BRIDGING MOLECULAR GENETICS & BIOPHYSICS IN CELL MOTILE SYSTEMS

W-Am-Sym II-1

MUTANTS OF FORCE-GENERATING ELEMENTS OF THE BACTERIAL ROTARY MOTOR. D.F. Blair and H.C. Berg, Department of Cellular and Developmental Biology, Harvard University, Cambridge, MA 02138.

Flagellated bacteria possess a remarkable motility system based on a reversible rotary motor linked by a flexible coupling (the proximal hook) to a thin helical propeller (the flagellar filament). The motor derives its energy from protons driven into the cell by chemical gradients or electrical fields. A summary will be given of recent work on the structure and function of the rotary motor, with an emphasis on the analysis of mutants of MotA and MotB, elements essential for force generation.

W-Am-Sym II-2

MOLECULAR GENETIC MANIPULATION OF CARDIAC MYOSIN: Leslie A. Leinwand, Albert Einstein College of Medicine, Bronx, N.Y. 10461

The complex nature of the myosin gene family in vertebrate muscle complicates its detailed in vivo genetic analysis. We have taken two molecular genetic approaches to study the components of sarcomeric myosin, the major force-producing motor of muscle. In the first, we have expressed the subunits of myosin in E. coli and have assayed them for several functions including thick filament assembly, subunit interactions, and ATPase activities. A small (193 amino acid [aa]) segment of the myosin rod was identified that is responsible for the 143Å periodicity characteristic of the intact molecule. However, larger segments (313aa including the above sequence) are necessary for the solubility properties of the myosin rod as well as its higher order structures. Coexpression of cardiac myosin heavy chain (MHC) segments and myosin light chain 1 (MLC1) identified 18 aa of the MHC that are necessary for MLC1 binding. Triple expression constructs including MLC2 are currently under analysis. Crude extracts of bacteria coexpressing aa 1-808 of the MHC and the entire MLC1 exhibit K^+-EDTA and Ca^{++} ATPase activities. However, these activities appear labile upon further purification. In order to complement these in vitro studies, we analyzed the ability of cardiac myosin to function in the complex motile behavior of Dictyostelium (Dd), the unicellular slime mold. We have made a myosin chimera consisting of the Dd head and the cardiac rod and transformed Dd myosin null cells (with J. Spudich, Stanford). The null cells are deficient in cytokinesis, development and the capping of cell surface receptors. Transformants expressing the Dd/cardiac myosin chimera remain deficient in cytokinesis and development, but are capable of capping cell surface receptors. These results demonstrate that the muscle and nonmuscle myosin rods are not entirely functionally interchangeable, and that distinct domains exist within the myosin rod.

W-Am-Sym II-3

A MINUS-END DIRECTED, KINESIN-RELATED MOTOR MOLECULE INVOLVED IN CHROMOSOME SEGREGATION. Richard A. Walker, Edward D. Salmon and Sharyn A. Endow. Dept. Biology, University of North Carolina, Chapel Hill, NC and Dept. Microbiology & Immunology; Duke University, Durham, NC. (Intro. by I.R. Gibbons.)

Molecular genetics has led to the identification of a kinesin-related Drosophila protein required for proper segregation of chromosomes in meiosis and in early mitotic divisions of the embryo. The predicted amino acid sequence of the protein, which is encoded at the claret locus, is 40-45% identical to the microtubule motor molecule, kinesin. The claret segregation protein was expressed in bacteria and tested for its ability to support gliding of microtubules on extract-coated glass surfaces. Results of these experiments indicate that the claret protein is a novel microtubule motor molecule. It supports microtubule gliding at velocities averaging 4 μm/min, approximately 10-fold slower than kinesin and 20-fold slower than cytoplasmic dynein. Contrary to predictions based on its amino acid similarity to kinesin, tests of motor directionality show that the claret protein moves along microtubules toward their minus ends, unlike kinesin but like dyneins. The claret motor protein also shows the unusual capability of causing microtubules to rotate as they glide, indicating that it can generate torque with forward movement.

The finding of minus-end directionality for a protein with sequence similarity to kinesin suggests that the kinesin and dynein motor domains are ancestrally related. The velocity of the claret motor is unlike either kinesin or dyneins, but is similar to the rates of chromosome movement observed in meiosis and mitosis. Ability to move toward the minus ends of microtubules suggests a role for the claret protein in moving chromosomes poleward in prometaphase and/or anaphase.

W-AM-Sym II-4

UNDERSTANDING THE IN VIVO FUNCTION OF KINESIN IN TERMS OF ITS MOLECULAR DESIGN. George S. Bloom[1], Janet L. Cyr[1], Mark C. Wagner[2], K. Kevin Pfister[3], David L. Stenoien[1], Nobutaka Hirokawa[4], Reiko Sato[4], Hiroshi Yorifuji[4] and Scott T. Brady[1]; Depts. of Cell Biology and Neuroscience[1], and Pharmacology[2], University of Texas Southwestern Medical Center; Dept. of Anatomy and Cell Biology, University of Virginia Health Science Center[3]; Dept. of Anatomy and Cell Biology, University of Tokyo School of Medicine[4].

Kinesin is a microtubule-stimulated ATPase that can cause microtubules to glide along substrata *in vitro*. During the past few years our laboratories have been engaged in a systematic effort to define the function of kinesin *in vivo*, and to understand the structural basis for that function. Using highly purified bovine brain kinesin we initially established that the native protein comprises two ATP-binding, ~124 kDa heavy chains and a pair of ~64 kDa light chains, and demonstrated that interchain disulfide bonds are not essential for the maintenance of quaternary structure. Multiple apparent isoforms of both heavy and light chains were found. Monoclonal antibodies made against the heavy and light chains have been used for several purposes. First, one of the heavy-chain specific antibodies was found to be a potent inhibitor of the transport of membrane-bounded organelles (MBOs) along microtubules in axoplasm isolated from giant squid axons. This indicated that kinesin probably functions *in vivo* as an organelle motility motor. Consistent with this interpretation have been the results of immunofluorescence and immuno-EM studies of squid axoplasm, cultured mammalian cells and mammalian peripheral nerve. In all of these sources, staining by anti-kinesins has been restricted to MBOs, and ligation studies of mammalian peripheral nerve have indicated that kinesin is preferentially associated with MBOs moving in the anterograde direction. High resolution EM and immuno-EM have also been used to examine the structure of purified kinesin. These studies indicated that the fully extended molecule is an ~80 nm long rod-shaped structure. At one end of the molecule are two ~10 nm diameter globular, microtubule-binding domains, which are formed by heavy chains of kinesin. The opposite end of kinesin contains a "fan-shaped" domain, where two light chain epitopes have been identified, and which is connected to the globular regions by a long, thin shaft. The available evidence suggests that the fan-shaped region and a nearby portion of the shaft are involved in binding to MBOs. Most recently, we have characterized the molecular structure of the kinesin light chains in much greater detail. Three distinct, but closely related light chain cDNAs have been cloned and sequenced from rat brain. The potential significance of isoforms for the functions of kinesin heavy and light chains is being investigated.

W-AM-G1

NEURONAL CALCIUM CHANNEL cDNAS FROM A MARINE RAY. W.A. Horne, I.P. Inman, T.L. Schwarz, R.W. Tsien. Department of Molecular and Cellular Physiology, Stanford CA 94305

N-type Ca^{2+} channels trigger the release of transmitter in many neuronal systems but little is known about their primary structure. The electric organ of the marine ray *Discopyge ommata* is the richest known source of binding sites for ω-conotoxin GVIA (a peptide toxin largely specific for N-type channels). We used PCR to amplify candidate Ca channel cDNA sequences from a λgt10 cDNA library (kindly provided by R. Scheller), derived from the electromotor nucleus that innervates the electric organ. Degenerate oligonucleotide primers were synthesized based on amino acid sequences (IGMQVFG & ATGEAWQ) flanking a 40-a.a. region between segments IVS5 and IVS6. This region is well-conserved among published Ca^{2+} channel sequences from skeletal muscle, heart and brain. When the PCR products were screened with a detection oligonucleotide probe based on a sequence (INRNNNF) within the predicted amplified region, we identified a band at ~120-b.p. After cloning the PCR products, two distinct 120-b.p. fragments were identified by sequencing (doe-1 and doe-2). doe-2 is highly homologous to DHP-sensitive Ca^{2+} channels from cardiac or skeletal muscle (Tanabe et al.). doe-1 is less closely related to these channels and much more similar to the rbA and rbB subtypes from rat brain (Snutch et al.). Using doe-1 to rescreen the cDNA library, we isolated a cDNA clone that codes for a region of a hypothetical Ca^{2+} channel extending from the NH_2 side of repeat III into the carboxy terminal region.

Some aspects of the doe-1 sequence are noteworthy. The IIIS4 and IVS4 segments contain charged amino acids in every third or fourth position, as expected for putative voltage sensors; there is an extra positive charge in IIIS4 relative to the DHP-sensitive channels. In its pattern of charged residues in IVS4, doe-1 is more similar to rbA and rbB than to rbC and cardiac and skeletal L-type Ca^{2+} channels. The regions of greatest deviation between all Ca^{2+} channels include the predicted extracellular loops between IVS1 and IVS2 and between IVS3 and IVS4. Based on homology to Na channels, the cytoplasmic loop between IIIS6 and IVS1 may be crucial for inactivation. Here doe-1 resembles rbA more closely than rbC or the cardiac or skeletal Ca^{2+} channels. It will be interesting to see if doe-1 and doe-2 are members of DHP-insensitive and DHP-sensitive subfamilies of Ca^{2+} channels, as suggested by sequence homology. If this is the case, our findings would imply that the divergence between these categories occured relatively early in evolution, in a common ancestor to rays and mammals.

W-AM-G2

EVIDENCE FOR THE EXISTENCE OF MUTUALLY EXCLUSIVE ALTERNATIVE SPLICING OF THE DIHYDROPYRIDINE-SENSITIVE VOLTAGE-DEPENDENT CALCIUM CHANNEL. W.J. Koch, P.T. Ellinor, R. Diebold, A. Schwartz. Department of Pharmacology and Cell Biophysics, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267-0575.

Recent pharmacological, electrophysiological, and molecular biological studies have provided evidence for the existence of tissue specific isoforms of the voltage-dependent calcium channel (VDCC). Based upon this, we have examined the primary structure of VDCC's from aorta, heart and brain. We have isolated a full length VDCC clone from rat aorta which corresponds to a transcript size of 8.6 kb. This sequence is similar to the cardiac channel in all but two regions. In the third transmembrane region (S3) of Motif IV we have found cDNA clones which encode for two different sequences within this specific region. These two forms of S3 appear to arise from the alternative splicing of a primary transcript since we have identified these two forms in rat aorta and in rabbit and human hearts. We have also cloned a distinct VDCC isoform from rat brain which also contains two forms of this S3 domain. The second region of significant difference between the cardiac and aorta transcripts is located in the carboxy termini and could represent a tissue-specific region. To prove that the two S3 regions are spliced products of a single gene, a rat genomic library was screened with our rat aortic VDCC cDNA. Two expressed S3 regions were found as two adjacent exons on a single gene locus separated by a 800bp intron. Another area of variability is also being examined at the genomic level.
Supported by grants RO1 HL43231, HL41496, HL22619, HL07382.

W-AM-G3

MOLECULAR DIVERSITY OF L-TYPE CA^{2+} CHANNELS IS CREATED BY ALTERNATIVE SPLICING.

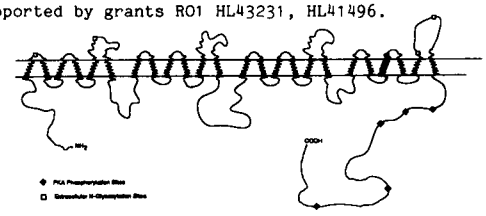
Edward Perez-Reyes, Xiangyang Wei, Antonio Castellano, and Lutz Birnbaumer. Department of Molecular Physiology and Biophysics, Baylor College of Medicine, Houston, TX.

The diversity of L-type calcium channels was probed using the polymerase chain reaction (PCR™, Cetus). In order to amplify related calcium channel genes, primers were based on non-degenerate regions that are conserved between the skeletal (CaCh 1) and the cardiac (CaCh 2) calcium channels. Using rabbit heart mRNA as template, we developed methods for: cDNA synthesis, PCR amplification, and for subcloning the products. Related sequences were amplified from human heart, hamster heart, mouse ovary, mouse BC3H1 cells, and hamster insulin-secreting (HIT) cells. Sequencing of various clones revealed the presence of alternate splicing in gene products coding for CaCh 1, CaCh 2, and a related calcium channel of neuroendocrine origin, CaCh 3. These variants differed in both the sequence of the 3rd membrane spanning domain of Unit IV (IVS3), and the linker between this segment and the next (IVS4). These results can be explained by the use of alternate exons of equal size, which accounts for the amino acid changes in IVS3, in combination with an alternate splice acceptor site or an exon skipping event, which produces the a linker of variable length. We conclude the following from these studies: 1) alternate splicing occurs in at least 3 of the 5 known calcium channel genes; 2) there may be tissue specificity to the use of alternate exons; 3) none of the calcium channel cDNAs is expressed solely in the tissue from where it was originally cloned, for example the "cardiac" gene is also expressed in brain, pancreas, and in ovary; 4) single cell types may express more than one calcium channel subtype; 5) single cell types may express more than one calcium channel gene; and 6) molecular diversity of α_1 subunits occurs through both the expression of distinct genes and alternate splicing of the primary transcript.

W-AM-G4

CLONING AND EXPRESSION OF RABBIT AND HUMAN CARDIAC ISOFORMS OF THE DIHYDROPYRIDINE RECEPTOR. D.F. Slish, G. Varadi, D.B. Engle, D. Schultz, and A. Schwartz. Department of Pharmacology and Cell Biophysics, Univ. of Cincinnati College of Medicine, Cincinnati, Ohio 45267-0575

The L type voltage dependent calcium channel (VDCC) is characterized by its sensitivity to the calcium channel antagonists (CCA). The VDCC from rabbit skeletal muscle has been extensively studied; its putative structure includes four polypeptides designated α_1, α_2/δ, β, and γ, each of which has been cloned. The α_1 subunit contains the binding domains for the CCA. Molecular cloning has shown that the α_1 shares structural similarity with other cation channels and is the channel forming subunit and voltage sensor. We have cloned the α_1 subunit from rabbit cardiac tissue. It shows 65.9% similarity with the skeletal muscle α_1, with greater similarity in transmembrane regions and divergence in the amino and carboxy terminus. Our cloned α_1 is 98.8% similar to rabbit cardiac α_1 of Mikami et. al., with an important divergence in the S3 segment of motif IV which is due to alternative splicing. Northern analysis shows two message sizes, 8.5 and 13 kb, in contrast to a 6.5 kb message in skeletal muscle RNA. The cloned normal human heart α_1 is 85-90% similar to the rabbit heart α_1 and varients due to alternative splicing have been identified.
Supported by grants RO1 HL43231, HL41496.



W-AM-G5

THE α_2 SUBUNIT OF L-TYPE VOLTAGE DEPENDENT CALCIUM CHANNEL ACTS AS AN ANCHORING PROTEIN WHEN α_1 AND α_2 CDNA's ARE COEXPRESSED IN LTK CELLS. G. Varadi, A.E. Lacerda, D. Schultz, Shaoqing Tang, A.M. Brown and A. Schwartz. University of Cincinnati and Baylor College of Medicine

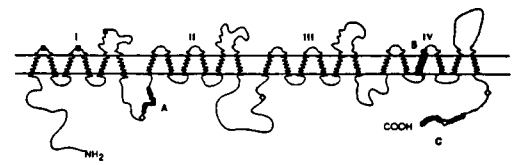
The dihydropyridine (DHP) sensitive Ca^{2+} channel in skeletal muscle is composed of α_1, α_2-δ, β and γ polypeptide subunits. Complementary cDNA's for each of these subunits have been cloned and their primary amino acid structures deduced. Expression of the α_1 subunit in Ltk mouse cells exhibited drug binding activity for organic calcium channel blockers and also a slow calcium current in which activation and inactivation kinetics differ from the calcium currents of the native skeletal muscle. In order to clarify the role of other subunits in expression of native Ca^{2+} channels, we co-transfected the α_2 and all other subunits into α_1 expressing L cells.

We have found that coexpression of α_1 and α_2 subunits produced approximately a twofold increase in peak Ca^{2+} currents. It also doubled the B_{max} for binding of DHP leaving the K_d unchanged. This observation suggests that expression of α_2 increases the number of functional α_1 subunits in the plasma membrane. The coexpression of α_1 cDNA with other subunits and their contribution in Ca^{2+} channel and drug binding activity will also be discussed.
Supported by grants RO1 HL43231, HL22619, HL07382.

W-AM-G6

MOLECULAR CLONING OF MULTIPLE SUBTYPES OF A NOVEL RAT BRAIN ISOFORM OF THE α1 SUBUNIT OF THE VOLTAGE DEPENDENT CALCIUM CHANNEL. P.T. Ellinor, A. Hui, R.J. Diebold, A. Schwartz. Department of Pharmacology and Cell Biophysics, University of Cincinnati College of Medicine, Cincinnati, OH 45267-0575.

We have isolated several cDNA's encoding for a novel voltage-dependent calcium channel α1 subunit from rat brain (RBα1). The complete nucleotide sequence of a 6975 base pair RBα1 cDNA encodes for a protein of 1634 amino acids and has a transcript size of 8.6 kb specific to brain. This brain channel clone exhibits notable differences throughout its entire transcript when compared to skeletal and cardiac clones, suggesting that it arises from a separate gene. The RBα1 clone encodes for an unusual amino terminus not reported for any other sequence. Multiple subtypes of this α1 isoform have been characterized that are indicative of alternative splicing of a primary transcript. Three variant forms of this transcript have been found which encode different forms of the cytoplasmic region between Motif I and Motif II (A). Two other species of clones differ only in the S3 region of Motif IV(B), and three clones have been found which encode different carboxy termini(C). The combination of these various subtypes leads to the potential generation of as many as 18 isoforms of this rat brain α1 subunit.
Supported by grants HL43231, HL41496, HL22619.



W-AM-G7

AFFINITY CHROMATOGRAPHY WITH IMMOBILIZED CALCIUM CHANNELS. A METHOD FOR ISOLATION OF CHANNEL MODULATORS. Pal L. Vaghy and Kathleen E. Rowader, Department of Pharmacology and Cell Biophysics, University of Cincinnati, College of Medicine, Cincinnati, Ohio 45267-0575.

Voltage-dependent calcium channels are regulated by both exogenous ligands, such as drugs and toxins, and by endogenous modulators (i.e. G proteins). The existence of putative endogenous 1,4-Dihydropyridine-like ligands has also been suggested. The goal of our study was to develop a simple and highly specific procedure for isolation and characterization of these modulators. Calcium channels purified from digitonin-solubilized skeletal muscle membranes were immobilized on Wheat germ Lectin-Sepharose 6MB. Ligands such as (+)[^{3}H]PN200-110, (-)[^{3}H] Desmethoxyverapamil and [^{3}H]Diltiazem reversibly bound to the immobilized calcium channels. This was shown by displacement with competing ligands, allosteric inhibitors and by dilution. During the binding, washing and dilution processes the channels remained on the column. Elution of bound ligand with dilution was used to regenerate functionally intact immobilized channels. The regenerated channels could be used for binding of another channel ligand. Alternatively, the immobilized channel-ligand complex could be eluted from the column with N-acethyl-glucosamine. This latter process served as part of the Wheat germ Lectin-Sepharose regeneration process. Our data suggest that functionally intact purified calcium channels are useful for the identification and affinity purification of exogenous and endogenous channel modulators when immobilized on Wheat germ Lectin-Sepharose. (Supported by NIH grant HL-41088 to P.L.V.).

W-AM-G8

THE BRAIN RYANODINE RECEPTOR: A CAFFEINE SENSITIVE Ca^{2+} RELEASE CHANNEL

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We have recently identified the presence of a high affinity [^{3}H]ryanodine receptor in rabbit brain membranes (McPherson, P.S. and Campbell, K.P., *J. Biol. Chem.* 265, 18454-18460, 1990). [^{3}H]Ryanodine binding is enriched in membranes from the hippocampus, but is significantly lower in membranes from the brain stem and spinal cord. The [^{3}H]ryanodine labeled receptor is solubilized from whole brain membranes using CHAPS phosphatidylcholine containing 1 M NaCl, and comigrates through sucrose gradients like the skeletal receptor as a large complex (~30S). Solubilized receptor is specifically immunoprecipitated by antibodies raised against the skeletal muscle ryanodine receptor. Here, we have isolated the brain [^{3}H]ryanodine receptor, and report that it displays a caffeine sensitive Ca^{2+} channel activity upon incorporation into lipid bilayers. Scatchard analysis in the presence or absence of caffeine indicates that caffeine increases the binding of [^{3}H]ryanodine to brain membranes without affecting the K_d. Brain membranes were solubilized and were subjected to heparin-agarose chromatography. [^{3}H]IP_3- and [^{3}H]ryanodine-receptors show clear migratory differences when the heparin eluate is subjected to centrifugation on linear sucrose density gradients. Peak [^{3}H]ryanodine binding fractions from the sucrose gradients were further purified by removing contaminating IP_3 receptor with concanavilin A and recentrifugation on sucrose gradients. Peak [^{3}H]ryanodine binding fractions from the second gradients contain a ~400,000 Da band on SDS-PAGE which is cross-reactive with a monoclonal antibody raised against the cardiac ryanodine receptor. Caffeine increases [^{3}H]ryanodine binding to the isolated ryanodine receptor with an EC_{50} similar to that for binding in membranes, but does not effect [^{3}H]IP_3 binding. When incorporated into lipid bilayers, the isolated brain ryanodine receptor displays a 107 pS slope conductance only in the presence of caffeine, and is blocked by the addition of ruthenium red. Thus, the brain ryanodine receptor is an ion channel which likely has a role in mediating the central nervous system effects of caffeine, and may be involved in the process of Ca^{2+}-induced Ca^{2+} release. K.P. Campbell is an Investigator of the Howard Hughes Medical Institute. Roberto Coronado is an established investigator of the American Heart Association.

W-AM-G9

Isolation of a Rat Brain Transcript with Homology to the β Subunit of the Skeletal Muscle Dihydropyridine Sensitive Calcium Channel.

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The dihydropyridine sensitive calcium channel enriched in skeletal muscle T-tubules, consists of four subunits (α_1, α_2, β and γ). All of these subunits have been recently cloned. Sequence information suggests that α_1 is the principle transmembrane subunit which contains the pore of the ion channel. The cDNA sequence of the β subunit predicts numerous consensus phosphorylation sites (Ruth et al., Science 245:1115) consistent with in vitro studies suggesting a regulatory role. We have isolated a cDNA for the γ subunit (Jay et al., Science 248:490) and the β subunit. Northern blot analysis using cDNA probes derived from the rabbit skeletal muscle β subunit message shows cross-hybridization, at high stringency, with a 3.4 kb message in both rabbit and rat brain. This is significantly larger than the 1.8 kb message observed in the skeletal muscle of both species. We have screened a rat brain cDNA library with probes made from the skeletal muscle β subunit cDNA clone. The largest insert isolated was 3.4 kb, corresponding to the full length message. Preliminary sequence analysis of this and smaller inserts predict a 63 kDa protein with high homology to the rabbit skeletal muscle β subunit. A segment of 50 amino acids extending from skeletal residue #208 to #258 is absent in the brain sequence. In addition, there is significant divergence at the C-terminus. This loss of homology occurs at #488 of the 524 residue skeletal protein. Beyond this site, the 35 remaining amino acids are substituted by 142 amino acids of brain sequence. As with the skeletal β sequence, there are numerous consensus phosphorylation sites suggesting that this is a homologous brain protein which has a calcium channel regulatory function analogous to that proposed for the skeletal muscle β subunit. Although there are nucleotide differences throughout the two sequences, we have not yet determined if the transcripts are from two different genes or if they can be accounted for by species differences. Current studies are aimed at delineating between these two possibilities. We are also pursuing in vivo expression to determine its putative regulatory function. K.P. Campbell is an Investigator of the Howard Hughes Medical Institute.

W-AM-H1

METABOLIC REGULATION OF INTRACELLULAR ION MOVEMENT: GATING CONTROL OF THE MAJOR CATION CHANNEL IN YEAST TONOPLAST MEMBRANES. Adam Bertl and Clifford Slayman. Department of Cellular & Molecular Physiology, Yale University, New Haven, CT 06510.

Controlled opening and closing of ion channels in cellular membranes has proven to be a major device for metabolic regulation. Size limitations have restricted direct observation of such gating mostly to cell plasma membranes or to bilayer (reconstituted) preparations, but the "giant" organellar membranes surrounding plant and fungal vacuoles (tonoplasts) provide an almost ideal system for direct study of *intracellular* channel behavior. Recent patch-electrode experiments on these storage organelles have identified several varieties of K channels (Tester, New Phytol. 114:305, 1990), non-specific channels (Hedrich et al., Bot. Acta 101:7-13,1988), and calcium channels which appear to be IP_3-modulated (Alexandre et al., Nature 343:567, 1990).

We have studied yeast tonoplasts as a potential membrane expression system for heterologous channels, and have found the predominant native channel species to have a conductance of ~120 pS, to be cation selective ($P_K:P_{Na}:P_{Cl}$ = 1:1:<0.1), and to be voltage-gated: the maximal open probability is ~0.7 at -80 mV (cytoplasm negative to the vacuolar sap), declining to ~0 at +80 mV. More importantly, gating of the channel is strongly modulated by calcium, but the calcium effect extends into the physiological range only in the presence of sulfhydryl reducing agents, such as dithiothreitol (DTT) and 2-mercaptoethanol (ME). Millimolar concentrations of these agents diminish the effective activating concentrations of Ca^{2+} from 1-10 mM to ~1 μM, while strong oxidizing agents (such as chloramine T) irreversibly disable the channels. Certain other mercaptide reagents, such as N-ethylmaleimide, are poor (weak, unreliable) inhibitors of channel gating, so the effects of DTT and ME appear preferentially related to oxidation state. Several potential physiological reductants have been tested, and at least one, glutathione (0.1-2.0 mM), has been found to mimic DTT and ME. The results suggest a simple mechanism whereby cellular metabolic status might regulate storage organelles, and also suggest a practical explanation--spontaneous oxidation--for the often observed "run-down" of isolated channels under the conditions of patch-recording.

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W-AM-H2

SUBSTANCE P MODULATES TWO IONIC CURRENTS THROUGH DIFFERENT TRANSDUCTION PATHWAYS IN LOCUS COERULEUS NEURONS. **K.Koyano, B.Velimirovic, J.J.Grigg, Y.Nakajima and S.Nakajima, (Intro. by G.M. Wahler), Dept. of Pharmacol., and Dept. of Anat.and Cell Biol. Univ.of Illinois at Chicago, Chicago, IL 60612**

In Alzheimer's patients substance P deficiency has been reported. Previously we have found that substance P depolarizes cultured cholinergic neurons from the nucleus basalis of Meynert by reducing an inward rectifying K-current and this effect is mediated through a pertussis toxin-insensitive G protein (Stanfield et al.,1985, Nature 315:498; Nakajima et al.,1988, PNAS,85:3643; Yamaguchi et al.,1990, J.Physiol., 426:499). Substance P also depolarizes locus coeruleus (LC) neurons (Masuko et al.,1986, J.Neurosci.,6: 3229); however, this depolarization cannot be explained simply by an inhibition of the inward rectifier. The present study was undertaken to determine which ionic currents underlie this effect. Membrane currents were recorded from cultured rat LC neurons using the whole-cell clamp method. In one third of the cells tested, substance P produced an initial inward current followed by a slow outward current at -84 mV in high K (10 mM) Krebs solution. The rest of the cells showed either the inward or the outward current only. The initial inward component was associated with a conductance increase in a voltage-independent manner. This current had a reversal potential near -10 mV, suggesting an increase in cation (Na / K) permeability. It was resistant to extracellular application of dTC (0.1 mM), APV (20 μM), MK-801 (10 μM), Co (2.4 mM), Cs (2.5 mM), TEA (5 mM), 4AP (5 mM), TTX (0.5-20 μM) and amiloride (50 μM). On the other hand, the slow outward component was caused by the inhibition of the inward rectifier. In GTPγS (260 μM) preloaded cells, the first application of substance P produced a transient inward current followed by a sustained, irreversible outward current. Further application of substance P continued to elicit an inward current but no longer evoked an outward current. This suggests that the initial inward current is caused by a process unrelated to G proteins, whereas the slow outward current (the inhibition of the inward rectifier) is mediated through a G protein.

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W-AM-H3

REGULATION OF SODIUM CHANNELS IN DEVELOPING TUNICATES

Yasushi Okamura, Jayhong Chong, and Gail Mandel
Introduced by Paul Brehm Department of Neurobiology & Behavior, SUNY, Stony Brook, NY 11794

The molecular cues or cellular interactions leading to the expression of a voltage-dependent ion channel are not known. We are using the voltage-dependent Na channel as a probe to address regulation of excitability in the developing nervous system. Such studies are difficult in vertebrates because of the complexity of cells involved in neuronal development, their small size, and by a lack of suitable cell lines where very early developmental events can be recapitulated. Therefore, we have exploited the advantages of the tunicate embryo, *Halocynthia roretzi*. In this species blastomeres cleavage-arrested at the 8-cell stage undergo normal neuronal development; the cells (80-200um in dia.) can be studied from fertilization until tadpole stage and are amenable to microinjection. The blastomeres can be cultured individually, or in combinations with other blastomeres.

The development of excitability in the tadpole of *H. roretzi* is due to the disappearance of an "egg" type Na channel (type I) and the sequential appearance of two other distinct Na channels, termed type II and type III. The induction of sodium currents II and III occurs over a short, critical time period,40 hours post-fertilization. The induction is dependent upon blastomere contact and new gene transcription. We have obtained several cDNA clones encoding portions of distinct tunicate sodium channels using the polymerase chain reaction. Sequence analysis of one of the cDNAs predicts a protein with 60% amino acid homology with vertebrate sodium channels in the putative trans-membrane domains. Northern blot analysis indicates that the corresponding tunicate sodium channel mRNA is 9.5- 10 kilobases, the same size as sodium channel mRNAs in rat brain. This mRNA is present at the gastrula and tadpole stages. Complementary DNAs that are unique to specific developmental stages have also been isolated. We are using the cDNAs as probes to determine how blastomere contact results in sodium channel gene expression and whether the expression of a specific sodium channel mRNA correlates with a specific set of functional properties in the cell.

W-AM-H4

CLONING AND CHARACTERIZATION OF AN OLFACTORY SPECIFIC CYCLIC NUCLEOTIDE GATED CATION CHANNEL

Evan Goulding, John Ngai, Andy Chess, Rich Kramer, Susan Colicos, Richard Axel, Steve Siegelbaum.
Columbia University College of Physicians and Surgeons, Howard Hughes Medical Institute.

We have isolated a functional cDNA clone encoding a cAMP/cGMP-gated channel from catfish olfactory epithelium. Clones encoding the cGMP-gated channel from a bovine retinal cDNA library were isolated using oligomeric DNA probes derived from the published sequence, these clones were then used to screen a catfish olfactory cDNA library. The retinal and olfactory channels are structurally quite similar; each comprises six putative transmembrane domains followed by a cytoplasmic tail region bearing significant sequence similarity to the cyclic nucleotide regions of mammalian cAMP- and cGMP-dependent protein kinases. There is 60% sequence identity between these two channels, with 85% identity in the putative cyclic nucleotide regulatory region. This channel is highly homologous with a cyclic nucleotide gated channel recently cloned from rat olfactory eithelium (Dhallan et al, 1990, Nature, 347).

We have expressed the olfactory channel protein in Xenopus oocytes and are studying its characteristics using inside-out patches. The channel has a unitary conductance of 25 pS. For cAMP, the channel has a Hill coefficient of 1.9 and a $K_{\frac{1}{2}}$ of 95 uM. The channel's affinity for cGMP is similar to the channel's affinity for cAMP, however the maximal current elicited by cAMP is 50-100% greater than that elicited by cGMP due to a higher maximal open probability. Open and closed time histograms have been composed from single channel recordings in the presence of varying concentrations of cAMP and cGMP. The open time histograms are fit by a single exponential with a time constant of 2 ms that is independent of cyclic nucleotide concentration. The closed time histograms are fit by two or three exponential components. There is a component with a brief time constant of 0.2 ms that is concentration independent and a component with a longer time constant which decreases with increasing cyclic nucleotide concentration.

W-AM-H5

EXTERNAL cAMP MODULATES CARDIAC Na^+ CHANNELS: EVIDENCE FOR EXTRACELLULAR cAMP RECEPTORS IN MAMMALIAN AND AMPHIBIAN MYOCYTES.

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Phosphorylation of the Na^+ channel by ß-adrenergic agonists or internal dialysis of cAMP results in the inhibition of I_{Na} channel (Catterall, Science, 242: 50, 1988 and Schubert et al. Science, 245: 516, 1989). Here, we show that external cAMP also regulates I_{Na}. In isolated whole cell-clamped rat, guinea pig, and frog ventricular myocytes, rapid (<10 ms) elevation of cAMP decreased I_{Na} 30-90% within 5-60s. The effect was dose-dependent (1-100 μM) and was measured in either 100 or 10 mM $[Na^+]_o$. In frog myocytes, 100 μM cAMP decreased I_{Na} by 40-90% reversably. cAMP shifted the steady-state inactivation of I_{Na} toward more negative potentials by 10-15 mV. Increasing the internal concentration of cAMP reduced the suppressive effect of external cAMP on I_{Na} suggesting a common mechanism mediating both effects. Inclusion of 50 μM GTP-rS in the internal solution did not alter the cAMP effect. 1 mM of GDP-ßS, on the other hand, suppressed the cAMP effect on I_{Na}. In mammalian atrial and ventricular myocytes, 100 μM cAMP also decreased I_{Na} rapidly but the effect was transient such that I_{Na} recovered fully within 180-200s. A second and third application of cAMP in mammalian cells failed to suppress I_{Na} suggesting desensitization of cAMP receptor. Phosphodiestrase inhibitor theophylline, GTP, GTP-rS all failed to alter the transient cAMP-response in mammalian myocytes. cAMP continued to suppress I_{Na} in the presence of adenosine or ATP, suggesting a different receptor type. We conclude that cAMP receptors exist in cardiac myocytes which allow for external modulation of Na^+ channels by cyclic nucleotides through a G-regulatory protein. (Supported by NIH grant HL 16152.)

W-AM-H6

AGONIST-INDUCED MODULATION OF IONIC CURRENTS AND CELLULAR Ca^{++} IN BOVINE ADRENAL CHROMAFFIN CELLS.

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The mechanisms by which humoral mediators modulate membrane excitability and intracellular free Ca^{++} concentration ($[Ca^{++}]_i$) in adrenal chromaffin cells are not well understood. We examined the effects of angiotensin II (AII) on voltage-dependent Na^+ channels, and AII and histamine (HA) on voltage-dependent Ca^{++} channels using the whole-cell voltage-clamp technique. Using 140 mM extracellular Na^+ as the charge carrier (outward K^+ currents were blocked with Cs^+ and TEA^+), 1 μM AII reduced Na^+ current peak amplitudes 25-50% without effecting the voltage-dependency of the current-voltage (I-V) relation. For Ca^{++} channels, Ba^{++} currents were recorded using 5 mM extracellular Ba^{++} as the charge carrier. Neither AII (1 μM) nor HA (100 μM) significantly effected the peak Ba^{++} current amplitudes but did shift the Ba^{++} I-V relation 5-10 mV towards more negative potentials. The agonist-induced shift in the I-V relations appeared to be more pronounced when GTP (10-100 μM) was present in the recording pipette, suggesting the involvement of a G-protein mediated transduction process. We also investigated the effects of AII on $[Ca^{++}]_i$ using the Ca^{++}-sensitive fluorescent dye Indo-1. In the presence of 2 mM extracellular Ca^{++}, AII (1 nM-1 μM) elevated $[Ca^{++}]_i$ with a time course that consisted of a high amplitude transient phase followed by a lower amplitude sustained phase. In the absence of extracellular Ca^{++}, the AII response consisted of just the transient phase (i.e. the sustained phase was dependent on extracellular Ca^{++} entry). Our results indicate that circulating mediators modulate voltage-dependent ionic conductances and induce the release of Ca^{++} from intracellular organelle(s) in chromaffin cells. Inhibition of Na^+ currents would surpress membrane excitability, and since secretion of catecholamines is dependent on extracellular Ca^{++} entry primarily during Na^+-dependent action potentials, AII may surpress neurogenic-induced secretion. The increase of $[Ca^{++}]_i$ by intracellular Ca^{++} release would further suppress excitabilty through the activation of Ca^{++}-dependent K^+ channels. The increased $[Ca^{++}]_i$ may have additional effects on other Ca^{++}-dependent processes in chromaffin cells (i.e. enkephalin and tyrosine hydroxylase synthesis). Supported by NSF Grant DCB-8812562.

W-AM-H7

PERTUSSIS TOXIN BLOCKS CALCIUM CURRENT INHIBITION BY NOREPINEPHRINE BUT NOT BY LHRH OR ATP IN FROG SYMPATHETIC NEURONS.

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LHRH, norepinephrine (NE) and ATP partially inhibit calcium current (I_{Ca}) and alter its kinetics of activation. These effects can be temporarily reversed by a strong depolarizing step (Fig.). The available evidence indicates G-proteins are involved (directly or indirectly) in coupling receptors with calcium channels. I have found that pertussis toxin (PTX) selectively blocks the NE-induced inhibition of I_{Ca}. Externally applied whole PTX (10 μg/ml for 24 hrs at room temperature) or internal dialysis of the A-protomer of PTX (1 μg/ml for 20 min) reduce I_{Ca} inhibition by NE but not LHRH or ATP (Fig.). This suggests that modulation of I_{Ca} by NE involves a G-protein distinct from that mediating inhibition by LHRH or ATP.

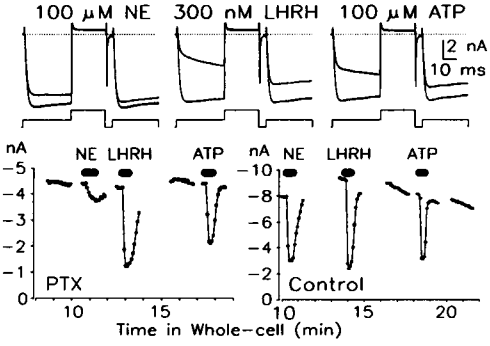

FIGURE: NE, LHRH and ATP inhibit I_{Ca} in control cells, but PTX selectively blocks the NE inhibition. The illustrated currents are from the PTX-treated cell shown below. The holding potential was -80 mV, with steps to -10 mV and +70 mV.

W-AM-H8

Alterations in the Plasma Membrane of Arterial Smooth Muscle Cells in Atherosclerosis.

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Recent studies from this laboratory have demonstrated elevations in membrane unesterified (free) cholesterol (FC) in arterial smooth muscle cells increases Ca^{++} and decreases K^+ movements across the membrane as well as decreases membrane fluidity and increases smooth muscle contractility and proliferation. Since atherosclerosis is characterized by elevations in serum cholesterol, we examined arterial smooth muscle obtained from atherosclerotic rabbits for Ca^{++} influx and K^+ efflux as well as membrane FC content. Rabbits were fed a high cholesterol diet (2%) for 10 weeks. Thoracic aortas were obtained following euthanasia with pentobarbital. Using aortic ring segments, Ca^{++} influx was measured with a standard $^{45}Ca^{++}$ influx protocol and K^+ efflux measured using $^{86}Rb^+$ as a marker for K^+ in a standard K^+ efflux protocol. Segments of aorta were also scraped to remove the intimal layer and the medial smooth muscle layer was dissected free. The smooth muscle was homogenized and subjected to differential ultracentrifugation to obtain a purified (>12-fold) plasma membrane fraction for cholesterol, phospholipid and protein assays. Ca^{++} uptake was increased in the atherosclerotic tissue compared to control (11.6±1.9 vs 22.2±2.9 µmole/Kg wet.wt./min.; $p<.02$) and ATP-dependent K^+ efflux suppressed 38% to Cromokalim, 79% to Pinacidil and 77% to ATP depletion with metabolic inhibitors ($p\leq.05$), while Ca^{++}-activated K^+ efflux was not affected. Membrane FC content increased 28.5% (75.2 vs 58.5 µg/mg protein) in the atherosclerotic tissue vs control, and the FC/PL ratio increased 81.4% (.684 vs .377). These results suggest that atherosclerosis is accompanied by an enrichment of the arterial smooth muscle plasma membrane with cholesterol, and this enrichment alters both calcium and ATP-dependent potassium channels. This finding supports the hypothesis that atherosclerosis is characterized by a lipid defect in the membrane phospholipid bilayer of arterial smooth muscle cells that accounts for its abnormal cation movements and cell function. Supported in part by PHS grant HL-30496 and grants from the American Heart Association, Southeastern Affiliate (T.N.T. & D.R.) and Delaware Chapter

W-AM-H9

PROTEOLYTIC DIGESTION WITH TRYPSIN REDUCES THE VOLTAGE-DEPENDENT CLOSING OF MIP CHANNELS INCORPORATED INTO PLANAR LIPID BILAYER MEMBRANES (BLM). G.R. Ehring, P. Lang and J.E. Hall. Dept. of Physiology and Biophysics, University of California, Irvine, 92717.

When incorporated into BLM lens MIP produces large conductance channels, which are open at membrane voltages near zero and closed at voltages of either sign with magnitudes greater than 80 mV (J. Gen Physiol. 96:631, 1990). In the lens MIP exists in two isoforms, which differ in phosphorylation state. The degree of phosphorylation modulates the voltage-dependent closing of MIP channels (Biophys J. 57:243a & 22a 1990). Lampe and Johnson have shown that the phosphorylation site is SER 243 (in press), which is near the carboxy-terminal end of the 263 amino acid MIP. With age, the carboxy-terminal is proteolytically degraded *in vivo* by endogenous proteases. We reasoned that the loss of the carboxy-terminus containing the phosphorylation site should reduce the voltage-dependent closing of MIP channels. To test this hypothesis we examined the effects of the protease, trypsin, on the closing kinetics of MIP channels already incorporated into BLM. SDS-PAGE on lens membrane fragments containing MIP determined that a concentration of 80 ug/ml trypsin was sufficient to digest MIP (MW 28 kD) to its 22 kD degradation product within 1 hour. Further digestion had no further effect. We incorporated MIP channels into phosphatidyl-ethanolamine-squalane BLM bathed in 200 mM KCl, 1 mM $CaCl_2$, 10 mM HEPES, pH 7.3. The voltage-dependent closing of the channels was assayed by studying the currents resulting from the application a family of 5 sec voltage pulses (+100 to -100 mV, increments of 20 mV) from a holding potential of 0 mV before and after the addition of trypsin. In control experiments, the current immediately after the application of the voltage pulse was a linear function of the applied voltage. As the applied voltage increased the current during the pulse decreased. At 100 mV the current at the end of the pulse was appoximately half that at the beginning of the pulse. The addition of trypsin reduced this voltage-dependent closure. The effects of trypsin were progressive and their time course was variable. When trypsin had only partially reduced voltage-dependent closure, the half times for decay of the current remained approximately the same as before the addition of the protease. Suggesting that the effects of trypsin the single channel level may be all or none. In addition to its effects on channel closure, trypsin also reduced the overall conductance of the bilayer suggesting that it may be destroying MIP channels or reducing their single channel conductance. Single-channel experiments are in progess to distinguish between these cases. Supported by NIH EY-05661.

W-AM-I1

ROLE OF SERINE AND THREONINE RESIDUES IN STRUCTURE AND FUNCTION OF BACTERIORHODOPSIN. T.Marti, H.Otto+, T.Mogi, S.Rösselet, M.P.Heyn+, and H.G.Khorana, Mass. Institute of Technology, Cambridge, MA 02139; and +Freie Universität Berlin D-1000 Berlin

22 Ser and Thr residues presumed to be located within and near the border of the transmembrane segments have been individually replaced by Ala or Val, respectively. Thr-89 was substituted by Ala, Val and Asp, and Ser-141 by Ala and Cys. Most of the mutants showed normal spectral and functional properties. These residues may nevertheless contribute to the structural stability of bR. Replacements of Thr-89, Thr-90 and Ser-141 revealed altered properties with respect to regeneration kinetics, absorption spectrum and dark-light adaptation. These mutants also showed reduced pumping activities, most likely due to a lower percentage of all-*trans* retinal in the light-adapted state. The data indicate that residues 89, 90 and 141 are in direct contact with the retinylidene chromophore. Photocycle measurements showed that in the mutants Thr-46→Val and Ser-226→Ala the decay of the M intermediate is significantly accelerated, thereby suggesting an interaction between these residues and Asp-96 which reprotonates the Schiff base. We conclude that none of the serines and threonines are obligatory for proton translocation by bR. (Supported by grants from the NIH, ONR, DFG and Swiss NSF).

W-AM-I2

Sequential Truncation of a Five Amino Acid Segment at the end of the Last Transmembrane Helix of the *Lac* Permease of *Escherichia coli* Edward McKenna, Dorothy Hardy, John C. Pastore, and H. Ronald Kaback Howard Hughes Medical Institute, University of California, Los Angeles, 405 Hilgard Avenue, Los Angeles, CA 90024-1570

Lac permease of *E. coli* is a 417 amino acid polytopic membrane protein that catalyzes the symport of a single β-galactoside with H^+. The proposed secondary structure predicts a polypeptide with a short cytoplasmic N-terminus, 12 hydrophobic transmembrane α-helices, and a 17 residue cytoplasmic C-terminal tail. Previous work [Roepe et al.(1989) *PNAS 86*,3992] localized a five residue sequence(residues 396-401) near the carboxyl end of the last transmembrane helix(XII) as a requirement for function and stability to proteolysis. In order to further define this region, individual site-specific mutants were constructed with stop codons placed sequentially at positions 396 through 401 of *lac* permease and the truncated *lac* Y genes were expressed from plasmid pT7-5. Sequential truncation of *lac* permease from amino acids 401 to 396 caused a progressive decrease in transport activity from a fully active to an inactive protein. Rates of transport, expressed as a percentage of wild-type *lac* permease, were 95%(S401stop), 70%(L400stop), 50%(T399stop), 30%(F398stop), <10%(V397stop), and <10%(S396stop) and the steady-state level of accumulation behaved similarly. The results suggest that the carboxyl end of putative transmembrane helix XII is involved in proper folding of *lac* permease and is, therefore, critical for l transport activity.

W-AM-I3

ENGINEERING A MEMBRANE TRANSPORT PROTEIN FOR FLUORESCENCE SPECTROSCOPY Thomas G. Consler and H.Ronald Kaback, Howard Hughes Medical Institute, Molecular Biology Institute, UCLA, Los Angeles, CA 90024-1570.

The *lac* permease of *E. coli* is a hydrophobic polytopic plasma membrane protein that catalyzes lactose/H^+ symport. This permease is encoded by the *lacY* gene, and it has been solubilized from the membrane, purified to homogeneity and demonstrated to be solely responsible for β-galactoside transport as a monomer. The wild type lactose permease has six tryptophan (Trp) residues. The fluorescence signal from all six Trp residues are represented by the overall fluorescence spectrum, thus obscuring the contribution by each individual Trp residue. Using site-directed mutagenesis, the lactose permease was redesigned for fluorescence spectroscopy. Thus, the native Trp residues were first replaced with phenylalanine (Phe) individually. Each of these mutant permeases retains significant lactose transport activity, indicating that the Trp to Phe substitutions do not dramatically alter the structure or the function of this membrane transport protein. Furthermore, all six Trp residues were replaced with Phe, creating a Trp-less permease (W6F; Menezes, et.al., PNAS, 87, 1638, 1990), without losing the ability to catalyze lactose/H^+ symport. The gene encoding W6F permease was then redesigned by site-directed mutagenesis using a template with specifically engineered unique restriction sites. This operation easily allows reintroduction of each of the six native Trp residues individually or insertion of single Trp residues at any desired position in the sequence of the permease. Single Trp containing permeases should be invaluable for identifying the conformationally dynamic regions of this membrane protein as well as domains that are influenced by substrate binding. In addition to the six native Trp residues, a single Trp was introduced into the W6F background at position E325W. This position was selected for its proximity to the mechanistically important residues in the permease, and thus has potential for being a localized spectroscopic probe in the protein.

W-AM-I4

THE MITOCHONDRIAL PHOSPHATE TRANSPORT PROTEIN (PTP) FROM THE YEAST *SACCHAROMYCES CEREVISIAE*. Hartmut Wohlrab, Anne Phelps, and Christian Schobert. Boston Biomedical Research Institute and Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02114.

We have cloned the gene of the mitochondrial phosphate transport protein (PTP) from *Saccharomyces cerevisiae*, a member of the mitochondrial anion transport protein gene family (1) and a receptor for the import of nuclear-coded proteins into mitochondria (2). Three peptides were prepared from the N-terminal blocked yeast PTP, which had been purified as a reconstitutively-active protein (3). Oligonucleotides, based on their sequences, were used to screen a Yep24-housed genomic library. Four independent clones were isolated. 2073 bases of the clone Y22 code for a 311-amino acid protein (Mr 32,814), which has similarities to the anion transport proteins: a triplicate gene structure and six hydrophobic segments. Typical for PTP, the triplicate gene structure possesses the X-Pro-X-(Asp/Glu)-X-X-(Lys/Arg)-X-(Arg/Lys)-X (X is an unspecified amino acid) motif and the very high homology only between the first and second repeat (4). The six hydrophobic segments harbor most of the 116 amino acids that are conserved between the yeast and the beef proteins. An N-terminal extended signal sequence, as found in the beef heart and rat liver proteins, is absent. The yeast protein has about 33% fewer basic and acidic amino acids and five fewer Cys residues than the beef protein. Of the three cysteines, only Cys-28, located in the first hydrophobic segment, is conserved. The protein is insensitive to N-ethylmaleimide since Cys-42 (beef) has been replaced with a Thr (4). Its retained mersalyl sensitivity must be due to one of the three cysteines. Coding and non-coding regions of the four independent clones are the same, suggesting the presence of only a single PTP gene in *S. cerevisiae*. (1) Wohlrab, H. (1986) *Biochim. Biophys. Acta* 853, 115; (2) Pain, D., et al. (1990) *Nature* 347, 444; Murakami, H., et al. (1990) *Nature* 347, 488; (3) Guerin, B., et al. (1990) *J. Biol. Chem.* (in press); (4) Phelps, A., et al. (1990) *Biochemistry* (in press). (Supported by NIH GM 33357).

W-AM-I5

FUNCTIONAL INTERACTION BETWEEN THE CYTOPLASMIC AND MEMBRANE-BOUND DOMAIN OF THE MANNITOL TRANSPORT PROTEIN OF ESCHERICHIA COLI.

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The mannitol specific transport protein, enzyme II^{mtl}, catalyzes mannitol transport and phosphorylation via the follwing reaction in which P-enolpyruvate serves as the initial phosphoryl group donor.

$$Mannitol_{out} + P\text{-}Hpr \xrightarrow{II^{mtl}} Mannitol\text{-}P_{in} + Hpr$$

Though in the overall reaction transport and phosphorylation are coupled events, mechanistically the two reactions are separate steps. The membrane bound domain translocates mannitol across the membrane, after which mannitol, still bound to the inwardly facing binding site is phosphorylated by the cytoplasmic domain. The main effect of phosphorylation of the enzyme is an activation of the transport activity of the translocator domain, which requires a conformational interaction between the two domains.
Data will be presented that support the hypothesis on the interaction between the two domains. Functional properties of the translocator domain were studied after chemical of mutational modifications in the cytoplasmic domain. Modification of Cys384, which is the phosphoryl group binding site in the cytoplasmic domain had two effects on the translocator domain, i. the binding affinity for mannitol was reduced and ii. the translocation activity had increased by a factor of 5 - 10.

W-AM-I6

THE MANITOL CARRIER OF E. COLI: PURIFICATION AND RECOMBINATION OF DOMAINS

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EII-mtl is a single 67 Kda polypeptide chain which catalyzes the transport and phosphorylation of mannitol. It consists of three domains, a membrane-bound domain in the N-terminal half and two cytoplasmic domains in the C-terminal half. These domains have been subcloned and expressed with retention of enzymatic activity. The N-terminal domain is the mannitol binding and translocating unit. The most extreme C-terminal domain has the function of EIII; it phosphorylates the middle cytoplasmic domain at a cysteine. The middle domain, in turn, couples the mannitol translocation and phosphorylation events. A similar motif has been reported for many other sugar-specific EII species. In EII-glc, EIII is free rather than covalently attached to EII as shown in figure 1.

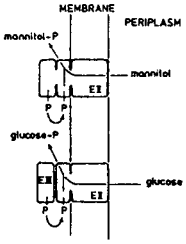

figure 1

The subcloning, purification and characterization of the middle domain of EII-mtl will be presented. Two chimeric proteins have been constructed; one consisting of the N-terminal domain of EII-mtl and the C-terminal domain of EII-glc and the other consisting of the N-terminal domain of EII-glc and the C-terminal domain of EII-mtl. The activities of these proteins alone and combined will be presented.

W-AM-I7

UNCOUPLING PROTEIN OF BROWN ADIPOSE TISSUE

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The Uncoupling Protein (UCP) of brown adipose tissue mitochondria is an H^+/OH^- translocator. At lower activity, UCP is able also to translocate anions, particularly Cl^-. The transport rate is linearly dependent on the membrane potential similar as in a channel and differently from a carrier, such as the ADP/ATP carrier, which has a logarithmic interdependence. This is interpreted in lower activation barriers of the H^+/OH^- translocation. Conformation changes and corresponding gating frequencies will be much lower with small solutes, such as H^+/OH^- in UCP than with large solutes, such as ATP in the ADP/ATP carrier. Surprisingly, the H^+ translocation rate does not change between pH 6 to 8. Obviously, with UCP exists an H^+ buffering supply mechanism. After removal of endogenous fatty acids H^+ translocation is dependent on addition of long chain free fatty acids. Recent studies show pH dependency of H^+ transport linked to the chain lenght of free fatty acids and their pK. The pK of FA increases with chain length from C_{10} pK 5 to C_{18} pK 7.5. These and other results can be integrated to a novel interpretation of free fatty acid function as a localized H^+-supply buffer. At the access channel, possibly on both sides of the central H^+ switch within UCP, $COOH^-$ groups of long-chain fatty acids supply H^+ thus facilitating sufficient high rates of H^+/OH^- translocation. These studies reveal that due to the lack of H^+ in the H^+ channel a H^+ translocation at a sufficient rate is virtually impossible unless localized H^+ buffers are supplied. This principle also holds for other H^+ translocators, e.g. for bacteriorhodopsin, where aspartyl groups provide localized intra channel H^+ supply. In UCP the access channels are obviously constructed not to contain intrinsic H^+ supplying COOH groups which therefore must be supplied by fatty acids for regulatory purposes. Thus studies on UCP reveal a general principle for H^+ translocation channels.

W-AM-I8

A Na^+-INDUCED CONFORMATIONAL TRANSITION PRECEDES Na^+ TRANSLOCATION IN THE MECHANISM OF Na^+-H^+ EXCHANGE. Eio Koh, James Kinsella, Linda Cheng, Kinya Otsu, Phillip Keller and Jeffrey Froehlich. N.I.A, N.I.H, Baltimore, MD 21224, and Charles H. Best, Institute, U. of Toronto, Toronto Ontario, Canada M5G 1L6.

Transient state kinetic investigations of the renal brush border Na^+-H^+ exchanger at 0°C (Otsu et. al. PNAS; 1989) have provided evidence for a slow conformational transition that separates H^+_o release (following H^+ translocation) from Na^+_o binding (preceding Na^+ translocation) at the extravesicular membrane surface, viz.

$$C_oH \overset{H^+_o}{\rightleftharpoons} C_o \rightleftharpoons C_o' \overset{Na^+_o}{\rightleftharpoons} C_o'Na$$

To further test this model, we examined the effect of varying the extravesicular pH on $^{22}Na^+$ uptake in the transient and steady state phases of the exchange reaction. Lowering the external pH at constant internal pH reduced the amount of Na^+ uptake in the presteady state (burst) and steady state (linear) phases while prolonging the duration of the lag phase which precedes the burst. Computer simulation of the time course of Na^+ uptake revealed that a minimum of 2 steps are required to account for the presence of the lag and the burst phases of the transport reaction. The faster of these steps, which gives rise to the lag, was assigned to the conformational transition, C_o to C_o', while the slower step was identified with Na^+ translocation. An analysis of the effects of extravesicular pH on the steady state Na^+ uptake indicated that H^+_o and Na^+_o compete for the same binding site (competitive inhibition), excluding mechanisms in which Na^+_o and H^+_o bind to different, slowly transforming, intermediate states (as above). Based on these results the following modification is proposed;

$$C_oH \overset{H^+_o}{\rightleftharpoons} C_o \overset{Na^+_o}{\rightleftharpoons} C_oNa \rightleftharpoons C_o'Na$$

where Na^+ binding to the unliganded carrier induces the conformational transition that precedes Na^+ translocation. The Na^+-induced transition may represent Na^+ occlusion by the transport protein or the formation of a tight Na^+ binding complex.

W-AM-I9

ELECTROGENIC PROPERTIES OF THE CLONED Na^+/GLUCOSE TRANSPORTER: TRANSPORT MODEL UNDER NON RAPID EQUILIBRIUM CONDITIONS. L. Parent, S. Supplisson, D.D.F. Loo, and E.M. Wright. Dept of Physiology, UCLA-School of Medicine, Los Angeles, CA 90024-1751.

Transmembrane currents associated with the cloned Na^+/glucose transporter were studied in cRNA injected *Xenopus Laevis* oocytes. Presteady-state carrier ($\tau_{\frac{1}{2}}$ = 10ms) and steady-state sugar specific currents were measured simultaneously in a single oocyte. A kinetic model was developed according to these experimental observations: i) sugar specific currents are not observed in absence of Na^+;ii) sugar specific I-V curves are sigmoidal; iii) the variation of sugar specific currents as a function of external Na^+ is best described by a Hill plot with an apparent coupling coefficient of 2; iv) phlorizin-sensitive Na currents are observed in absence of sugar which suggests some internal Na leak; v) $K_{0.5}$ and i_{max} for αMDglucose and Na^+ are dependent on voltage and on the concentration of the co-substrate; vi) presteady-state carrier currents are observed as transient outward currents. Altogether, these results suggest that the current is carried by the empty carrier. The above described transporter properties were described by a 6-state, simultaneous and ordered kinetic model assuming a maximum expression of 10^{11} carriers per oocyte. Carrier translocation in the membrane as well as Na and sugar binding and dissociation are treated as a function of their individual rate constants, and empty carrier translocation and/or Na binding are considered to be voltage dependent. Steady-state current equations were derived using the King-Altman method which gave 15 King-Altman patterns and 90 terms. Presteady-state carrier currents were simulated by numerical integration of the current derived equations using the individual transporter rate constants. The model accounts quantitatively for the following experimental results: i) the sigmoidal shape of the sugar specific I-V curves, ii) the decrease in $K_{0.5}$ for αMDG observed at 0 mV as the external Na^+ is increased, iii) the decrease in Na dependence of $K_{0.5}$ for αMDG as Vm is increased to -150 mV, iv) the absence of Na dependence in I_{max} for αMDG between 10 and 100 mM Na, v) the voltage and sugar dependence of $K_{0.5}$ and i_{max} for Na^+ ions, vi) the presteady-state outward currents being a function of external Na and αMDG. **We conclude that:** 1) Na binding and empty carrier translocation are both voltage dependent and 2) to account for presteady-state and steady-state currents, carrier translocation rates must fall between 1 and 10 sec^{-1}. Translocation rates are therefore 10 to 1000 times slower than all Na and sugar association and dissociation rates pointing toward rapid equilibrium conditions. While we cannot rule out a more complex kinetic model, the electrical properties of the cloned Na^+/glucose transporter at the steady-state and presteady-state level are adequately described by a 6-state, simultaneous, and ordered kinetic model.

W-AM-I10

I_{NaCa} CURRENT TRANSIENTS IN GIANT CARDIAC MEMBRANE PATCHES WITH STEP ION CONCENTRATION CHANGES. **D.W. Hilgemann & A.Collins** / Dept. of Physiology, UTSW Med.Ctr., Dallas, TX, 75235.

Na^+/Ca^{2+} exchange current transients (I_{NaCa}) were studied in giant excised cardiac membrane patches with external (pipette) solutions to allow activation of 1) only $+I_{NaCa}$ (5 mM Ca^{2+}, 0 Na^+), 2) both + and $-I_{NaCa}$ (2 mM Ca^{2+}, 150 mM Na^+), and 3) only $-I_{NaCa}$ (5 mM Mg^{2+}, 2mM EGTA, 150 mM Na^+) by appropriate 'cytoplasmic' (bath) solution changes (<0.4 s). $+I_{NaCa}$ inactivation rate is strongly $[Na]_i$-dependent (rate constants, 0.15 to 1 s^{-1} with increasing $[Na]_i$). Steady state inactivation of $+I_{NaCa}$ and the steady state $+I_{NaCa}$, itself, have identical $[Na]_i$ dependencies, while the quasi-instantaneous current is half-activated at lower $[Na]_i$. When patches are bathed in Ca^{2+}-free, Na^+-free solution, $+I_{NaCa}$ activates fully within 0.5 s upon application of Ca^{2+}/Na^+-containing solutions and is followed by $[Na]_i$-dependent inactivation, as usual; no $+I_{NaCa}$ is detected in switches to Ca^{2+}-free solution with 100 mM Na^+. Thus, secondary activation by $[Ca^{++}]$ exists independently of $[Na]_i$-dependent inactivation and is fast in absence of $[Na]_i$. $-I_{NaCa}$ is similarly available within 0.5 s upon application of $[Ca^{2+}]_i$. In the absence of $[Na]_i$ simple $[Ca^{2+}]_i$-dependence of $-I_{NaCa}$ is found without inactivation (K_d, 2.5-5 μM). $-I_{NaCa}$ does however undergo $[Na]_i$-dependent inactivation, with similar time- and $[Na]_i$-dependence as $+I_{NaCa}$ inactivation. These results document existence of multiple inactive exchanger states, depending on ion binding configurations of the exchanger, and pin point the entrance into $[Na]_i$-dependent inactivation to a state fully sodium loaded with cytoplasmic-orientated binding sites. Progress in resolving time courses of fast $[Ca]_i$-dependent state transitions and half-cycles of exchange with a rapid solution switcher will be reported.

W-AM-J1

ELECTROSTATIC EFFECTS ON PROTEIN STABILITY

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Electrostatic interactions involving ionizable groups are important contributors to protein stability. One of the characteristic properties of small globular proteins arising from these electrostatic interactions is the pH-dependent stability in aqueous solution: most native proteins are unstable in extremely acidic or alkaline solutions. The denaturation energy of a native protein can be decomposed into pH-dependent terms (electrostatic interactions) and pH-independent terms (including van der Waals interactions, hydrogen bonds of non-ionizable protons, hydrophobic interactions, and conformational entropy). We have developed a procedure to calculate the total electrostatic energy and the pH-dependent terms of the denaturation energy for any protein of known structure. Firstly, the pK_a's of the ionizable groups in a protein have to be derived by calculating the desolvation energies, the polar interactions, and the pairwise charge-charge interactions of the ionizable groups. This is done with the Finite Difference Poisson-Boltzmann method. The average charges of the ionizable groups between pH=0 and pH=14 are then determined by a hybrid method which includes the Tanford-Roxby self-consistent iteration and a statistical mechanics averaging. Once the average charge calculation is completed, the total electrostatic energy of the protein as a function of pH is calculated. This procedure has been applied to hen egg-white lysozyme and T4 lysozyme to account for the pH-dependent stability observed in experiments with either wild-type or mutant proteins.

W-AM-J2

COMPUTATIONAL STUDY OF THE ROLE OF CHARGED GROUPS IN THE FOLDING TRANSITION OF THE C-PEPTIDE.

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A computational study of the role of all ionizable groups of the C-peptide in its helix-coil transition is performed within the framework of continuum electrostatics. The method employed in our computations involves a numeric solution of the Poisson equation with the Boundary Element Method. Our calculations correctly predict the experimentally observed trends in the helix-coil equilibrium of the C-peptides, and suggest that the mechanisms involved are more complex than usually presumed in the literature. Our results suggest that: electrostatic interactions in the unfolded conformation are often more important than in the helix; total electrostatic contribution to the helix-coil transition due to the side chains of the C-peptide destabilizes the helix; changes in the helix stability produced by the changes in the ionization state of the side chains are dominated by side chain effects; the effect of the helix dipole on the energetics of the helix-coil transition of the C-peptide is either minor or similar to other contributions in magnitude; while the formation of a salt bridge is electrostatically favorable, formation of the hydrogen bond between a charged and a polar side chains is not.

W-AM-J3

SIMULATION OF THE UNFOLDING OF AN α-HELIX IN WATER

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We report a 1 ns molecular dynamics (MD) simulation on an 18-residue peptide which forms part of the H-helix of myoglobin. The calculations were carried out using the AMBER3.0 set of programs and the united atom AMBER/OPLS force-fields. The peptide in the helical conformation found in the X-ray structure of the protein was surrounded with TIP3P water to a thickness of at least 8A along the three axes, energy-minimised, and heated from 0 to 300K using temperature regulated MD; then followed the main part of the simulation: 1 ns at 300K (constant temperature MD). The coordinates were saved at regular intervals and analysed. The overall result of the simulation was the slow unwinding of a good α-helix with intact $i \leftarrow i+4$ hydrogen bonds to a more disordered structure, with the disturbance (loss of helicity as followed by changes in the hydrogen bond pattern, the values of the main chain torsions, and that of θ, the virtual torsion involving α-carbons) generally moving from the C-terminus towards the N-terminus. The first hydrogen bond to break was Tyr-15←Ame-19, about 30 ps into the simulation, and by ~550 ps, only two helical hydrogen bonds remained. A variety of mechanisms are involved in the breaking of the α-helical hydrogen bonds. Some of the intermediates involved are transient 3_{10}-helical structures, bifurcated hydrogen bond structures intermediate between α and 3_{10}, and water-inserted hydrogen bonds (M. Sundaralingam & Y.C. Sekharudu, Science 244, 1333-1337, 1989) of the $i \leftarrow i+4$ or $i \leftarrow i+3$ type. It was common for the intermediates to have more than one H_2O molecule hydrogen bonding to a donor or an acceptor on the peptide, and vice versa. The detailed sequence of events in the breaking of some of the α-helical hydrogen bonds will be presented and the implications of the simulation on the mechanism of protein folding will be discussed. Results of a similar simulation on an analogue of the ribonuclease C-peptide will be reported; here the helix persists for a much longer time.

W-AM-J4

PROBABLE NON-LOCAL INTERACTIONS IN GLOBULAR PROTEINS.

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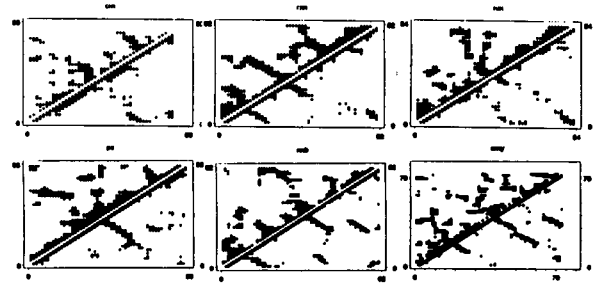
Idealized lattice models of C^α backbones are used to explore non-local interactions in small globular proteins. Starting from the protein's amino acid sequence and a randomly generated non-compact (Rg^2 > 10X the native structure) C^α chain, a simplified Monte Carlo algorithm is used to determine important long-range (|I-J|>3) residue-residue contacts. For the six proteins studied, crambin (46aa), rubredoxin (52aa), ferrodoxin (54aa), trypsin inhibitor (58aa), neurotoxin (62aa) and amylase inhibitor (74aa), between 64 to 78% of native non-local interactions were found. Distance plots of the final structures indicated similarities with those of the native folded structures (see below, bottom:native, top:calculated, cutoff distance < 7.5Å). These structures provide starting conformations for constructing models with more complete atomic details.



W-AM-J5

CONFORMATIONAL SEARCH USING MONTE CARLO-MINIMIZATION PROCEDURE.

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A Monte Carlo-minimization (MCM) procedure has been developed for the search of low energy conformations of peptides by combining Monte Carlo torsional sampling, steepest descent and conjugate gradient minimizations, and Metropolis algorithm, using the flexible-geometry force field implemented in DISCOVER (1). Compared with the original version of the Monte Carlo-minimization procedure (2), new features have been introduced to improve its efficiency in sampling the conformational space of a peptide. The lowest-energy structure of Met-enkephalin obtained in test runs starting from random conformations, using DISCOVER potentials, is structurally similar to the global minimum-energy conformation obtained using the ECEPP potential function, indicating convergence of the procedure for the penta peptide. Applications of the MCM method to bradykinin, bradykinin analogues and a cell-adhesion peptide, have located lower-energy structures compared with previous results using other methods. The MCM procedure can also be used as a structural refinement method for larger peptides, by performing the Monte Carlo-minimization iterations starting from specific candidate structures of interest, and has been applied to the study of low energy conformations of endothelin.

(1) Registrated trademark of BIOSYM Technologies, Inc.
(2) Z. Li and H.A. Scheraga (1987), "Monte Carlo-minimization approach to the multiple-minima problem in protein folding", Proc. Natl. Acad. Sci. USA, 84, 6611.

W-AM-J6

XRAY-BASED MICROSCOPIC MODEL OF PROTEIN DENATURATION, Peter Leopold, Dept. of Physics, Univ. of Calif., San Diego.

In an effort to reproduce protein denaturation and identify unfolding pathways, we present an Ising model for the denaturation phase transition of small globular proteins. The model consists of a Monte Carlo simulation performed on crystallographically-derived secondary bonds. The free energies of secondary bonds depend on bond type (disulfide, hydrophobic, etc.) and on the entropies of the random loops and coils in local structure. Bonds are entropically coupled to "neighboring" bonds by a set of self-scaling rules that remain invariant during the unfolding transition.

The Hamiltonian has the form

$$H(\vec{b}) = -\sum_{i=1}^{i=N} \epsilon_i b_i - T\sigma(\vec{b})$$

where

$\vec{b}$ = *ordered array of N bonds,* b_i,
ϵ_i = *enthalpy of bond* b_i,
$\sigma(\vec{b})$ = *total loop and chain entropy for* $\vec{b}$.

Estimates of backbone dihedral partition functions are used in calculat ing local loop and chain entropies.

We present an analytical solution of the Hamiltonian for simple secondary and supersecondary structural motifs and show agreement with Zimm-Bragg theory. In addition, we present temperature dependent unfolding and heat capacity curves for a variety of small globular proteins and mutants. Finally, we discuss the implications of these results on unfolding pathways.

W-AM-J7

ANALYZING FOLDING REACTION OF THE ALPHA SUBUNIT OF TRYPTOPHAN SYNTHASE BY COMPUTER SIMULATION

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As protein folding reactions are probed in ever increasing detail, the relationship between the observed relaxation times and the rate constants for individual steps become complex. The resolution of these microscopic rate constants is critical for a quantitative evaluation of the effects of mutations on folding and an assessment of folding of an individual amino acid in folding. Computer simulation of folding mechanisms provides a realistic solution to this problem. We applied this approach to analyze the reversible, urea induced folding reaction of the α subunit of tryptophan synthase from E. coli. Based on a previously proposed model (Hurle and Matthews, Biochimica et Biophysica Acta: **913** (1987) 179-84), we obtained analytical expressions relating the microscopic rate constants and the experimentally measurable relaxation times and amplitudes (Ikai and Tanford, J. Molecular Biology: **73** (1973) 145-63). Estimates of the rate constants were obtained by simulating both unfolding and refolding reactions. These rate constants could be combined with plausible estimates of the optical properties of various species in the reaction to predict the equilibrium unfolding transition curse. Given the complexity of the kinetic folding model for the α subunit, simulation appears to provide a more accurate way to determine the effects of mutations on folding. This work was supported by NIH grant GM 23303.

W-AM-J8

EVIDENCE OF DIFFUSION/COLLISION/COALESCENCE PROCESS IN THE FOLDING OF STAPHYLOCOCCAL NUCLEASE AND THE NATURE OF THE COALESCENCE REACTION.

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The minimal kinetic model for folding of the acid and the alkaline unfolded S. nuclease has been determined to be, $D_3 \rightarrow D_2 \rightarrow D_1 \rightarrow N_0$, in which the three Ds are the three sub-states of the unfolded protein and N_0 is the native state. The time constants for these reactions were τ_{10} = 140 ±8 ms, τ_{21} = 840±30 ms and τ_{32} = 30±3 s, at pH 7.0, 0.125 NaCl, 25°C. Of these reactions, only the τ_{21} reaction was dependent on the viscosity of the solution in a manner consistent with the Diffusion/Collision/Coalescence Model, when the rate was compared in media of fixed dielectric constants. When the viscosity of the medium was fixed, its rate was found to depend on the dielectric constant of the solution in a manner suggesting that there was a cooperative change in the stability of the merged cluster at the dielectric constant of approximately 70. The activation energy of the τ_{21} reaction was 23.4 kJ mole^{-1}, which is comparable to that for a diffusion event. Both D_1 and D_2 sub-states exhibited non-random, sheet-like chain conformations but structures were not detected by differential scanning calorimetry. These results and other kinetic and spectroscopic data favor the framework model but do not exclude the hydrophobic collapse model. Data also suggest that long range electrostatic interactions may be involved in the chain condensation processes. [Supported by NIH Grant GM37304]

W-AM-J9

JASEP: A NEW JOINT ALGORITHM FOR SECONDARY STRUCTURE PREDICTION

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The classical problem of secondary structure prediction is approached by a new joint algorithm (JASEP) that combines the best aspects of six different methods including Chou-Fasman, homology modeling, information theory and neural network methods. Steps in the predictive scheme include (i) Optimizing the threshold for assigning a structural type from the predictive score of each method; (ii) weighting each method (its set of scores) by the correlation coefficient for each structural type; (iii) combining the scores from different methods, and (iv) comparing the scores for α-helical , β-strand and coil conformational states to assign secondary structure unambiguously at each residue position. In this process, each component method is also refined by optimizing its predictive power (as measured by the correlation coefficient). It is seen that a combination of three generically different, strong predictors is superior to any single predictive algorithm. While this approach is generally applicable to any protein class, the present application to twenty α/β proteins demonstrates good predictive power (with correlations, $Q_{7,\alpha}$ = 0.391; $Q_{7,\beta}$ = 0.417; $Q_{7,c}$ = 0.393 for α-helical, β-strand and coil conformations). By the criterion of correlation coefficient (Q_7) for each type of secondary structure, this joint algorithm performs better than any of the component methods and better than any other method reported in the literature.

W-AM-K1

CYTOCHROME *C* REVERSIBLY UNFOLDS ON BINDING WITH CARDIOLIPIN IN BILAYERS. Paul J. R. Spooner, and Anthony Watts. Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, U.K.

Cytochrome *c* exhibits a highly specific type of binding with the mitochondrial lipid cardiolipin and solid state NMR measurements have been conducted to determine if this interaction produces any significant changes in the structural and dynamic characteristics of the protein. Cytochrome *c* was labeled with deuterons at a variety of specific sites and ^2H NMR relaxation measurements on labeled protein in solution showed that the backbone sites retained in [*amide*-^2H]cytochrome *c* and the histidine residues labeled in [ε-^2H]*histidine* cytochrome *c* were *immobile* on the spectroscopic time-scale and can provide a realistic measurement of the correlation time for overall protein re-orientation as well as an accurate value of quadrupole coupling constant for these sites. Binding with cardiolipin bilayers disorders the surface sites of interaction on the protein labeled in [ε-N,N,-C^2H_3]*lysyl* cytochrome *c* and perturbs backbone structure to instantaneously release all deuterons in [*amide*-^2H]cytochrome *c*. On the other hand, measurements on the cardiolipin complex with [ε-^2H]*histidine* cytochrome *c* show that overall re-orientation of bound protein was slow in the spectroscopic time scale (τ_c > 10-7s). Additional measurements on the protein-lipid complex prepared in ^2H_2O indicated that no stable α-helices can exist in the protein backbone with a life-time longer than around 10^{-6} s and that these effects of cardiolipin on the protein structure could be reversed by cooling the complex to a point where a fraction of the lipid appeared immobile from ^{31}P NMR measurements. The ^{31}P NMR relaxation of lipid in the cytochrome *c*-cardiolipin complex is dominated by interaction with the protein heam which appears to be altered in its electronic configuration and possibly more exposed to the external environment. The results describe pronounced changes in cytochrome *c* on binding to cardiolipin bilayers which involve a reversible unfolding of the backbone structure in the protein.

W-AM-K2

BIOPHYSICAL STUDIES OF THE INTEGRAL MEMBRANE PROTEIN FOLDING PATHWAY.
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We have made seven synthetic peptides corresponding to each of the individual transmembrane α-helices from the protein bacteriorhodopsin (BR). These peptides have been reconstituted into phospholipid vesicles, and their structures are being characterized using a variety of biochemical and biophysical techniques. Proteolysis protection experiments have been used to establish the physical location of the peptides relative to the lipid bilayer. CD spectroscopy has been performed on the vesicles in solution in order to establish the secondary structure of the peptides. Finally, macroscopically oriented protein/phospholipid multilayers have been made by drying down the vesicles onto suitable transparent supports for spectroscopic studies; both CD spectroscopy and polarized Fourier transform infrared spectroscopy have been used to characterize the orientation of the protein secondary structure relative to the phospholipid bilayer in these samples. These experiments demonstrate that most of the peptides form stable membrane-spanning α-helices in isolation from the remainder of the BR molecule. The results provide evidence in favor of a two-stage model of integral membrane protein folding in which independently stable α-helices could be inserted into the lipid bilayer in the initial phase of the folding pathway, followed by their association within the plane of the bilayer in a second physically distinct process. Furthermore, we have observed that one of these synthetic peptides, that corresponding to the "C" helix of BR, is soluble at significant levels in aqueous buffers free of either detergent or phospholipid. This peptide associates spontaneously with phospholipid vesicles, and the details of the association are being studied using fluorescence spectroscopy, CD spectroscopy, and polarized infrared ATR spectroscopy.

W-AM-K3

DIMENSIONALITY AND ELECTROSTATICS PRODUCE AN APPARENT COOPERATIVITY IN THE BINDING OF BASIC RESIDUES ON PEPTIDES TO ACIDIC LIPIDS IN MEMBRANES.
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Several groups have shown that the activation of protein kinase C depends in a sigmoidal manner on the mole % PS in a membrane; the Hill coefficient, α_H > 5. When binding reactions take place in solution, α_H > 1 implies cooperativity. In other words, α_H > 1 implies the association constant for one or more successive binding steps increases as saturation proceeds. It is not generally recognized that when reactions occur at the surface of a membrane, both nonspecific accumulation in the aqueous diffuse double layer and reduction of dimensionality can produce an apparent cooperativity or α_H > 1. A simple theoretical model to describe the binding of peptides with basic residues to membranes with acidic lipids combines the Gouy-Chapman-Stern theory of the diffuse double layer, the Boltzmann relation and mass action equations. We tested the model by making filtration and equilibrium dialysis measurements of the binding of the pentavalent peptides Lys_5 and PKC(19-36), the pseudosubstrate region of protein kinase C, to membranes formed from mixtures of PC and either PS or PG. The fraction of bound peptide was a sigmoidal function of the mole % negative lipid in the membrane. The data could be described by either our simple model or by a Hill equation with α_H = 5. As expected theoretically from our model, but not from the Hill treatment, decreasing the salt concentration produced a large increase in the fraction of bound peptide because it increased the magnitude of the negative potential at the surface of the membrane. The intrinsic microscopic association constant of a basic residue on a peptide with a monovalent acidic lipid in a membrane was of order 10 M^{-1} or the net change in free energy was about 1.5 kcal/mol. The two main limitations to our simple model are that the effective valence of these large peptides is < 5 and that the activity coefficient of the negative lipid in the membrane appears to increase about a factor of 3 as the mole % negative lipid increases.
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W-AM-K4

EFFECT OF HIGH PRESSURE ON SEVERAL CLASSES OF MEMBRANE-BINDING PROTEINS
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Light scattering or fluorescence energy transfer was used to detect the ability of high hydrostatic pressure, upto 2.7 kbar, to dissociate an array of membrane-binding proteins from phospholipid vesicles. Three vitamin K-dependent, peripheral membrane-binding proteins (bovine factor X, bovine protein Z, and bovine prothrombin), which require calcium for binding to phospholipids, were observed to dissociate from small unilamellar vesicles (SUVs, 30 nm diameter) composed of 25% phosphatidylserine (PS) and 75% phosphatidylcholine (PC) at comparable midpoint dissociation pressures (0.3-0.6 kbar). These processes were completely reversible. The midpoint dissociation pressure for the factor X and SUV interaction was similar over a 25-fold range of SUV concentration under circumstances where the percent saturation of the vesicles with factor X was constant. Factor X was also shown to dissociate from large unilamellar vesicles (LUVs, 100 nm diameter, 25% PS: 75% PC) with a midpoint dissociation pressure of 0.5 kbar, which is not significantly different from the value of 0.4 kbar observed when SUVs were used. These results suggested that pressure-induced dissociation of factor X from the membrane arose from factors other than simple alterations in K_D.

A second group of membrane-binding proteins which bind calcium, but in a phospholipid-dependent manner, varied in their susceptibility to pressure-induced dissociation from SUVs. The proteins include protein kinase C, a 64 kD protein, and a 32 kD protein. The 32 kD protein dissociated from vesicles under high pressure (midpoint of 0.8 kbar) while protein kinase C and the 64 kD protein did not. All three proteins, however, were completely released from the vesicle surface upon addition of EDTA.

The ionic, peripheral membrane-binding protein, factor V_a light chain, which constitutes a third type of membrane-binding protein that interacts with acidic phospholipids in a calcium-independent manner, also failed to dissociate from the membrane under high pressure.

Although these three types of proteins could clearly be separated into two groups, one set being susceptible to pressure-induced dissociation from membranes and the other not, this behavior did not seem to correlate precisely with other aspects of the interaction (e.g. calcium-requirement). Further work will be needed to identify the factor(s) distinguishing dissociable versus non-dissociable interactions. (Supported by NIH grant HL15728)

W-AM-K5

EXTENSIVE SEGREGATION OF ACIDIC PHOSPHOLIPIDS IN MEMBRANES INDUCED BY THE BINDING OF PROTEIN KINASE C AND RELATED PROTEINS. Mohammad D. Bazzi & Gary L. Nelsestuen, Dept. of Biochemistry, University of Minnesota, St. Paul, MN 55108.

Protein kinase C and two other proteins with molecular weights of 64 and 32 kDa, purified from bovine brain, had similar striking phospholipid- and calcium-binding properties. These proteins constitute a class of proteins that bind calcium in a phospholipid-dependent manner; free proteins showed no detected calcium binding, but each protein bound 8-10 calcium in the presence of membranes. Other measurements suggested that the binding of these proteins to membranes induced extensive segregation of acidic phospholipids in the membranes. Addition of these protein to vesicles containing 15% fluorescently-labeled phosphatidic acid dispersed in phosphatidylcholine resulted in rapid and large quenching of the fluorescence signal. Light scattering measurements showed that quenching of the fluorescence signal coincided with binding of these proteins to membranes. In contrast, the binding of these proteins to vesicles composed entirely of fluorescent phospholipids did not change the fluorescence signal of the lipid. Thus, the fluorescence quenching appeared to result from self-quenching of fluorophores which become clustered upon binding the proteins. Fluorescence quenching was examined using vesicles with variable contents of fluorescent phospholipids. Binding of these proteins to vesicles containing 5-50% fluorescent phospholipid showed differing levels of fluorescence quenching that closely approximated the theoretical behavior expected for complete segregation of fluorescently-labeled phospholipids in the outer layer of the vesicles. Similar results were obtained using fluorescently-labeled phosphatidylglycerol. These results suggested a model in which calcium may function as a bridge between the proteins and phospholipids. Since these proteins bound 8-10 calcium ions in the presence of phospholipids, they may each induce clustering of a related number of acidic phospholipids. This property, which was very striking for this class of proteins, was barely detectable with other classes of proteins that bind to acidic phospholipids. Thus, protein kinase C is a member of a larger class of proteins that display striking and unique membrane-binding behavior. (Supported by grant GM-38819).

W-AM-K6

MEMBRANE INSERTION AND LATERAL DIFFUSION OF FLUORESCENCE LABELED CYTOCHROME C OXIDASE SUBUNIT IV SIGNAL PEPTIDE IN CHARGED AND UNCHARGED PHOSPHOLIPID BILAYERS.

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The association of the fluorescence labeled mitochondrial signal peptide coxIV-25 with artificial bilayers was characterized by fluorescence spectroscopy, binding and lateral diffusion experiments. In the presence of small unilamellar vesicles of phosphatidylcholine (POPC) and phosphatidylglycerol (POPG), NBD fluorescence was enhanced and shifted to shorter wavelengths. A partition coefficient of approx. 2000 M^{-1} could be derived from titration experiments with POPC. Apparent partition coefficients of 10^4-10^5 M^{-1} were determined with different mol% of POPG. Lateral diffusion coefficients (D) of the peptide in membranes were measured by fluorescence recovery after photobleaching. In bilayers of POPC, the peptide exhibited high Ds ($8*10^{-8}$ cm^2/s, 21°C) which were 1.5-1.6 times greater than those of NBD-labeled phospholipids ($5*10^{-8}$ cm^2/s, 21°). However, in the presence of 20 mol% POPG the Ds of the peptide ($3*10^{-8}$ cm^2/s, 21°C) were 1.5-1.8 fold reduced compared to those of the lipid. Taken together, these experiments suggest two different states of association: The peptide is only loosely bound to POPC membranes where it diffuses in the vicinity of the choline headgroups, but strong interactions between the basic residues of the peptide and acidic phospholipids cause a deeper insertion into the bilayer and, as a consequence, reduced lateral mobilities.

W-AM-K7

INTERACTIONS OF LYSIN WITH MEMBRANE PHOSPHOLIPIDS, Keelung Hong[1], Tetsuo Hoshino[1], Demetrios Papahadjopoulos[1] and Victor D. Vacquier[2]; [1]Cancer research Institute, University of California, San Francisco, CA 94143; [2]Marine Biology Research Division, Scripps Institution of Oceanography, University of California at San Diego, La Jolla, CA 92093.

Lysin, a protein of Mr-16K from the acrosome granule of abalone, displays potent activity in dissolving the egg vitelline layer for facilitating the sperm to penetrate the egg envelope. The primary structure of this cationic protein projects some hydrophobic domains in the predicted secondary structure. Lysin acts by hydrophobic interaction with egg vitelline layer. Lysin not only binds to phospholipid membranes but also induces fusion of negatively charged liposomes. The results of the experiments which were designed for monitoring the interactions of lysin with membrane phospholipids by fluorescence spectroscopy are presented. Even though lysin can cause a non-fusing phosphatidylcholine liposomes to release encapsulated carboxyfluorecein, there was no change of the tryptophan fluorescence of lysin upon binding to membrane. We found a larger than 10 nm blue shift of tryptophan emission maximum when lysin bound to the negatively charged membranes which were susceptible to lysin-mediated fusion. This blue shift illustrates a conformational change of lysin upon binding to anionic phospholipid. Quenching of tryptophan fluorescence of lysin by including brominated phosphatidylcholine in the negatively charged liposomes reveals clearly that lysin penetrates into the bilayers and the depth of penetration is estimated at least 6A from the headgroup-hydrocarbon boundary. Energy transfer of tryptophan fluorescence of lysin to the surface-labeled acceptor of lipid in the membranes has also been observed only in liposomes susceptible to lysin-mediated fusion. Our observations suggest that positively charged lysin initially interacts with liposome through charge-charge interaction and consequently a conformational change may enable lysin to be partially embeded in the hydrophobic region of the bilayer. This embeded lysin may use its second electrostatically positive segment exposed at the surface to associate with the second bilayer and the penetration of lysin into bilayers will destabilize the bridged two bilayers and fusion occurs.

W-AM-K8

Specificity in the Protein Kinase C:Lipid Interaction

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The effects of surface charge, ionic strength, and pH on the cooperative interaction of phosphatidylserine with the Ca^{2+}/lipid-dependent protein kinase C have been examined. The highly cooperative binding to, and activation by, phosphatidylserine has led us to propose a model in which protein kinase C interacts with membranes by cooperatively sequestering phosphatidylserine around a membrane-interacting domain. This contribution explores the role of non-specific and specific interactions in the phosphatidylserine-dependent regulation of protein kinase C. Binding of protein kinase C to detergent:lipid mixed micelles or model membranes is regulated by surface charge, whereas activity is regulated specifically by a cooperative interaction with phosphatidylserine. If protein kinase C is pre-bound to micelles containing phosphatidic acid (a lipid that does not activate protein kinase C), enzymatic activity continues to display a marked sigmoidal dependence on phosphatidylserine. This result suggests that 1] surface charge is sufficient to recruit protein kinase C to the membrane, and 2] phosphatidylserine is cooperatively recruited to membrane-bound protein kinase C. Similar experiments altering ionic strength and pH are consistent with electrostatic interactions playing a role in the initial protein kinase C:membrane interaction, followed by cooperative sequestration of phosphatidylserine.