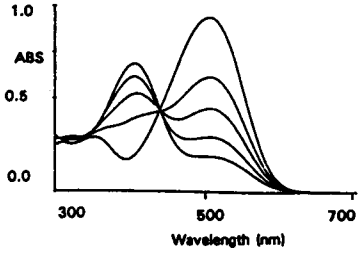
M-Pos511

A NEW WAVELENGTH-RATIOMETRIC Ca^{2+} PROBE WITH LONG WAVELENGTH EXCITATION AND EMISSION. ((E.U. Akkaya and J.R. Lakowicz)) Univ of Maryland, School of Medicine, Center for Fluorescence Spectroscopy, Department of Biological Chemistry, 108 N. Greene Street, Baltimore, MD 21201. (Spon. by J.R. Lakowicz)

We have synthesized a fluorescent Ca^{2+} probe that contains the highly selective Ca^{2+} chelator BAPTA (1,2-bis-(2-amino-phenoxy)ethane-N,N,N',N',-tetraacetic acid) as an integral part of the fluorophore. On binding to Ca^{2+} (K_d=1.8 μM), emission is mostly unchanged; but the absorbance peak shifts dramatically (from 508 nm to 407 nm) to shorter wavelengths. Hence, wavelength-ratiometric measurements are possible using two excitation wavelengths. Both Ca^{2+}-bound and free forms of the probe can be excited at wavelengths where autofluorescence would not be problem in an intracellular environment. As with all polycarboxylate probes, conversion to a cell permeable AM (acetoxymethyl) ester form is feasible. While the quantum yield of the probe is low, we see this probe as the first example of a series of related probes. We intend to optimize its spectral properties by chemical modification of the basic structure. We also appreciate the potential of our approach in the development of wavelength-ratiometric probes for other cations of biological significance.



M-Pos512

QUANTIFICATION OF CHANGES IN MITOCHONDRIAL SPECIFIC PYRIDINE NUCLEOTIDE FLUORESCENCE FROM SKELETAL MUSCLE CELLS.

((K.N. Richmond*, P.C. Johnson*, and R.M. Lynch*+))Departments of Physiology* and Pharmacology+, University of Arizona, Tucson AZ 85724

Adjustments in cell metabolism elicit changes in pyridine nucleotide (PN) fluorescence from skeletal muscle *in vivo*. The changes in fluorescence are assumed to reflect shifts in the mitochondrial NAD^+/NADH redox couple. To test this assumption the distribution of PN fluorescence in enzymatically dissociated single cells from rat spinotrapezius muscle was compared to that for a mitochondrial selective fluorescent probe (Rhodamine 123) using 3-D microscopic imaging techniques. Images were acquired using a liquid cooled CCD camera attached to an Olympus IMT-2 microscope equipped with a motorized focus which provides precise movement in the Z plane. Fluorophore distributions were analyzed by acquiring through-focus image sets consisting of 15 optical sections which were then processed to reduce out of focus blurring using a deconvolution algorithim based on regularization theory. From Rhodamine 123 images the cell volume occupied by mitochondria was estimated to be 21%. This is consistent with reported values for other red muscles analyzed by electron microscopy. A comparison of PN fluorescence to the mitochondrial distribution indicated 30-35% of the PN fluorescence is associated with mitochondria in the resting state. Preliminary studies indicate that increases in PN fluorescence elicited by the mitochondrial inhibitor cyanide also are greatest in these areas. This observation is consistent with the interpretation that changes in PN fluorescence at the tissue level are indicative of changes in the mitochondrial metabolic state. Current studies will quantify mitochondrial PN fluorescence during perturbations in muscle cell metabolism.

M-Pos513

IMAGING SUBCELLULAR STRUCTURES WITH THE SCANNING FORCE MICROSCOPE (SFM).

((L. I. Pietrasanta†*, A. Schaper†, F. J. Barrantes* and T. M. Jovin†)) †Department of Molecular Biology, Max Planck Institute for Biophysical Chemistry, P. O. Box 2841, W-3400 Göttingen, Federal Republic of Germany. *Instituto de Investigaciones Bioquimícas (INIBIBB), C. C. 857, 8000 Bahía Blanca, Argentina.

An SFM (NanoScope from Digital Instruments) was used for high resolution imaging of subcellular structures of cultured rat mammary carcinoma cells. We adjusted the conditions of sample preparation so as to selectively visualize the microvilli, the nucleus, the mitochondria, and the various components of the cytoskeletal system, with high resolution and no apparent distortion. The microvilli are well preserved after fixation in glutaraldehyde vapor and are clearly distinguished as abundant 1-2 µm protrusions from the cell surface. To observe the intracellular organelles, the plasma membrane was removed with non-ionic detergent in a buffer system. The nuclei are very well defined; if the nuclear envelope is removed, features of the chromatin are visible. Invariably, the nucleoli appear as higher, discrete structures. The mitochrondria are concentrated primarily in the perinuclear space and exhibit a well defined filamentous shape. The cytoskeletal network is resolved as a complex mesh in which the three major components are distinguishable: actin fibers, microtubules, and intermediate filaments. In all cases, positive identification of the substructures was achieved by imunofluorescence, immunogold, and specific fluorescence probes.

Similar studies are being conducted on cellular systems with specific cell surface receptors.

M-Pos514

MEASUREMENT OF MICROSTRUCTURE, ADHESION AND UNBINDING OF GIANT VESICLES BY QUANTITATIVE REFLECTION INTERFERENCE CONTRAST MICROSCOPY.

((J.Rädler)) Physics Department, Technical University Munich, D-8046 Garching/Munich, Germany. (Spon. by E.Sackmann)

Reflection Interference Contrast Microscopy (RICM) allows to study the membrane-substrate separation distance of giant phospholipid vesicles close to an underlying surface with nm resolution. Osmotically swollen vesicles can be regarded as rigid colloidal particles interacting with substrates via long range electrostatic, VdW and gravitational forces. The interaction was determined from the Brownian motion of vesicles tracked by image processing. In the case of adhering vesicles the bound contour shapes were reconstructed from the interference pattern depending on the area to volume ratio and the bending elasticity modulus. Membranes attracted by a weak potential exhibited thermally excited surface undulations. The time-space correlation function was measured and compared with the scaling behaviour predicted for undulation forces and the unbinding transition. A new contrast variation technique based on RICM is presented which allows optical density mapping of bilayers close to a surface with an out of plane resolution of 0.2nm and a lateral resolution of 0.2µm.

MOLECULAR MECHANISMS OF MEMBRANE FUSION

Tu-AM-Symi-1

MEMBRANE FUSION PROTEINS: FROM VIRUS TO SPERM.

((J.M. White)), Univ. of California, San Francisco, CA 94143

The processes of virus-cell and cell-cell fusion are topologically identical. In both cases, membrane leaflets that face the extracellular environment make initial contact. We have therefore hypothesized that proteins involved in important cell-cell fusion events, such as sperm-egg fusion and myoblast fusion, resemble viral membrane fusion proteins. This hypothesis will be discussed.

Most viral membrane fusion proteins share the following characteristics: They are composed of one or two type I integral membrane glycoproteins that form higher order oligomers. They contain a fusion peptide, a relatively hydrophobic stretch of amino acids, in a transmembrane-anchored subunit. Proteolytic processing activates fusion potential. The fusion glycoprotein complex is also involved in binding to the host cell membrane. These features will be illustrated in the context of recent models for how the well characterized influenza virus hemagglutinin promotes membrane fusion.

A sperm surface protein called PH-30 was implicated in the process of sperm-egg fusion based on the ability of an anti-PH-30 monoclonal antibody to prevent sperm-egg fusion. Biochemical, cell biological, and molecular biological studies have revealed that PH-30 shares many properties with viral membrane fusion proteins: PH-30 is a complex of two type I integral membrane glycoproteins. Proteolytic processing of PH-30 correlates with the acquisition of fertilization competence. One subunit of PH-30 contains an apolar sequence that resembles viral fusion peptides. The other subunit contains a receptor binding domain. Possible roles of PH-30 in sperm-egg binding and fusion will be discussed.

Tu-AM-Symi-2

A DISSECTION OF STEPS LEADING TO VIRAL ENVELOPE PROTEIN-MEDIATED MEMBRANE FUSION.

((Robert Blumenthal)) NCI, NIH, Bethesda, MD, 20892.

The viral fusion process involves a range of steps before the final merging of membranes occurs. Our studies deal with a number of key questions concerning the fusion process such as: How does triggering the event by a pH or temperature change, or receptor binding affect conformation of the viral envelope protein? Do the viral proteins form oligomeric complexes at the site at which fusion is to occur? Can we identify intermediate fusion steps or structures? What sorts of molecular rearrangements occur before, during and after the fusion event? These questions are approached by developing kinetic assays for fusion of fluorescently-labeled virus with a variety of target membranes using spectrofluorometric and video microscopic techniques. We have studied the mode of action of the hemagglutinin (HA) protein of influenza virus, as well as other viral envelope proteins using both intact virus and viral proteins expressed on surfaces of cells. We could identify a number of intermediates in the kinetic pathway, which include a committed pre-fusion state, a fusion junction which allows redistribution of lipids and small aqueous molecules, and a wide opening which allows entry of the nucleocapsid into the cells. The molecular structure of the various intermediates is examined by a combination of biochemical, biophysical and genetic approaches.

Tu-AM-SymI-3

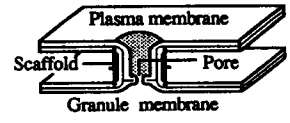
PROPERTIES OF THE FUSION PORE IN EXOCYTOSIS AND VIRUS ENTRY. ((W.Almers[1*], L.Breckenridge [2], A.E.Spruce [3], A.Iwata [3], F.W.Tse [3])) [1] Max-Planck-Inst. f. Medical Research, Heidelberg, Germany, [2]Univ. of Glasgow. England, and [3]Univ. of Washington, Seattle, WA

In synaptic transmission, Ca opens a "fusion pore" that connects the inside of a synaptic vesicle and the extracellular space, releases transmitter and initiates fusion of vesicle with plasma membrane. The speed of transmitter release (< 1ms) suggests that the pore is in place before the action potential arrives, and formed by a macromolecule extending across both vesicle and plasma membranes. We have recorded currents through nascent fusion pores during exocytosis in mast cells. The pore opens abruply to a conductance, g, of about 300 pS, and an estimated diameter of 2 nm. Later, g increases more gradually as the pore dilates. We have suggested that the abrupt opening of the pore is analogous to that of an ion channel, while the more gradual increase in g reflects dilation caused by recruitment of lipid into the pore circumference. The model predicts that the pore should allow lipids of the two bilayers to mix, but not before the pore has dilated. This was tested on fusion pores formed by HA, the fusion-catalyzing envelope protein of *Influenza*. Fibroblasts expressing HA on their cell surface were decorated with single RBC's and allowed to fuse to them. We stained the outer leaflet of the RBC bilayer with fluorescent lipid and followed lipid flux from RBC to fibroblast by video imaging. Simultaneously, we measured g. Lipid flux was readily seen once g had grown beyond 1 nS, but was undetectable while g < 0.5 nS. Evidently flux requires a threshold pore size. Our results suggest that the smallest and earliest fusion pores are surrounded by a continuous ring of protein.

Tu-AM-SymI-4

MECHANO-ELECTRICAL GATING OF THE EXOCYTOTIC FUSION PORE. ((J. Fernandez, J. Monck and A. Oberhauser)) Mayo Clinic,Rochester,MN 55905.

The exocytotic fusion pore, the earliest aqueous connection between the lumen of a secretory vesicle and the extracellular medium, has an unknown molecular structure. The patch clamp technique has been used to monitor, at high resolution, the activity of single exocytotic fusion pores in mast cells. Based on our data and the results of bilayer fusion experiments, we suggest that at rest, a scaffold of proteins connects the granule and the plasma membranes (Monck and Fernandez, J. Cell Biol., Vol. 119, December, 1992). On activation by GTP binding proteins of the rab family (Oberhauser, Monck, Balch and Fernandez, Nature, 1992, in press) this scaffold draws the granule and plasma membranes together at a narrow focal point, by-passing the short-range repulsive interbilayer hydration forces. The two closely apposed, highly curved membranes fuse into a single bilayer in much the same way as two tensed or depleted phospholipid bilayers fuse to form a hemifused bilayer. A pore formed in this bilayer can expand irreversibly, fluctuate, or close (Nanavati, Markin, Oberhauser and Fernandez, Biophys. J. 63:1118-1132, 1992). Pore opening itself is a reversible event triggered by an electromechanical stimulus on the hemifusion bilayer (Oberhauser and Fernandez, Biophys. J., 1992, 61:421a; submitted); collapse of the hemifusion bilayer causes complete irreversible fusion. Our current efforts are directed towards identifying the scaffold proteins utilizing PCR cloning strategies in combination with the patch clamp measurements.



Tu-AM-SymI-5

ROLE OF LIPIDS AND PROTEIN IN FUSION PORE FORMATION. ((Joshua Zimmerberg)), LTPB, NICHD, NIH, Bethesda, MD 20892.

By which process does a small fusion pore composed of contiguous bilayer membranes form in a small appositional area? Capacitance measurements of *beige* mouse mast cells suggests small pore intermediates of variable size which continuously grow to reach semi-stable conductances between 1 and 20 nS. Correlated morphological and electrophysiological data from mast cells suggest that the semi-stable state is the same as that captured in freeze-fracture replicas -- of hourglass configuration with contiguous fracture planes joining the granule and plasma membranes with no apparent proteinaceous specializations. The continuous transitions in conductance are consistent with a lipid/protein complex mediating fusion. Consistent with this, a naturally occurring class of lipids, lysolipids, inhibit exocytotic fusion pore opening in mast cells, pH-induced baculoviral syncytia formation, GTP-induced rat microsome fusion, and Ca^{++}-triggered sea urchin egg exocytosis. Inhibition is reversible, does not correlate with lysis, and is not attributed to any chemical moiety of lysolipids. Since we find fusion of sea urchin egg secretory granules to vesicles composed of purified lipids, as in viral fusion, proteins in one membrane can complex with lipids in another membrane to form a lipid/protein complex to mediate fusion.

MOLECULAR RECOGNITION AND BINDING

Tu-AM-SymII-1

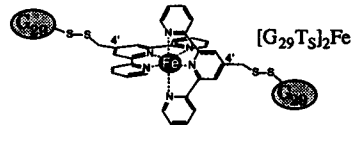
UNDERSTANDING THE BASIS FOR MINOR GROOVE BINDING SPECIFICITY. ((D.E. Wemmer)) Department of Chemistry, University of California, Berkeley, CA 94720.

Over the past few years the structures of a number of complexes of minor-groove binding small molecules with A-T rich DNAs have been determined with NMR and x-ray diffraction. The most extensively studied are netropsin and distamycin, together with a number of synthetic variants. Two of these variants have been shown to bind specifically to sites containing G-C base pairs. The basis for this change in specificity will be discussed in light of structural and energetic studies of both A-T and G-C specific binding molecules. These studies also provide information about the ability of different DNA sequences to adapt to different binding modes.

Tu-AM-SymII-2

SYNTHETIC DIMERIZATION DOMAINS USED TO CONSTRUCT DNA BINDING PROTEINS WITH ALTERED SPECIFICITY. ((Bernard Cuenoud and Alanna Schepartz)) Department of Chemistry, Yale University, 225 Prospect Street, New Haven, CT 06511.

The bZIP motif is characterized by a coiled coil domain that mediates protein dimerization and a basic domain that contacts DNA. These two domains are separated by a six residue "linker" whose sequence is not conserved across bZIP families. Molecules were formulated in which the GCN4 coiled coil is replaced by a series of stereochemically defined metal complexes which systematically alter the relative orientation of the basic domain peptides. Both the affinity and the specificity of the peptides are modulated by seemingly small changes in orientation. One molecule, $[G_{29}T_S]_2Fe$ binds a GCN4 target site with a K_d of 0.13 nM, and upon binding its target exhibits the same DNase I footprint and increase in helicity as GCN4. Dramatically decreased binding is observed with complexes exhibiting altered orientations of the basic domains. Although GCN4 recognizes the CRE and AP1 sites with similar affinity, $[G_{29}T_S]_2Fe$ prefers the CRE by a factor of 300. That the affinity and specificity of basic domain peptides are modulated by seemingly small orientational changes suggests that the detailed architecture of the zipper-linker boundary plays a dominant role in defining bZIP:DNA interactions.



Tu-AM-SymII-3

DESIGN OF NOVEL THYMIDYLATE SYNTHASE INHIBITORS USING ITERATIVE CRYSTALLOGRAPHIC ANALYSIS. ((D.A. Matthews, R.J. Bacquet, C.A. Janson, S.H. Reich, M.D. Varney, S.E. Webber, and W.W. Smith)) Agouron Pharmaceuticals, San Diego, CA 92121

We report successful applications of an iterative cycle of ligand design, synthesis, biochemical evaluation and crystallographic analysis of the protein-ligand complex in the development of enzyme inhibitors. Several series of structurally distinct compounds have been designed as inhibitors of thymidylate synthase. The ability to study in detail the binding mode of each successive molecule in a series greatly facilitates the analysis of those forces which dominate receptor-ligand interactions. The methodology described may speed the discovery of lead compounds and assist their development into drugs.

Tu-AM-SymII-4

SOLVENT EFFECTS ON RECOGNITION AND BINDING ((Kim A. Sharp)) Dept. of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, PA 19104-6059

Specific binding and recognition implies a substantial negative free energy of interaction of the right ligand in the right conformation, compared to both the unbound state and other ligands and or conformations. This results from a balance between intermolecular interactions and molecule solvent interactions. The latter predominantly consists of electrostatic and hydrophobic interactions. The electrostatic contribution to solvation depends on the molecular charge distribution, the shape of the molecule and the distribution of ions around it. The hydrophobic solvation term depends on the solvent accessible area buried upon binding and also upon the shape of the molecule through the curvature of the buried area. Ways to treat these effects and applications to protein-protein and protein-nucleic acid binding will be discussed.

POTASSIUM CHANNELS II

Tu-PM-A1

FAST INACTIVATION BY THE BALL PEPTIDE IN *Shaker* B K CHANNELS IS HIGHLY TEMPERATURE DEPENDENT. ((M. Nobile, R. Olcese, Y.C. Chen, L. Toro, and E. Stefani)) Dept. Molecular Physiology & Biophysics. Baylor College of Medicine. Houston, TX 77030

The energy profile of the interaction between the ball peptide (BP) and the internal mouth of *Shaker* B K channels was investigated. Lowering the temperature had a major effect on the fast inactivation process. Macroscopic currents in the cut-open oocyte showed the following changes after lowering the temperature (20 to 10 ^{o}C): 1) Peak amplitude decreased with a Q_{10} of 1.5; 2) the activation time constant decreased with a Q_{10} of 3.14; 3) the decay time constant decreased with a Q_{10} of 7.2, and 4) the recovery from inactivation was less temperature dependent (Q_{10} = 1.57) than the installation of inactivation. At 0 mV the ratio between the peak amplitude and the steady-state level of the current was 4.0 at 20 ^{o}C and 1.44 at 5 ^{o}C. These findings indicate that the fast inactivation process has a high temperature dependence. To further investigate whether the high energy step was due to the interaction between the BP and the internal mouth of the channel we investigated in excised inside-out macropatches the temperature dependence of the inactivation induced by the synthetic peptide. The BP (93 μM) added to the internal side of Shaker-B IR (inactivation removed, delta 6-46) induced rapid inactivation (decay time constant of 20 ms at 40 mV). Lowering the temperature from 20 to 15 ^{o}C greatly slowed down the current decay, practically removing fast inactivation. These results suggest that the energy step resides mainly in the interaction between the inactivating peptide and the channel and not in the hypothetical bending of the chain. Supported by grants HL47382, HL37044 and AHA-Natl. Center 900963.

Tu-PM-A2

Model for Channel Activation Based on the Kinetics of Wild-Type and V2 Mutant *Shaker* 29-4 Potassium Channels. ((N.E. Schoppa, K. McCormack, and F.J. Sigworth)) Dept. of Cell. and Mol. Physiology, Yale Univ., New Haven, CT.

Mutating the second leucine in the conserved heptad repeat region of *Shaker* 29-4 potassium channels to valine (V2) causes a ~60 mV depolarizing shift and reduction in voltage sensitivity of channel activation without altering the total amount of charge moved in the activation process (Schoppa et al., *Science* 255: 1712, 1992). An explanation for this surprising result based on the steady state properties of V2 and the wild-type channel (WT) is that the V2 mutation has only a small effect on "early" steps but shifts the equilibrium of a "late" step in the channel opening process by ~60 mV. Several lines of kinetic evidence from ionic and gating currents at least qualitatively support this hypothesis. First, ionic tail currents at all membrane potentials decay much more quickly in V2 than in WT, and at very negative membrane potentials, where tail currents should reflect the rate constants of the final transition in the opening process, the V2 mutation shifts the voltage dependence of the decay time constants by about +80 mV. Secondly, while the gating currents of WT show a prominent second exponential component at potentials where the opening of these channels is the most voltage sensitive, V2 gating currents decay monoexponentially. Finally, while single channel closed times of WT exhibit many exponential components, V2 exhibits only 2 or 3 closed time components, consistent with the notion that the V2 mutation has separated activation transitions in their voltage dependence. We are exploiting this fact in a more thorough kinetic analysis of V2 and WT ionic and gating currents.

Tu-PM-A3

CONSERVED CYSTEINE RESIDUES IN S2 AND S6 ARE NOT ESSENTIAL FOR EXPRESSION OF Kv2.1 (DRK1) IN *XENOPUS* OOCYTES.
((Roger D. Zühlke, Hui-juan Zhang and Rolf H. Joho)) Department of Cell Biology and Neuroscience, The University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75235-9039.

The wealth of data derived from functional expression of cloned voltage-gated potassium (K^+) channels contrasts markedly with the virtual absence of structural information about the channel protein. Recombinant DNA techniques have been used to correlate biophysical properties with putative channel domains. However, there is still a considerable lack of structurally meaningful data. Comparative sequence alignments of known voltage-gated K^+ channels revealed two conserved cysteine residues in the putative transmembrane segments S2 and S6 (C232 and C393 in Kv2.1). If these residues were connected by a disulfide bridge, it would put a structural constraint on possible channel models by placing S2 next to S6, and it would also imply an essential role in the folding process and/or in the maintenance of functional integrity of the channel. We used site-directed mutagenesis to investigate this possibility. Replacement in Kv2.1 of C232 with serine, phenylalanine or tyrosine did not affect expression levels when measured in *Xenopus* oocytes with a two-electrode voltage-clamp technique. Substitutions of C393 with serine or glycine were well tolerated, however, the bulky residue phenylalanine or the charged residue arginine in position 393 led to loss of function. We suggest that a disulfide bridge between C232 and C393, although possible, is not essential for functional expression of Kv2.1 in *Xenopus* oocytes. Whether disulfide bridges within or across channel subunits, linking S2 to S6, are indeed present, and how other mutations of these cysteine residues affect channel function is currently under investigation.
(Supported by NS28407 to R.H.J.).

Tu-PM-A4

MUTATIONS IN THE S6 DOMAIN AFFECTING CONDUCTANCE, TEA AND BARIUM BLOCKADE IN THE PERMEATION PATHWAY OF THE *SHAKER* POTASSIUM CHANNEL. ((G.A. Lopez, Y.N. Jan and L.Y. Jan)) Howard Hughes Medical Institute and Departments of Physiology and Biochemistry, University of California San Francisco, San Francisco, CA 94143-0724.

In order to identify regions of the potassium channel protein which may contribute to the internal mouth and pore lining structure, we have made use of a chimeric channel. The chimeric channel contained part of the sixth transmembrane domain (S6) and several adjacent residues from NGK2, transplanted into the N-terminal deletion mutant of *Shaker B*. These two channels differ in several pore properties including single-channel conductance, blocking by internal and external TEA, and block by internal Ba^{++}. NGK2 has a high sensitivity for external TEA and a low affinity for internal TEA, whereas *Shaker* has the opposite sensitivities. In addition, the single-channel conductance of NGK2 is approximately three times of that of *Shaker*. The chimeric channel showed an internal TEA sensitivity and single-channel conductance similar to NGK2. We have also found that the NGK2 channel, like the chimera, is extremely sensitive to block by internally applied Ba^{++} whereas *Shaker* is less sensitive to block. The block by Ba^{++} is extremely voltage dependent in the NGK2 and chimeric channel. Because the S6 domain from NGK2 transferred several pore properties typical of NGK2 to the *Shaker* channel, we propose that the S6 domain is a structural component of the ion conducting pore.