2. Characterization of line tension, by forming transient pores in cell membranes; these pores have been generated by optoporation of vesicles derived from various lipids and line tensions calculated from the rate of pore closing. Results reveal interesting differences between the vesicles that are a function of hydrocarbon chain length and phase behavior of the membrane region.
3. Deformation and fusion of vesicles induced by a weak optical force generated from a focused laser beam; these studies provide information on the membrane bending and curvature.

Current studies are also focused on the inclusion of mechanosensitive proteins such as gramicidin in the vesicle bilayer region to gain further insights into mechanotransduction. Significant results of these studies will be presented.

Membrane Transporters & Exchangers

408-Pos An EPR Spectroscopy Analysis of the Magnesium Transport System CorA in the Closed-State

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Board B240

In bacteria, magnesium uptake is primarily mediated by the CorA family of membrane proteins of which the ortholog from *Thermotoga maritima* has been recently crystallized, revealing an unprecedented fold. Using a cystein mutagenesis scan approach, we carried out mobility, solvent and O_2 accessibility measurements on 102 positions within the CorA sequence. The scan starts from residue 246, located at the very beginning of the stalk helice connecting the cytoplasmic domain to the transmembrane domain, and ends-up at the C-terminal residue after TM2. Each mutant was individually spin-labelled and reconstituted into liposomes in the presence of a saturating concentration of Magnesium. Solvent accessibility data were mapped on the crystal structure of CorA and the structure was relaxed to fully satisfy the EPR constrains using an *in silico* pseudo-atom approach based on a modified CHARMM force-field, method detailed by P. Sompornpisut *et al.*

We also provide molecular constrains to build the connecting loop between TM1 and TM2 (the most conserved motif), absent in all the crystal structures. Our modeling procedure uses an *ab-initio* step (using Rosetta), with a second step were models are later constrained to satisfy EPR derived data. Co-(III) hexamine, a structural analog of the fully hydrated Mg^{2+} has been showed to inhibit in vivo CorA mediated Mg^{2+} accumulation, a property that has been interpreted as CorA and related transport systems must initially bind a hydrated cation. Based on that observation, we have built our model so that the extracellular loops can accommodate an ion of the size of a hydrated Mg^{2+}.

Together, these results provide structural and dynamic insights on CorA embedded in a native environment; this model will serve our on going study on conformational changes associated with gating.

409-Pos Determining The Osmotic Permeability Coefficient Of Single Transporters And Channels In Plasma Membranes Of Epithelial Cells Via Fluorescence Correlation Spectroscopy (FCS)

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Board B241

Osmotic water flow dilutes the solution it enters. Since the effect is maximal in stagnant water layers close to epithelia, the actual osmolyte concentration which acts as a driving force is unknown. As a result, the epithelial water permeability is usually underestimated. Calculation of the turnover rate per transport protein is additionally hampered by uncertainties in determining transport protein concentration in plasma membranes. We applied fluorescence correlation spectroscopy (FCS) to carry out spatially resolved measurements of dye dilution in the aqueous solution close to MDCK monolayers which stably expressed AQP1. A mathematical model was developed to derive the velocity of water flow and osmolyte concentrations in the immediate vicinity of the epithelial from the time-dependent dye distribution. The validity of the approach was confirmed by steady state measurements of osmotic water permeability which were undertaken by scanning ion sensitive microlectrodes. By moving the observation volume of the confocal microscope to the plasma membrane, the transport protein concentration was determined by FCS. For this purpose, we tagged the human sodium glucose co-transporter (hSGLT1) to EGFP. The osmotic permeability coefficient of single hSGLT1 molecules was determined using this procedure in combination with aqueous dye dilution measurements.

410-Pos Characterization of the Proteolytic Cleavage of the hCTR1 Copper Transporter that Occurs in the Absence Of O-glycosylation at Ser27

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Board B242

The human copper transporter hCTR1 is a homotrimeric plasma membrane protein. The hCTR1 protein contains an extracellular amino-terminus of 65 amino acids that contains N-linked polysaccharides at Asn15. We showed that hCTR1 also contains O-linked polysaccharides at Thr27. In the absence of O-glycosylation at

Thr27, hCTR1 is proteolytically cleaved, leaving a partly functional hCTR1protein lacking the first 30 amino acids.

We are investigating the cellular location of the proteolytic activity and the cleavage site in the hCTR1 protein. Previous work indicates that cleavage occurs within a five amino acid sequence (ASHSH, residues 29–33), which does not contain obvious protease cleavage sites. We constructed a series of hCTR1 double mutants that contain:

1. a T27A substitution (which causes cleavage), and
2. various substitutions in the ASHSH sequence.

We are overexpressing these mutants to determine which amino acids in the ASHSH peptide are required for cleavage. Results thus far indicate that A29 and S30 are most important for cleavage. The cleavage patterns of all double mutants will be presented, as well as mutations that delete or change the entire ASHSH sequence.

We are also investigating where cleavage takes place in cells expressing mutant (cleaved) hCTR1. We looked for the cleaved, (FLAG-tagged) amino-terminal glycopeptide in cell lysates, in purified and fractionated membranes, and in the growth media. We observed low levels of a 10–12 kDa glycopeptide in the growth media. The peptide was isolated using both anti-FLAG IP and pull-down with lectin beads that bind to the N15-linked polysaccharides in the cleaved glycopeptide. Further experiments will be presented to clarify whether hCTR1 lacking O-linked glycosylation is cleaved in the plasma membrane (thus releasing the glycopeptide), as our initial results suggest.

411-Pos The Role Of The Lateral Membrane Packing Density For The Function Of The ABC Transporter P-Glycoprotein (ABCB1)

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Board B243

P-glycoprotein (ABCB1) is an ABC transporter that moves structurally very diverse drugs from the inner to the outer plasma membrane leaflet at the expense of ATP hydrolysis. It is highly expressed in membranes with protective functions and plays an important role in the absorption and excretion of drugs. ABCB1 functions in membranes of different lipid composition, exhibiting different lateral packing densities, π_M. Since little quantitative data were available on the lateral membrane packing density, π_M, and its role for ABCB1 function, we estimated the lateral membrane packing density for different Pgp containing membranes and investigated the influence on the concentration of half maximum activation of ABCB1 and the rate of ATP hydrolysis. The former varies by more than one order of magnitude whereas the latter varies only slightly. Pgp is highly size-adaptable and moreover facilitates the transport of charged compounds across the membrane. This is in marked contrast to uncatalyzed flip-flop or diffusion into the cell that decreases exponentially with increasing lateral packing density of the membrane and increasing cross-sectional area of the drug, A_D, and does not allow for the translocation of compounds in their charged form.

412-Pos Exploration of the Ribose Transport Pathway in the Rbs ABC Transporter Through Cofactor Variation

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Board B244

ATP Binding Cassette (ABC) transporters are transmembrane transporters that use the energy released by ATP hydrolysis to transport a wide array of substrates. They are found in all kingdoms of life, and are complicit in various genetic conditions, such as cystic fibrosis, macular degeneration, and multi-drug resistance. The *E. coli* ribose transporter (RbsABC) is a multisubunit ABC transporter complex with a periplasmic ribose binding domain, a transmembrane domain dimer, and a cytoplasmic nucleotide binding domain. The ribose transport complex has been shown to assemble and disassemble into distinct combinations of the subunits based on the presence of cofactors (ATP and analogues, ADP, orthovanadate, and magnesium), suggesting a series of steps for how the subunits associate and subsequently transport ribose. What is unknown, however, is the step (or steps) at which ribose is transported through the membrane. To answer this question, we incubated different forms of the ribose transport complex with radio-labeled ribose and assayed binding using equilibrium dialysis. This allowed us to determine the affinity to ribose of several different forms of the complex, representing progressive steps on the pathway. We observed a reduction in affinity of the complex for ribose when the complex was in the presence of AMP-PNP, a non-hydrolyzing ATP analogue, compared to the complex when no cofactors were present. We are currently endeavoring to observe the effects of mutagenesis on key residues to further explore how they affect ribose binding and transport.

413-Pos Enhacement Of D-glucose Uptake Rate By Toxins Inhibitors Of Protein Phosphatases In Two Hepatic Cellular Models

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Board B245

D-Glucose is transported into cells through Na+/ Glucose symporters or the widely extended group of facilitative transporters generally named GLUT. GLUTs are transmembrane proteins with different isoforms with a tissue-specific expression. Here we present a kinetic study of D-glucose uptake in two cellular models; an hepatocyte cell line that only express GLUT 1 (Clone 9) and primary cultured rat hepatocytes that express GLUT 2. D-glucose uptake assays were performed by using 2-NBDG, a fluorescent D-glucose derivate that gets into cells by the same transporters than D-glucose.

Time-course of D-glucose uptake was faster and higher in primary hepatocytes than in the cell line. Additionally we tested the effect of three self-related toxins on the D-glucose uptake rate of the two cell types. Microcystin-LR (MC-LR) and Okadaic Acid (OA) that exhibit clear protein phosphatase-2A and -1 inhibition effect, enhanced D-glucose uptake in primary hepatocytes. However, only Okadaic Acid increased the glucose transport into Clone 9 cells because MC-LR needs to get into the cells by bile acid transporters absent in the cell line. Methyl Okadaate, a toxin with poor or lack of protein phosphatase-1 and -2A inhibition action, did not induce any change in the uptake rate of D-glucose into any of the cells used in our study.

414-Pos Lactose permease mutants: A comparative molecular dynamics study

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Board B246

LacY from E. coli is a lactose/H+ symporter. The conservative replacement of cysteine by glycine at position 154 has profound effects on the functional and physical properties of LacY. This mutant form of the transporter, C154G LacY, binds ligand at least as well as the wild type (WT), and yet its transport ability is impaired. Furthermore, its physical properties are drastically altered, exhibiting much greater thermal stability and a reduced tendency to aggregate compared to WT; properties that made it more amenable to crystallographic trials. The mutation site is located in TM5 at the crossing point of TM1 and TM5. At position 154, the mutant glycine is in close proximity to glycine 24 in TM1. The loss of activity has been attributed to the "unnatural" juxtaposition of the two glycine residues, which decreases the distance between TM1 and TM5 at the crossing point, increasing the strength of interaction between the two helices, thereby limiting the functionally important dynamic behaviour of this helix. We present a comparative molecular dynamics simulation study of multiple mutants at positions 24 and 154 of LacY. These simulations reveal that reduced bulk at the TM1 - TM5 crossing point compared to wild type does not lead to increased inter-helical association, as assessed by various criteria, including inter-helical contacts and inter-helical interaction energy. Instead, the mutants exhibited differences in their global conformational behaviour that indicate the mutations may be exerting their effect by a more subtle mechanism than increased inter-helix association. We also investigate mechanism by which only few amino acids substitutions at position 24 are able to successfully rescue activity in the C154G mutant. The simulations reveal that mutations at position 24 could lead to the altered dynamics of adjacent functionally important residues as has been previously proposed.

415-Pos Modeling Multiple Conformations of Lactose Permease in a Lipid Bilayer

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Board B247

The lactose permease of *E. coli* (LacY), which catalyzes the coupled symport of a galactoside and an H^+, is a paradigm for the Major Facilitator Superfamily of transport proteins. A combination of biophysical and biochemical findings with the 3D structures has led to a model for transport in which the sugar-binding site has alternating access to either side of the membrane. We have performed atomistic molecular dynamics simulations of LacY in a lipid bilayer in excess water with the protein chain in several conformations. The LacY crystal structures exhibit a single "inward-facing" conformation in which the protein chain is organized as two pseudo-symmetrical 6-helix bundles surrounding a large hydrophilic cavity open to the cytoplasm. Simulation trajectories starting with the inward-facing conformation provide insights into conformational differences between wild-type LacY and the C154G mutant, which strongly favors the inward-facing conformation. Connections are established between the specific environment of each transmembrane segment and both the protein topology and spatial organization through an analysis of the dynamic neighborhood of each amino acid residue. An experimental determination of the boundaries of the transmembrane segments based upon biophysical and biochemical data matches well with the interface between the hydrocarbon core and polar region of the lipid bilayer. Furthermore, the helix boundaries in the crystal structure fit well with the interface between the lipid bilayer polar region and the aqueous medium. Several lines of experimental evidence indicate that, upon substrate binding, LacY assumes multiple conformations. We have constructed alternative conformers to the crystal structure, including an outward-facing conformation employing inter-residue distance constraints based upon experimental data. The combination of these simulation trajectories contributes to the development of a detailed model of the conformational changes associated with the transport mechanism.

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416-Pos The Yeast Mitochondrial Citrate Transport Protein: Identification of the Lysine Residues Responsible for Inhibition Mediated by Pyridoxal 5′-Phosphate

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Board B248

We have previously concluded that the mitochondrial inner membrane citrate transport protein (CTP) contains at least two substrate binding sites which are located at increasing depths within the substrate translocation pathway. ***Site one,*** the more external of the two sites, consists of three residues (K83, R87, and R189), while ***site two*** is located near the middle of the membrane bilayer and consists of five residues (K37, R181, K239, R276, and R279). In the present

study, we examined the role of the three lysine residues located within these binding sites, as well as selected lysines located within other portions of the transporter, in mediating the inhibition of transport conferred by the lysine-selective reagent, pyridoxal 5′-phosphate (PLP). Our data with 7 lysine to cysteine substitution mutants reveal different roles for these wild-type lysine residues in mediating the sensitivity to inhibition by PLP. Thus, upon replacement of K83 with cysteine, 85% of the PLP-mediated inhibition of CTP function is lost. Moreover, replacement of either K37 or K239 with cysteine, caused a loss of 25% of the observed inhibition caused by PLP. In contrast, replacement of non-binding site lysines (i.e., K45, K48, K134, K141) with cysteine, resulted in little change in the PLP inhibition. Based upon these results, we conducted docking calculations which has led to the proposal of a binding mode for PLP. In combination, our data support the conclusion that PLP exerts its main inhibitory effect by binding to residues located within the two substrate binding sites of the CTP, with K83 being the primary determinant of the total PLP effect since the replacement of this single lysine abolishes nearly all of the observed inhibition by PLP.

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417-Pos Detecting Transport-related Conformational Changes In The Glutamate Transporter Homologue, Glt_{Ph}

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Board B249

Glutamate transporters (EAATs) are essential in clearing glutamate from the synapse, allowing precise control over excitatory synaptic function. Much has been learned in recent years about how these proteins function, however little is known about how the structural basis of this function; how do protein conformational changes lead to transport activity? A homolog of these proteins, Glt_{Ph} from *Pyrococcus horikoshii*, is functionally similar to the mammalian proteins; co-transporting substrate and sodium ions whilst having an uncoupled chloride conductance. Importantly, however it has been crystallized and its structure determined, making it an excellent model for understanding how structure and function are related in the EAATs. In order for transport to proceed, conformational changes must occur that allow alternating access of the substrate binding site to both the internal and external solutions. Using fluorescein maleimide labeling accessibility studies on single cysteine mutants of Glt_{Ph} we have identified regions of the protein involved in this process. Several residues become more accessible to fluorescent labeling in the presence of both aspartate and TBOA, a non-transported competitive inhibitor. Further investigation into the kinetics of labeling and changes in protease susceptibility will allow us to clarify the conformational changes that drive the transport cycle. Surprisingly, our accessibility studies implicate heretofore unexpected areas of the Glt_{Ph} protein in the conformational changes associated with substrate and inhibitor binding.

418-Pos EPR Spectroscopy of the Glutamate Transporter Homologue GltPH

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Board B250

The remarkable ability to maintain low levels of glutamate in the extracellular space following signaling events is achieved mainly by excitatory amino acid transporters (EAATs) located in the plasma membranes of both glial cells and neurons. Abnormal glutamate transporter function is implicated in Parkinson's disease, Alzheimer's disease, cerebral ischemia, epilepsy, and amyotrophic lateral sclerosis, underscoring the importance of understanding how these transporters function. Our research is centered on elucidating the structural and functional properties of glutamate transporters to reveal novel (i.e. small-molecule) approaches for treating these neuropathological conditions. To this end, we utilize EPR (electron paramagnetic resonance) to gain information on conformational changes associated with glutamate transport. Recently published crystal structures highlight conformational changes that occur in the extracellular gate during the binding of glutamate to the transporter, but do little to provide much information regarding conformational changes that occur in either the extra- or putative intracellular gates during the glutamate translocation step. We are using EPR measurements to determine conformational changes in both the extracellular and putative intracellular gates during the process of glutamate translocation. We have expressed and purified the bacterial glutamate transporter homolog, GltPH, reconstituted the protein in liposomes, and have demonstrated the ability to site-specifically spin-label the protein in liposomes. We are currently using EPR spectroscopy to gain information on subtle movements of the extra- and putative intracellular gates associated with glutamate translocation. EPR, which allows for the detection of changes in distances (5−20Å) between spin labels, allows us to define the relationships between proximity of the extra- and intracellular gates relative to each other during the process of glutamate translocation. By using this technique, we are gaining detailed information on the mechanism of glutamate transporter function.

419-Pos Hydroxy Fatty Acid - mediated proton transport

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Board B251

The transport mechanism of long-chain hydroxy fatty acids (HFA), which are important in anti-inflammatory action and neuroprotection, across plasma membranes is in dispute. According to two main hypotheses, protonated HFAs traverse across the membranes spontaneously similar to long chain fatty acids (FA, (1)) or their transport is facilitated by proteinaceous carriers (2). By using scanning pH-sensitive microelectrodes, we here demonstrate that the protonated

HFAs, such as 2-hydroxyhexadecanoic, 16-hydroxyhexadecanoic, 12-hydroxydodecanoic and 9,10,16-trihydroxyhexadecanoic acids, are able to move across planar lipid bilayers without protein assistance. The spontaneous permeability of 12-hydroxydodecanoic acid is at least two orders of magnitude larger than FA-permeability, facilitated by the uncoupling protein (3, 4). In concurrence with the theoretical model of weak acid transport, the pH value at maximum proton flux was almost equal to the pK of the studied HFA.

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420-Pos Dimeric Structure of Human Na+/H+ Exchanger Isoform 1 Overproduced in Sacchromyces Cerevisiae

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Board B252

The Na^+/H^+ exchanger isoform 1 (NHE1) is an integral membrane protein that regulates intracellular pH and cell volume by extruding an intracellular H^+ in exchange for an extracellular Na^+. Besides various physiological roles, human NHE1 (hNHE1) is implicated in heart disease and cancer cell migration. Although details of NHE1 regulation and transport are being revealed, little information is available on the intact protein structure. Our development of a successful overexpression and purification system for hNHE1 in *Saccharomyces cerevisiae* has enabled its structural characterization. Over-production of His-tagged protein and purification via Ni-NTA agarose and calmodulin sepharose chromatography yielded 0.2 mg of pure protein per liter of yeast culture. Reconstitution of hNHE1 in proteoliposomes demonstrated that the protein was active. Circular dichroism spectroscopy of purified hNHE1 revealed secondary structure of 41% α-helix, 23% β-sheet, and 36% random coil. Size exclusion chromatography indicated protein-detergent micelles sized in excess of 200 kDa, consistent with an hNHE1 dimer. Electron microscopy and single particle reconstruction of negatively stained hNHE1 showed a compact globular transmembrane domain and an apical ridge, which was assigned as the cytoplasmic domain. The reconstruction showed a clearly dimeric transmembrane domain, where each monomer compared well with the known crystal structure of the bacterial Na^+/H^+ exchanger, NhaA.

421-Pos ν-Calpain Selectively Targets Ca^{2+}-regulation in the Heart, Brain, and Kidney Splice Variants of NCX1

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Board B253

μ-Calpain (μCAPN) is a Ca^{2+}-activated protease abundant in mammalian tissues, including heart, brain, and kidney. Recently, Bano et al. (*Cell 120*:275–285, 2005) have reported that μCAPN cleaves and inactivates NCX3, a Na^+-Ca^{2+} exchanger subtype prominently expressed in skeletal muscle and neuronal tissues, whereas NCX2 was not affected. Here, we examined the effects of μXAΠN on alternatively-spliced variants of NCX1, commonly referred to as the heart (NCX1.1), kidney (NCX1.3), and brain (NCX1.4) subtypes. These exchangers are differentially regulated by Na^+ and Ca^{2+}. For all three, Na^+_i-dependent (I_1) inactivation of outward currents manifests as a decay of peak current to lower steady-state levels. However, for NCX1.1 and NCX1.4, Ca^{2+}_i-dependent regulation exhibits two distinct effects. Specifically, there is an overall activation of Na^+-Ca^{2+} exchange currents (I_2 regulation) and an alleviation of Na^+_i-dependent (I_1) inactivation as cytoplasmic Ca^{2+} is progressively elevated. With NCX1.3, however, the activation by cytoplasmic $[Ca^{2+}]_i$ is not associated with alleviation of I_1. Treatment of the cytoplasmic surface of patches with α-chymotrypsin eliminates all regulatory mechanisms. In this study, NCX1.1, NCX1.3 and NCX1.4 were expressed in *Xenopus* oocytes and outward NCX currents were measured using the giant excised patch technique. Following a 1-min exposure to 24 μg/ml μCAPN, Ca^{2+}-dependent (I_2) regulation of peak currents was ablated, whereas Na^+_i-dependent inactivation was unaffected. Moreover, higher $[Ca^{2+}]_i$ levels progressively eliminated Na^+_i-dependent inactivation in NCX1.1 and NCX1.4 but not in NCX1.3, similar to that observed prior to μCAPN treatment. Following μCAPN treatment, limited proteolysis with α-chymotrypsin fully deregulated all exchangers. Our results show that μCAPN selectively targets the Ca^{2+}-dependent (I_2) mechanism of NCX1 splice variants.

422-Pos Effect of ν-Calpain on CALX1.1, a Na^+-Ca^{2+} Exchanger from *Drosophila melanogaster*

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Board B254

CALX1.1, a Na^+-Ca^{2+} exchanger (NCX) from *Drosophila melanogaster*, is unique among NCXs in that its activity is inhibited by cytoplasmic Ca^{2+}. This unique regulatory phenotype can be eliminated by limited proteolysis of the intracellular surface of the exchanger with α-chymotrypsin (α-ChT). μ-Calpain is a Ca^{2+}-activated thiol protease distributed in a wide variety of tissues and has been postulated to inactivate NCX3 in neuronal cells under

conditions of excitotoxicity (Bano et al. *Cell 120*:275–285, 2005). In contrast, we have found that, in giant, excised membrane patches, mammalian NCXs are irreversibly *activated* by treatment with μ-calpain. Given the unique Ca^{2+} regulatory profile of CALX1.1, we examined the effects of μ-calpain on this exchanger to determine whether similar or distinct effects would be observed as compared with mammalian NCXs. CALX1.1 was expressed in *Xenopus laevis* oocytes and exchange currents were measured using the giant, excised patch clamp technique. Following a 30 s exposure to 18–96 μg/mL μ-calpain, outward Na^{+}-Ca^{2+} currents became less sensitive to the inhibitory effects of regulatory Ca^{2+}. For inward currents, where cytoplasmic Ca^{2+} serves as both a regulatory and a transport substrate, CALX1.1 also exhibits a Ca^{2+}-dependent inactivation of currents. Following μ-calpain treatment, the extent of inward current inactivation was also reduced. No current inactivation or further alterations in regulatory properties were detected following repeated application of μ-calpain. However, subsequent treatment of patches with α-ChT invariably abolished all ionic regulation of both outward and inward currents. Our results are consistent with the notion that μ-calpain affects NCXs by selectively targeting their Ca^{2+}-dependent regulatory mechanisms. It remains unknown whether similar deregulation by μ-calpain occurs within intact cellular systems.

423-Pos Dual Effect Of Na^{+}_{I} On Ca^{2+} Influx Through The Na^{+}/ca^{2+} Exchanger In Dialyzed Squid Axons. Experimental Data Confirming The Validity Of The Squid Axon Kinetic Model

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We have proposed a steady state kinetic model for the squid Na^{+}/Ca^{2+} exchanger which differs from other current models of regulation in that it takes into account into single kinetic scheme all ionic (Ca^{2+}_{i}- Na^{+}_{i}-H^{+}_{i}) and metabolic (ATP) regulations of the exchanger and in which the Ca_i-regulatory plays the central role in regulation. Although the integrated ionic-metabolic model predicts all squid steady state experimental data on exchange regulation a critical test for the validity of it is the predicted dual effect of Na^{+}_{i} on the steady state Ca^{2+} influx through the exchanger. To test this prediction, an improved technique for estimating isotope fluxes in squid axons have been developed which allows sequential measurements of ion influx and effluxes. With this method we report here two novel observations of the squid axon Na^{+}/Ca^{2+} exchanger:

1. At pH_i (7.0) and in the absence of MgATP, intracellular Na^{+} has a dual effect on Ca^{2+} influx: inhibition at low concentrations followed by stimulation at high $[Na^{+}]_i$. reaching levels higher than those seen without Na^{+}_{i};
2. In the presence of MgATP, the biphasic response to Na^{+}_{i} disappears and is replaced by a sigmoid activation.

Furthermore, the model predicts that Ca^{2+} efflux is monotonically inhibited by Na^{+}_{i}, more pronouncedly without than with MgATP. These results are predicted by the proposed kinetic model. Although not fully applicable to all exchangers, this scheme might provide some insights on expected net Ca^{2+} movements in other tissues under a variety of intracellular ionic and metabolic conditions.

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424-Pos The Na/Ca Exchanger is a Mechanosensitive Transporter

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Na/Ca exchange (NCX) activity was measured as Na-dependent Ca or Ba fluxes using the fluorescent probe fura-2 in transfected Chinese hamster ovary cells expressing the "non-regulated" mutant Δ(241-680). Cells were preincubated with gramidicin to clamp cytosolic Na at the desired concentrations, usually 100 mM. NCX activity was initiated either by

(a) superfusing the cells with Ca- or Ba-containing assay medium for 20 s, or
(b) aspirating the preincubation medium and rapidly replacing it with the assay medium.

Using the superfusion procedure, Ba uptake was biphasic, displaying a high rate of Ba uptake while the solution was being applied, but declining 5- to >10-fold following the cessation of solution flow. Ba efflux in exchange for extracellular Na or Ca (Ba/Ca exchange) was similarly biphasic. Ca uptake was also biphasic, showing a rapid increase in cytosolic [Ca] to a peak value, followed by a decline in $[Ca]_i$ to a lower steady-state value. Reinitiating solution flow multiple times led to multiple stimulations of exchange activity. When NCX activity was initiated by the rapid aspiration-replacement approach, the rapid phase of Ba influx was greatly reduced in magnitude and Ca uptake became nearly monophasic. Solution superfusion did not produce any obvious changes in cell shape or volume. NCX-mediated Ba and Ca influx were also sensitive to osmotically-induced changes in cell volume. NCX activity was stimulated in hypotonic media and inhibited in hypertonic media. The osmotically-induced changes in activity occurred within seconds and were rapidly reversible. We conclude that NCX activity responds to mechanical effects induced by either solution flow or osmotic volume changes.

425-Pos The Second Calcium Binding Domain Of The Sodium-Calcium Exchanger Has An Essential Role In Regulation

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The Na^+-Ca^{2+} exchanger plays a central role in cardiac contractility by maintaining Ca^{2+} homeostasis. The exchanger is composed of nine membrane-spanning segments and a large intracellular loop containing two Ca^{2+}-binding domains (CBD). CBD1 has been shown to be involved in binding regulatory Ca^{2+} but a role of CBD2 has never been defined. Here, we present structures of CBD2 in the Ca^{2+}-bound (1.7 Å resolution) and Ca^{2+}-free (1.4 Å resolution) conformations. Like CBD1, CBD2 has a classical immunoglobulin fold but, unlike CBD1, it coordinates two Ca^{2+} ions instead of four. In the absence of Ca^{2+}, Lys585 stabilizes the structure by coordinating two acidic residues (Asp552 and Glu648), one from each of the Ca^{2+} binding sites. Stabilization by Lys585 prevents the substantial protein unfolding that is seen in the Ca^{2+}-free form of CBD1. To identify the role of CBD2, we mutated each of the acidic residues that coordinate the Ca^{2+} ions and examined the effects of these mutations on regulation of exchange activity. Three mutants (E516L, D578V, and E648L), at the primary Ca^{2+} site, completely remove Ca^{2+} regulation, placing the exchanger into a constitutively active state. These are the first data defining the role of CBD2 as an essential regulatory domain in the Na^+-Ca^{2+} exchanger.

426-Pos Conformational Changes of the Ca2+-Binding Domain of the Na+-Ca2+ Exchanger Monitored by FRET in Transgenic Zebrafish Heart

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Board B258

The Na/Ca exchanger (NCX) is the major Ca extrusion mechanism in cardiac myocytes. The activity of the cardiac Na/Ca exchanger is dynamically regulated by intracellular Ca. Previous studies indicate that Ca binding to a high-affinity Ca-binding domain (CBD1) in a large intracellular loop induces conformational changes which could be detected by FRET. We generated transgenic zebrafish with cardiac-specific expression of the Ca binding domain (CBD1) linked to YFP and CFP. With this transgenic fish model, we were able for the first time to monitor conformational changes of the Ca regulatory domain of NCX in intact hearts. Treatment with the positive inotropic agents ouabain and isoproterenol increased Ca transients as well as the changes in FRET efficiency between contraction and relaxation. These results indicate that Ca regulation of the Na/Ca exchanger changes with inotropic state. The transgenic fish models will be useful to further characterize the regulatory properties of the Na/Ca exchanger in vivo.

427-Pos Development Of A Na^+/H^+ Exchange Model In Cardiac Myocyte

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The Na^+/H^+ exchanger plays a central role in the regulation of intracellular pH and cell volume in cardiac muscle cells. Many experimental studies have been conducted to clarify the properties of Na^+/H^+ exchange. The experimental data might be divided into 3 categories;

1. The dependence of the Na^+/H^+ exchange on $[Na^+]_i$, $[Na^+]_o$, $[H^+]_i$, and $[H^+]_o$ investigated using cardiac myocytes.
2. The forward and reverse modes studied under more drastic situation.
3. The turnover rate of individual Na^+/H^+ exchange molecule.

Despite a pile of those experimental findings, no kinetic model has been established yet which could explain compatibly all the above measurements. Here, we have developed a model of the Na^+/H^+ exchange. First, 6- state and 8- state model schema were assumed to embody the process of ion binding and releasing. The intracellular H^+ modulation site was also considered with Hill coefficients of 2 and 3. Then, Levenberg-Marquardt fitting method was applied to the experimental data of category (1) to search possible parameter sets of rate constants and dissociation constants for ion binding. The data of category (1) were only used from sheep Purkinje fiber at 37°C. Plenty of local minima of chi-square function were sought out by starting different points within a wide parameter range. Subsequently, the parameter sets were selected among the local minima, which satisfied both the data of category (2) and (3). As a consequence, comparison of the chi-square revealed that the 8-state model with Hill coefficient 3 was most appropriate. In addition, 12 parameter sets could be obtained to satisfy all the experimental results. By implementing this new Na^+/H^+ exchange model in the whole cell model, mechanisms underlying the pH homeostasis will be analyzed under physiological and pathophysiological conditions.

428-Pos Ca^{2+}-Dependent Conformational Changes of Cardiac Na^+-Ca^{2+} Exchangers Detected by FRET

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Board B260

Ca^{2+}-dependent conformational changes occurring in the large cytoplasmic loop of the Na^{+}-Ca^{2+} exchanger (NCX1.1) were monitored by changes in FRET between exchangers tagged with CFP or YFP. The protein fluorophores were inserted at positions 266, 371, 467 and 548 within the large cytoplasmic loop. As determined by electrophysiology, all fluorescent NCXs are active with properties generally similar to untagged exchangers. CFP- and YFP-tagged exchangers were coexpressed in Xenopus oocytes from which plasma membrane sheets were isolated (Ottolia et al, AJP, 292(4):C1519-22, 2007). Upon addition of Ca^{2+}, the pair NCX-266CFP+NCX-266YFP showed decreased FRET in a dose-dependent manner. Similar results were observed when NCX-266CFP was coexpressed with either NCX-371YFP or NCX-467YFP. However, the Ca^{2+} dependency of FRET measured between NCX-266CFP and NCX-467YFP showed a decreased sensitivity to Ca^{2+}. Micromolar concentrations of either Mg^{2+} or Ba^{2+} did not evoke FRET changes. Preliminary data indicate that Ca^{2+} does not induce changes in FRET between the pairs NCX-371CFP+NCX-371YFP or NCX-467CFP+NCX-467YFP.

These results indicate that

1. The full-length NCX undergoes Ca^{2+}-dependent conformational changes,
2. The large cytoplasmic loops of adjacent exchangers are in close proximity supporting the recent evidence for oligomerization of NCX and
3. Ca^{2+} changes the distance between the large intracellular loops.

Finally, our data suggest that during Ca^{2+} regulation the N-terminal portion of the large cytoplasmic loop of one exchanger moves with respect to the Ca^{2+}-binding domains of an adjacent NCX.

429-Pos Intermolecular Crosslinking of Na^{+}-Ca^{2+} Exchanger Proteins: Evidence for Dimer Formation

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The cardiac Na^{+}-Ca^{2+} exchanger (NCX1) is modeled to contain nine transmembrane segments (TMS) with a pair of oppositely oriented, conserved sequences called the α-repeats that are important in ion transport. Residue 122 in the α-1 repeat is in proximity to residue 768 in TMS 6 and the two residues can be crosslinked. We examined substrate specificity of this intramolecular crosslink and find that crosslink formation is favored by the presence of extracellular Na^{+}. We also find evidence that NCX1 can form dimers. At 37 ºC in the absence of extracellular Na^{+}, copper phenanthroline catalyzes disulfide bond formation between cysteines at position 122 in adjacent NCX1 proteins. Dimerization was confirmed by histidine tag pull-down experiments that demonstrate the association of untagged NCX1 with histidine-tagged NCX1. Dimerization occurs along a face of the protein that includes parts of the α-1 and α-2 repeats as well as parts of TMS 1 and TMS 2. We do not see crosslinking between residues in TMS 5, TMS 6, or TMS 7. These data provide the first evidence for dimer formation by the Na^{+}-Ca^{2+} exchanger.

430-Pos Activation of Na+-Ca2+ Exchange by Protein Kinase A in Scallop Muscle Membranes

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Board B262

In an isolated membrane fraction, Ser^{621} in the CBD2 subdomain of the large cytoplasmic (f) loop of sea scallop adductor muscle Na^{+}-Ca^{2+} exchanger (NCX-SCA) is known to be a substrate for protein kinase A (PK-A). Accessibility of the site to PK-A is blocked under conditions where the Ca^{2+} regulatory sites are occupied. Measurements of Na^{+} gradient driven $^{45}Ca^{2+}$ uptake have now been carried out, and show a consistent ~ 40% increase in Ca^{2+} accumulation rate after treatment with the membrane vesicles with PK-A. Whether this effect involves solely Ser^{621}, or whether activation also requires phosphorylation of Thr^{366} and/or Ser^{374}, which lie in consensus sequences for PK-A phosphorylation N-terminal to the Ca^{2+} regulatory domain, remains to be determined. Treatment of scallop muscle membranes with chymotrypsin caused a ~ 5-fold activation of enzyme activity, just as with vertebrate exchangers; but trypsin, which is known to excise a 37 kDa segment from the f loop of NCX-SCA, destroyed the enzyme activity. Calpain had the same effect as trypsin. If vertebrate exchangers are affected by calpain in the same way, these results would be consistent with the malfunctioning of NCX3 in ischemic stroke being due to its inactivation rather than its activation.<br

431-Pos Regulation of Cardiac Na Transporters by Endocytosis

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Relatively little is known about the trafficking of cardiac transporters and channels. Except for metabolite transporters, cardiac ion channels and transporters do not appear to be harbored in internal membrane storage depots. Accordingly, surface densities can be regulated only by transcription, translation, and the removal of proteins from the surface membrane. We report on new model systems to attempt to study transporter removal from and insertion into the surface membrane. In BHK cells, chemical labeling under a wide variety of conditions identified three factors that promote internalization of Na/Ca exchangers (NCX1); high cytoplasmic Na, metabolic stress, and high PIP2. In patch clamped BHK cells, massive endocytosis and loss of NCX1 currents can be induced by sequentially removing cytoplasmic ATP, inducing a Ca transient,

and then reintroducing ATP. In whole hearts, chemical labeling suggests that NCX1 is internalized in response to multiple interventions that induce 'ischemic preconditioning' of the heart, and NCX1 currents in myocytes are correspondingly decreased in preconditioned cells. Na/K pump currents are highly stable in excised giant patches, but they run down substantially under our whole myocyte conditions. In rodent myocytes, both NCX1 and Na/K pump currents can run down completely in a few minutes during patch clamp with large-diameter pipettes, and capacitance decreases in parallel. Decreases of both capacitance and Na transporter currents can be blocked by GTP-gamma-S, and sometimes partially reversed. Run-down is not blocked by GDP-beta-S, and in some cases appears enhanced. Finally, we report on progress with NCX1 fusions with photo-convertible proteins (Dendra2) to monitor NCX1 mobility (and eventually trafficking) by optical methods.

432-Pos Examination of Ionic Regulation of NCX1 and NCX2 Using Chimaeric Exchangers

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Board B264

Sodium-calcium exchangers (NCX) are ion counter-transporters that play an important role in trans-sarcolemmal Ca^{2+} removal in most cell types. Alternatively spliced variants of NCX1 are often expressed in a tissue-specific manner and differ only in a small portion of their large cytoplasmic loop. Specific combinations of mutually-exclusive and cassette exons are associated with profound effects on ionic regulation in NCX1. While the NCX2 exchanger exhibits a unique ionic regulatory profile compared to NCX1, no alternative splicing has been identified for this exchanger. To investigate the role of the first mutually exclusive exons in NCX1 (A and B) and their analogous counterpart in NCX2, chimeric exchangers were constructed which interchanged these exons between NCX1 and NCX2. Additional chimeras were then created interchanging the respective XIP regions from NCX1 and NCX2. Chimaeric constructs were expressed in *Xenopus* oocytes and outward Na^+-Ca^{2+} exchange activity was assessed using the giant, excised patch technique. Substitution of exon A from NCX2 with the corresponding A or B exons from NCX1 greatly reduced the rate and extent of Na^+-dependent inactivation, independently of intracellular [Ca^{2+}]. However, replacement of both exon A and the XIP region from NCX2 with the corresponding regions from NCX1.4 re-establishes a wild-type NCX2 profile. Moreover, this construct gains an additional phenotype of NCX1.4, that of alleviation of Na^+-dependent inactivation at higher [$Ca^{2+}{}_i$]. The first mutually exclusive exon is therefore critical in determining Na^+ and Ca^{2+}-dependent regulatory properties in NCXs and appears to interact with other regulatory regions of the cytoplasmic loop (i.e. the XIP region) to achieve distinctive regulatory phenotypes.

433-Pos Na-dependent Inactivation of NCX in Transfected CHO Cells

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NCX activity was compared in CHO cells expressing either the wild-type NCX or mutants that are either resistant (K229Q) or hypersensitive (F223E) to Na-dependent inactivation (mutants kindly provided by Drs. D. Nicoll and K. Philipson, UCLA). NCX activity was measured as Ca uptake in fura-2 loaded cells treated with gramicidin to clamp cytosolic Na at the concentrations indicated. At 10 mM Na, no significant differences in Ca uptake were observed between the wild-type and mutant exchangers. At 140 mM Na, the wild-type and the K229Q mutant showed robust Ca uptake, while the activity of the F223E mutant was strongly inhibited. The activity of the F223E mutant could be restored either by increasing the concentration of Ca in the assay medium, or by reducing the concentration of extracellular Na. Na-dependent inactivation is promoted by PIP2 depletion and by low pH. In cells expressing the muscarinic acetylcholine receptor (kindly provided by Dr. D. Hilgemann, UT Southwestern Medical Center), extensive PIP2 depletion could be induced by applying 100 μM carbachol. PIP2 depletion with carbachol actually enhanced the activity of the wild-type NCX at 10 mM Na, but had little effect at 140 mM Na; the K229Q mutant behaved similarly. Carbachol induced a further inhibition of NCX activity in the F223E mutant. The cytosolic pH was reduced to 6.4 by treating the cells with the combination of monensin and nigericin (10 μM each). At pH 6.4 and 140 mM Na, the NCX activities of the wild-type exchanger and the F223E mutant were strongly inhibited; the K229Q mutant was less strongly inhibited. We conclude that in transfected CHO cells the wild-type NCX is resistant to Na-dependent inactivation under normal physiological conditions, and becomes susceptible to inactivation only at low cytosolic pH.

434-Pos Both NCX and NCKX families regulate Ca^{2+} homeostasis in Human Aortic Smooth Muscle Cells

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Two families of Na^+/Ca^{2+} exchangers have been demonstrated in mammals: NCX, where Ca^{2+} movement is dependent solely upon sodium; and NCKX, where Ca^{2+} movement is also dependent on potassium. While both families have been studied extensively in the heart and brain, very little is known about NCX and NCKX in blood vessels. Therefore, we sought to characterize the expression and function of Na^+/Ca^{2+} exchange in human aortic smooth muscle cells (hASMC). To identify the role of Na^+/Ca^{2+} exchangers in the regulation of Ca^{2+} homeostasis, cytoplasmic free Ca^{2+} ($[Ca^{2+}]_{cyt}$) in hASMC was measured with a digital Ca^{2+} imaging system. After

voltage-gated Ca^{2+} channels and Na^+ pump were pharmacologically blocked by nifedipine (10 μM) and ouabain (1 mM), $[Ca^{2+}]_{cyt}$ signal induced by membrane depolarization was largely inhibited. However, in the presence of nifedipine and ouabain, when the cells were superfused using a Li^+ solution with 0 K^+ and 0 Na^+ to respectively inhibit the activity of NCKX and activate the reverse mode of NCX, a large increase in $[Ca^{2+}]_{cyt}$ signal was observed. Also, when NCX was inhibited by KB-R7943 (10 μM) and K^+ (5 mM) was restored to stimulate the activity of NCKX, a large increase in $[Ca^{2+}]_{cyt}$ signal was detected. After both NCX and NCKX were inhibited by KB-R7943 and 0 K^+, superfusion of 0 Na^+ did not cause any increase in $[Ca^{2+}]_{cyt}$. Expression of NCX1 and NCKX3 was detected in hASMC. Taken together, our data provide evidence for the expression and function of NCX1 and NCKX3, which may contribute to controlling $[Ca^{2+}]_{cyt}$ homeostasis in human blood vessels.

435-Pos Upregulation Of Cardiac Sarcolemmal NCX Current By Insulin Involves XIP Interaction Domain

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Board B267

The cardiac sarcolemmal Na^+/Ca^{2+} exchanger (NCX) plays an important role in Ca^{2+} extrusion and is thus essential for Ca^{2+} homeostasis. The large cytoplasmic domain (f-loop) participates in the regulation of NCX by various intracellular factors. Previously, residues 562-679 of the f-loop were show to be determinants of NCX inhibition by exchanger inhibitory peptide (XIP) (Maack et al, *Circ. Res*. 2005). Here we show that deletion of the same region eliminates the enhancement of NCX current by insulin, which has been reported to alter myocardial contractility. NCX current (I_{NCX}) was measured in adult guinea pig myocytes, either freshly isolated (FM) or cultured for up to 48 hrs (CM), and in myocytes after adenoviral expression of canine NCX1.1 with the 562–670 f-loop deletion ($NCX_{D562–679}$). I_{NCX} was recorded by whole-cell patch clamp as the Ni^{2+}-sensitive current at 37°C with intracellular Ca^{2+} buffered. Insulin (1 microM) increased I_{NCX} (at +80mV) in FM (n=9) and in CM (n=6) by 110% and 64%, respectively ($p<0.05$). The effect of insulin was decreased in myocytes expressing $NCX_{\Delta562–679}$ (+42%; n=7; N.S.) when XIP was not included in the pipette solution, and was further suppressed (+11%; n=9; N.S.) when XIP (100 microM) was included to block any remaining native guinea-pig I_{NCX}. The insulin effect on I_{NCX} was only partially inhibited by wortmannin (100nM), suggesting that the mechanism involves additional pathways besides phosphatidylinositol-3-kinase activation. The finding that the 562–670 f-loop domain is implicated in both XIP and receptor-mediated modulation of NCX highlights its important role in acute physiological or pathophysiological regulation of Ca^{2+} balance in the heart.

436-Pos Inactivation of cardiac mitochondrial K /H exchange by quinine exposes matrix acidification and K influx by putative Ca -dependent K channels

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Introduction: We reported previously that low doses of NS1619, a putative opener of mitochondrial Big K^+ channels (mBK_{Ca}) initiates preconditioning in isolated hearts and increases respiration and reactive oxygen species (ROS), without altering membrane potential in isolated, non phosphorylating mitochondria. Our aim was to test if mBK_{Ca} opening stimulates K/H exchange (KHE) to alter matrix pH and K^+ in K^+-containing buffer.

Methods: Mitochondria were isolated from guinea pig hearts by differential centrifugation and the final pellet was suspended in isolation buffer with K^+ or pH sensitive dyes PBFI and BCECF, respectively, for 10 min at 25°C. Excess buffer dyes were then washed out by centrifugation. The K^+ ionophore valinomycin (0.25 nM) and CCCP (4 μM) were used to verify K^+ and H^+ influx in experiments with and without KHE inhibition (KHEI) by 500 μM quinine.

Results: KHEI increased matrix pH, indicating active KHE and proton leak. Valinomycin increased and then decreased matrix pH without KHEI and increased K^+ influx (by 30%) only with KHEI. Without KHEI, NS1619 had no effect on pH or K^+; with KHEI, NS1619 showed a dose dependent increase in K^+ influx; at 30 μM there was a small increase in K^+, but at 50 and 100 μM, NS1619 increased K^+ influx by 6% and 15%. With KHEI, NS1619 caused a decrease in pH.

Conclusion: This study demonstrates that opening putative mBK_{Ca} channels with NS1619 increases matrix K^+ if quinine is present to block KHE. Without inhibition of KHE, K^+ influx is balanced by K^+ efflux via KHE. The recovery of matrix pH with NS1619 after inhibition of KHE could be due to an electrophoretic proton leak or faster respiration induced by NS1619.

437-Pos Effects of Different Inhibitors on the Sarcoplasmic Reticulum Ca-ATPase

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Board B269

Sarcoplasmic reticulum (SR) Ca-ATPase is a well characterized cation transport ATPase that is obtained with vesicular fragments of SR. SR native vesicles were adsorbed on a thiol/phospholipid bilayer anchored to a gold electrode, the so-called solid supported membrane (SSM), and the SR Ca-ATPase was activated by concen-

tration jumps of an appropriate substrate through a rapid solution exchange (1,2).

By employing the SSM method we detected pre-steady state charge movements within a SR Ca-ATPase cycle, obtained with SR vesicles adsorbed on a SSM and subjected to Ca^{2+} jumps in the absence of ATP and to ATP jumps in the presence of Ca^{2+}. These charge movements are attributed to the binding of calcium ions to the enzyme in the ground state and to their release from the enzyme phosphorylated by ATP (2).

We have performed electrical measurements to compare systematically the effects of various inhibitors on the ion transport mechanism of the SR Ca-ATPase. Our results show that the charge movements generated by the SR Ca-ATPase are affected by these compounds demonstrating different degrees of potency and specificity. From this preliminary study we may conclude that the mechanism of inhibition involves stabilization of intermediate states of the SR Ca-ATPase cycle, whereby progress and completion of the cycle are impeded.

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438-Pos Using Embedded Vesicles In Hydrogels For Localized Drug Delivery For Ovarian Cancer

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Board B270

In United States ovarian cancer is the fourth leading cause of death from gynecologic malignancies. In this study, we seek to develop a new treatment therapy for ovarian cancer patients. We have designed a localized drug delivery system that consists of two components: a non-ionic surfactant vesicle and a crosslinked three-dimensional polymer network. The cross-links provide a mesh-like cavity to encapsulate the vesicles. This vesicle/hydrogel system provides control over drug dosage and the release time. It also provides stability to the vesicles and an additional control over the release rate. The non-ionic vesicles were prepared by hydration of amphiphilic films with PBS buffer solution and fluorescent marker (mimics and tests the drug release rate). After hydration, the vesicles were extruded to constrict their size distribution. Unentrapped dye was removed using ultracentrifugation and gel filtration. The release rate of the dye was measured from intact vesicles when exposed to high ion and low ion environments. The dye encapsulated vesicles were placed in a semi-permeable cellulose membrane and immersed in a solution of either PBS or Milli-Q water. Diffusion of the dye through the membrane was determined at different time intervals by using fluorescence spectroscopy. Results showed that the release of the dye from the bilayer vesicles without the hydrogel network support took place within the first 12 hrs of the experiment. It also indicated that drug release from vesicles is highly dependent on ion concentration because the release rate was found to be higher in Milli-Q water than in PBS solution which has a pH similar to that of the vesicles. The final outcome of this study will contribute to the development of a low cost and improved method for the release of drugs in intracavitary ovarian cancer treatment.

439-Pos Looking For An Optimum Transepidermal Liposome Drug Delivery System: Characterization Of Mechanical Properties Of Liposomes And Polarity Properties Of Transdermal Pathways In Human Skin

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Board B271

Skin is the principal barrier and protection against the external environment and responsible for keeping the water balance of the body. The outer part of the skin, epidermis, is the first obstacle to overcome in order to penetrate the skin. Epidermis is composed of four different regions showing highly complex compositional features. Also, epidermis shows water and pH gradients that are relevant for the proper function of the skin as a barrier. Within the last decade transepidermal drug delivery has gained importance based on kinetic as well as compliance arguments. The use of liposomes as a vehicle for drug delivery through the skin has been intensively studied since 1980. One of the important characteristics of liposomes for transdermal drug delivery is the membrane elasticity. However, no quantitative data regarding this last parameter has been described in the literature for the various liposome formulations utilized in transdermal drug delivery. In order to perform a proper characterization of vehicles for transepidermal drug delivery, it is necessary to focus on two different aspects of the system. One is to characterize the skin and its physical properties which will influence liposome penetration, and secondly to characterize the mechanical properties of the carriers (i.e. liposomes). We have been able to characterize areas of human skin with different polarities using Laurdan generalized polarization (GP) images, particularly in areas corresponding to liposome penetration pathways. Secondly the mechanical properties of liposomes of particular compositions were characterized and correlated with the extent of liposome penetration in human skin. These studies demonstrate for the first time a quantitative correlation between physical properties of the tissue and those corresponding to the carrier used for transepidermal drug delivery.

440-Pos Doxorubicin Decreases Creatine Transport In Hl-1 Cardiac Myocytes Expressing The Human Creatine Transporter

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Board B272

Doxorubicin (DXO) is a widely used chemotherapeutic agent commonly used to treat leukemia as well as solid tumors. A major side-effect of its administration is cardiotoxicity that may cause cardiomyopathy and heart failure (HF) in a significant number of patients (Minotti, 2004 Pharmacol Rev *56*, 185–229). The molecular mechanisms leading to HF in this setting are poorly understood. DXO circulating at therapeutic levels in isolated perfused heart preparations alters high-energy phosphate metabolism via the creatine (Cr) energy shuttle. The Cr kinase isoform distribution is shifted, and enzymatic activity is decreased (Tokarska -Schlattner, 2005 Am J. Physiol Heart Circ. Physiol *289*, H37–H47). We have characterized the effect of DXO treatment on Cr transport in HL-1 murine cardiac cells expressing the human Cr transporter (CrT) protein. We demonstrate that DXO diminishes Cr transport in a dose and time dependent manner. A statistically significant (n=4 ANOVA, $p<0.05$ Fisher LSD) decrease in Cr transport is detectable after 24 hrs of incubation with 50nM DXO, a concentration that is forty fold less than the 2 μM plasma concentration typically achieved after administration of a bolus of DXO during the course of chemotherapy. Kinetic analysis reveals that incubation with 100nM DXO causes a statistically significant increase in Km (45.5 ± 1.7 vs 37.7 ± 1.85 μM in controls, n=4, $p<0.05$, t-test), and tends to decrease Vmax (6.07± 0.7 vs 4.7 ± 0.48 nmol/mg protein in controls, $p = 0.16$). The decrease in Vmax is mirrored by a decrease in abundance of total CrT protein. We conclude that a decrease in Cr transport may be responsible for the bioenergetic derangements observed in DXO-associated heart failure.

Intercellular Communication & Gap Junctions

441-Pos The Relevance of Non-Excitable Cells for Cardiac Pacemaker Function

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Aging and sinus node disease induce an increased ratio of cardiomyocytes to fibroblasts in the sinoatrial node and it has been demonstrated that cardiomyocyte spontaneous activity can be attenuated when intercellularly coupled to and depolarized by fibroblasts. Our goal was to determine if the mere physical separation of neighboring cardiomyocytes by interspersed fibroblasts modulates spontaneous activity and conduction velocity (υ) independent of their depolarizing effect.

The spontaneous activity and υ in multicellular cardiomyocyte preparations (HL-1 monolayers) were monitored with microelectrode arrays. Myocyte+fibroblast (WT- or Cx43(−/−)-fibs) co-cultures were established by either 'mixing' the fibroblasts between the cardiomyocytes or by 'layering' them onto confluent cardiomyocyte monolayers. This way the effect of fibroblast infiltration could be separated from their effect on cardiomyocyte V_m. WT-fibs reduced the beating frequency of HL-1 cells in mixed (62±5%; n=13) and layered (63±4%;n=10) preparations. HL-1+Cx43(−/−)-fibs co-cultures exhibited decreased heterocellular coupling and attenuated cardiomyocyte V_m depolarization; however, the decreased beating frequency persisted in 'mixed' cultures (61±6%; n=30). Only in layered HL-1+ Cx43(−/−)-fibs co-cultures the fibroblasts' effect on spontaneous activity (7±2%;n=16) could be prevented. In contrast, although υ in mixed and layered cultures correlated negatively with the amount of WT-fibs, the decrease of υ in both mixed and layered co-cultures of HL-1+Cx43(−/−)-fibs was significantly attenuated. Comparable to uncoupled cardiomyocyte preparations, in both mixed co-cultures of HL-1+WT and HL-1+Cx43(−/−)-fibs, the co-efficient of the inter-beat interval (C_{IBI}) was significantly increased (694±198% and 587±112%). However, C_{IBI} remained comparable to control in 'layered' HL-1+WT-fibs or +Cx43(−/−)-fibs cultures. The data demonstrate that fibroblasts modulate cardiomyocyte excitability through intercellular coupling and cardiomyocyte depolarization; however, their major effect on the spontaneous activity is mediated by separating neighboring cardiomyocytes, which is, for the heterocellular resistances tested, independent from the intercellular coupling established.

442-Pos Movement of M3 Helices During Gating of Cx43 Gap-Junctional Hemichannels by PKC-Mediated Phosphorylation

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Board B274

The decrease in permeability to large solutes through gap-junctional channels (GJC) and hemichannels (GJH) in response to lowering pH and phosphorylation is believed to occur through a ball-and-chain mechanism, with the C-terminal domain (CTD) acting as the ball. It has been proposed that decreases in cytoplasmic pH and phosphorylation cause a movement of the CTD with association to other intracellular domains, which results in a decrease in the effective channel pore size. Our goal was to determine whether a ball-and-chain mechanism could explain the regulation of Cx43 GJH permeability by PKC-mediated phosphorylation. We studied purified and reconstituted GJH formed by Cx43 variants that contain only one cysteine residue at predetermined positions (I156C, V164C, V167C and a Cys added at the C-terminal end), for labeling with acceptors probes (fluorescein, ATTO-465 or ABD). We then measured Angstrom distances between probes in homologous residues in different