feedback mechanisms yet to be defined, involving proteins and lipids that affect membrane curvature, dynamics (fusion/fission), and (probably) tethering. Events associated with apoptosis are a good example, with tBid-cardiolipin interactions strongly implicated in the changes observed in inner-membrane curvature and internal mobilization of cytochrome c. Another example is the response of the mitochondrial inner membrane to reactive oxygen species (ROS). Hyperoxic conditions induce a transition in cristae of *Drosophila* flight muscle mitochondria to close-packed dilated tubes (diameters ~30 nm), creating the appearance of a "swirl" in these mitochondria. These "swirl" cristae resemble in several aspects those found in other mitochondria under conditions of elevated ROS, such as the tubular cristae of steroidogenic mammalian tissue and the paracrystalline (cubic) cristae of fasting amoeba. It is suggested that formation of wide, tubular and (in some cases) fused inner-membrane compartments may be a general protective response to oxidative stress, facilitating the clearance and/or reducing the damaging effects of ROS on the inner membrane.

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1763-Plat Morphological Changes In Neuronal Mitochondria Assessed With A Laser Spinning-disk Confocal Microscopy

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Changes in mitochondrial matrix volume play an important role in the regulation of metabolic activity, organelle motility, and programmed cell death. However, monitoring of mitochondrial remodeling in live cells with a conventional laser point-scanning microscopy is complicated due to long exposures to laser light and a significant photobleaching of samples. Spinning-disk confocal microscopy (SDCM) provides an excellent opportunity to monitor mitochondrial remodeling in live cells owing to a much faster acquisition of images and less photobleaching. In cultured hippocampal neurons, SDCM allowed multiple acquisitions of serial images of individual neuronal mitochondria (z-stacks) without a substantial change in their morphology. Subsequent image processing consisted of 3D blind deconvolution, fluorescence intensity thresholding, and isosurface 3D reconstruction. In order to obtain a quantitative assessment of mitochondrial remodeling in our experiments, the SDCM and image processing were calibrated using green fluorescent microspheres with Ø 175 nm (Molecular Probes). Using SDCM and cultured hippocampal neurons expressing mitochondrially targeted enhanced yellow fluorescent protein (mito-eYFP),

we evaluated the effects of FCCP and openers of mitochondrial ATP-sensitive K^+ channels on mitochondrial morphology. Additionally, we quantitatively assessed remodeling of neuronal mitochondria resulting from an exposure of cultured hippocampal neurons to excitotoxic glutamate.

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1764-Plat Functional comparison of the Mg2+ permeable channels Lpe10 and Mrs2 in mitochondria

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The eukaryotic Mrs2 and the yeast Lpe10 proteins are located in the inner mitochondrial membrane and belong to the CorA family of magnesium transporter. These proteins include two transmembrane domains, of which the first one ends with the conserved gly-met-asn motif and cytosolic N- and C-termini. Lpe10 shares 30 % identity in amino acid sequence with Mrs2 but only the latter protein includes a basic C-terminal extension.

To functionally characterize Lpe10 channels we performed magfura-2 microscopy on isolated mitochondria with either overexpression or deletion of Lpe10 that significantly increased, or decreased respectively, Mg2+ influx in comparison to wild-type mitochondria. Similar results were observed by modulation of Mrs2 protein level. Side-directed mutagenesis of the highly conserved gly-met-asn motif abolished Mg2+ influx of Lpe10. Moreover cross-linking and blue-native polyacrylamide gel electroporesis (BN-PAGE) revealed that both Lpe10 and Mrs2 form multimers, suggesting assembly to homomeric channels. Electrophysiolical patch-clamp measurements on giant lipid vesicles fused with inner mitochondrial membrane particles of the yeast Mrs2 or Lpe10 overexpressing cells enabled to record single Mrs2 and Lpe10 channels. We have recently shown (Schindl et al., Biophys. J., 2007) that Mrs2 overexpression yielded a Mg2+ selective ion-channel with a conductance of about 155 pS that could be inhibited by Cobalt-hexaammine. Analysis of Lpe10 channels is currently under investigation and will be additionally discussed.

We conclude that both Lpe10 and Mrs2 form oligomeric complexes in the mitochondrial inner membrane, constituting Mg2+ permeable channels.

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1765-Plat Electrostatic Funneling of Nucleotide Binding in the Mitochondrial ADP/ATP Carrier

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The mitochondrial ADP/ATP carrier (AAC) is responsible for the exchange of ADP and ATP across the inner mitochondrial membrane. AAC switches between a cytoplasm-open state (c-state) and a matrix-open state (m-state) during nucleotide translocation. Although the structure of AAC in the c-state has been solved recently, the mechanism of nucleotide binding and the nature of conformational changes underlying the transition from the c-state to the m-state are not understood. Here we performed 150 ns molecular dynamics simulations on an AAC monomer embedded in a lipid bilayer. The simulations reveal a strong positive electrostatic potential, induced by the heavily charged AAC, which is found to be crucial for nucleotide binding; ADP molecules placed in different initial orientations are strongly attracted by the potential and funneled into the binding site of AAC where they converge into the same final orientation. To study the conformational change of AAC during ADP translocation, an ADP molecule was pulled through AAC towards the mitochondrial matrix. Our simulations reveal concerted outward rotation of the three odd-numbered helices of AAC, which opens the matrix half of the protein and allows for the translocation of the nucleotide. The discovered strong positive potential of AAC and its importance in substrate binding inspired us to examine other members in the mitochondrial carrier family (MCF). We calculated the net charges of 974 yeast membrane proteins including the 35 yeast MCF members. The results show that a significant positive net charge is a common feature shared by most MCF members. In addition to its significance in substrate binding, as demonstrated here for AAC, the positive net charge of mitochondrial carriers might also play an important role in partitioning of the proteins into the negatively charged inner mitochondrial membrane.

1766-Plat Effects of 4′-chlorodiazepam on Cellular Excitation-Contraction Coupling and Ischemia-Reperfusion Injury in Rabbit Heart

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Recent evidence indicates that the activity of energy-dissipating ion channels in the mitochondria can influence the susceptibility of the heart to ischemia-reperfusion injury. Ligands to the mitochondrial benzodiazepine receptor inhibit mitochondrial inner membrane ion channels and have been shown to protect the heart against arrhythmias and infarction. However, the effects of these compounds on cellular electrophysiology and Ca2+ handling have not been well characterized. In this study, we describe the effects of 4′-chlorodiazepam (4-ClDzp), a well-known ligand of the mBzr, on the physiology of both isolated cardiomyocytes and intact hearts. In isolated rabbit cardiomyocytes, 24μM 4-ClDzp evoked a significant reduction in the time to 50 and 90% repolarization of the cardiac action potential, associated with a decrease in inward currents. The peak intracellular calcium transient was also significantly smaller in

4-ClDzp. In intact perfused normoxic rabbit hearts, 4-ClDzp mediated a dose-dependent negative inotropic response, consistent with the notion that 4-ClDzp was reducing calcium influx. Hearts that underwent 30 minutes of global ischemia and 30 min of reperfusion were protected against reperfusion arrhythmias and post-ischemic contractile impairment when 4-ClDzp (24μM) was administered throughout the protocol. Moreover, a single bolus dose of 4-ClDzp given at the onset of reperfusion also effectively eliminated arrhythmias and restore contractile function after ischemia. In contrast, hearts treated with cyclosporin-A, a classical blocker of the mitochondrial permeability transition pore, were not protected against reperfusion arrhythmias. The findings indicate that the effects on 4-ClDzp on both mitochondrial and sarcolemmal ion channels contribute to protection against post-ischemic cardiac dysfunction. Of clinical relevance, the compound is effective when given on reperfusion, unlike other preconditioning agents.

1767-Plat Development Of The RyR-mitochondrial Calcium Coupling During Differentiation Of Cardiac Myoblasts

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Myoblasts and myocytes both display local Ca2+-coupling between the endo/sarcoplasmic reticulum (ER/SR) and mitochondria, supporting the propagation of IP3 receptor (IP3R)/ ryanodine receptor (RyR)-mediated Ca2+ signals to the mitochondrial matrix. However, it is unclear how the ER/SR-mitochondria local interactions are coordinated with the Ca2+-release channel switch (from IP3R to RyR) during differentiation. Here, H9c2 myoblasts and myotubes were used to model differentiation; mitochondrial morphology, IP3R/RyR-mediated cytoplasmic Ca2+ ([Ca2+]c)-signals and the ensuing mitochondrial matrix [Ca2+] ([Ca2+]m) rise were analyzed. Myoblasts contained relatively few, luminally connected elongated mitochondria, whereas myotubes were abundant in discrete, globular mitochondria. Ultrastructural (TEM) analysis showed that the ER/SR-mitochondrial associations involved a larger fraction of the mitochondrial surface in myotubes than in myoblasts. To study the ER/SR-mitochondrial interface in vivo, a FRET-generating ER-mitochondrial inducible linker system is currently evaluated. Fluorescence imaging of [Ca2+] signals using fura2 or ratiometric-pericams confirmed that activation of the IP3R-pathway by vasopressin and stimulation of RyRs by caffeine were effective only in myoblasts and myotubes, respectively to evoke a substantial [Ca2+]c or [Ca2+]m response. Similar results were obtained by direct stimulation of the corresponding receptors by IP3 or caffeine in permeabilized cells. Overexpression of skeletal muscle RyR1 in myoblasts did not affect the overall shape and mitochondrial morphology, providing a minimum model to study the role of RyR expression in the Ca2+-coupling. Based on pericam measurements of caffeine-induced [Ca2+] signals, essentially every cell displaying RyR1-dependent Ca2+ release also displayed a rapid [Ca2+]m response. Thus, as RyRs replace IP3Rs during muscle differentiation, the local coupling between RyR and mitochondrial Ca2+ uptake sites also appears. Expression of the RyR seems to be sufficient to induce their interaction with the mitochondria, though this process is accompanied by major changes in the mitochondrial morphology during myoblast differentiation.

Platform AK: Myosin & Myosin-Family Proteins

1768-Plat Role of the Specific Switch-2 Structure of Myosin 5 in Its Working Mechanism

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Myosin 5 is a processive vesicle transporter: as a two-headed single molecule, it can take numerous steps along the actin filament without detachment. The kinetic basis of this stepping is an ATPase mechanism in which strongly actin-bound states are predominant. The switch-2 loop is an essential element of the ATP binding site of myosins. Its consensus sequence is LDIXGFE where X is variable among classes (X=A/S in myosin 2; Y in myosin 5). A conformational change of switch-2, the open-closed transition, brings catalytic residues into place and is therefore necessary for ATP hydrolysis, whereas a closed-open transition may be required for product release. To examine the role of the specific switch-2 sequence in the myosin 5 mechanism, we created constructs containing point mutations at the X position of switch-2 (Y439S, Y439A, Y439E). Applying fluorescence spectroscopic, steady-state and transient kinetic methods we found that mutations favoring the open state caused an increase of the basal ATPase activity by accelerating phosphate release. Interestingly, all mutants exhibited decelerated actin-activated ADP release and ATPase activity compared to the wild-type. It was suggested that it is the bulky Y439 that causes switch-2 to adopt a unique structure in the rigor state of myosin 5. Correspondingly, we found that the mutants show higher temperature dependence of actin binding, more resembling muscle myosin 2. Our results imply that switch-2 plays a role in the processive mechanism of myosin 5 through modulation of actin binding and product release.

1769-Plat Role of the Upper 50 kDa Domain in Coupling the Actin- and Nucleotide-Binding Regions of Myosin V

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Myosin V is an excellent model to examine structural transitions associated with energy transduction because its high actin affinity allows examination of actin-induced conformational changes in the weak and strong actin binding states. Our previous work has demonstrated that labeling myosin V in the upper 50 kDa domain with the biarsenical dye FlAsH can serve as an acceptor for fluorescence resonance energy transfer (FRET) studies with mant labeled nucleotides and IAEDANS actin. These FRET studies suggest that myosin V can adopt a conformation in which the