Th-AM-H7

HYDROLYSIS RESISTANT GTP ANALOGUES STIMULATE EXOCYTOSIS IN BOVINE ADRENAL CHROMAFFIN CELLS. ((I.M. Robinson, A.F. Oberhauser, K. Okano, and J.M. Fernandez.)) Dept. of Physiology and Biophysics, Mayo Clinic, Rochester, MN 55905.

We have used the whole-cell patch clamp technique to measure secretion in adrenal chromaffin cells. Using the patch pipette to perfuse the cell, it was possible to stimulate an exocytotic response with the non-hydrolysable GTP analogue, GppNHp. When cells were stimulated by GppNHp in the presence of 30 nM $[Ca^{2+}]_i$, only 6 of 9 cells responded. In contrast, 100% of cells responded when stimulated in the presence of 200 nM $[Ca^{2+}]_i$. Perfusion of the cells with either 30nM or 200 nM Ca^{2+}-buffered solutions did not stimulate a secretory response in the absence of guanine nucleotides. The response to GppNHp could be inhibited by either GDPβS or by GTP. This suggests that a sustained activation of a GTP-binding protein is sufficient to cause exocytotic fusion. This response was also observed when the cells were stimulated in a nominally Ca^{2+} free extracellular solution, suggesting that the response was independent of Ca^{2+} entry into the cell. Use of the caged form of GTPγS has shown that the cells still secrete in response to GTP analogues after 20 minutes in the whole-cell configuration, a time in which cytosolic factors of molecular weights smaller than 110 kDa would have leaked out of the cell. The use of this technique enabled the recording of individual step increases in cell membrane capacitance of 2-3fF. These steps are presumably due to the incorporation of individual secretory vesicles into the plasma membrane. When amperometry was used concomitantly with capacitance measurements to study the release of catecholamines from the cells, many "step"-increases in capacitance were accompanied by simultaneous spikes in the amperometric recordings. The maximum rate of capacitance change induced by GTPγS was accompanied by the highest frequency of amperometric spikes. These two results argue against the possibility that the guanine nucleotide analogues are merely exerting their effect on cell membrane capacitance by inhibiting endocytosis.These results suggest that a GTP-binding protein can stimulate exocytosis, is not free to diffuse and as such could be one of the components of an hypothesised fusion pore scaffold.

Th-AM-H8

ASSOCIATION OF ENDOGENOUS Go-ALPHA SUBUNIT WITH THE PURIFIED N-TYPE VOLTAGE-DEPENDENT CALCIUM CHANNEL. ((M.W. McEnery*, A.M. Snowman#, and S.H. Snyder#)) *Dept. of Physiol. and Biophysics, Case Western Reserve Univ. Sch. of Med., Cleveland, OH 44106 and #Dept. of Neuroscience, The Johns Hopkins Univ. Sch. of Med., Baltimore, MD 21205.

Modulation of the omega-conotoxin GVIA (CTX) sensitive N-type voltage-dependent calcium channel (VDCC) by numerous neurotransmitters and guanine nucleotides suggests a dynamic interaction between activated G-protein alpha subunits and the N-type VDCC. Our previous report on the purification of the N-type VDCC based upon its ability to bind [^{125}I]CTX with picomolar affinity (McEnery, et al., (1991) Proc. Natl. Acad. Sci. (USA) **88**, 11095; McEnery (1993) Methods in Pharmacol. **7**: 3) and the observation of Gs-alpha associating with L-type VDCC (Hamilton, et al., (1991) J. Biol. Chem. **261**: 19528), suggested a possible association of N-type VDCC with an endogenous G-alpha subunit. The addition of the G-protein activator AlF_4- modulated the [^{125}I]CTX binding characteristics of the solubilized N-type VDCC. Further immunological analyses employing G-alpha subunit-specific antibodies to monitor the cofractionation of G-alpha with [^{125}I]CTX binding activity throughout the purification procedure indicate the selective recovery of Go-alpha in the purified N-type VDCC preparation, as neither Gs-alpha, Gi-alpha nor G-beta/gamma could be detected. Furthermore, Go-alpha associated with N-type VDCC acted as a substrate for pertussis toxin-dependent ADP-ribosylation only upon the addition of exogenous G-beta/gamma subunits. These results strongly suggest a high affinity complex between an activated Go-alpha and N-type VDCC maintained throughout biochemical purification of the N-type VDCC.

Th-AM-H9

REGULATION OF THE EXOCYTOTIC FUSION SCAFFOLD BY GTP AND Ca^{2+} BINDING PROTEINS. ((A.F. Oberhauser, I.M. Robinson, V. Balan and J.M. Fernandez)) Dept. of Physiology and Biophysics, Mayo Clinic, Rochester MN 55905.

We have recently proposed that the formation of the exocytotic fusion pore is directed by a macromolecular protein scaffold. We suggested that Ca^{2+} and GTP-binding proteins of the rab3 family are some of the components of the scaffold. We investigated the interaction between the Ca^{2+} and GTP binding proteins by perfusing patch-clamped mast cells with caged Ca^{2+} compounds and guanine nucleotides and found that a rapid burst of exocytotic fusion can be induced by flash photolysis of DM-nitrophen (10 mM plus 3 mM Ca^{2+}) in the presence of 1.5 mM GTP. Each burst is composed of 30-50 fusion events (measured as stepwise increases in cell membrane capacitance). Exocytotic fusion stopped a few seconds after the UV exposure, reflecting the return of $[Ca^{2+}]_i$ to resting levels (measured with 100 μM fura-2) as the uncaged Ca^{2+} diffused out of the cell and was replaced with fresh caged Ca^{2+}. In experiments in which GTP was omitted from the pipette solution, no fusion events were observed. Similar to the effect of GTPγS, the secretory response induced by Ca^{2+} does not washout: flash photolysis of caged Ca^{2+}, after as long as 25 min of cell perfusion, is still able to induce complete degranulation, indicating that the target Ca^{2+} binding protein is closely associated with the scaffold regulating exocytosis. We also studied the role of other types of proteins in exocytosis by perfusing mast cells with synthetic peptides that have been shown to inhibit exocytosis in other cell types. Peptides for the C2 domain of synaptotagmin (1 mM) and for the most conserved region of the annexins (0.5 mM) did not inhibit the exocytotic response elicited by GTPγS or the combination of Ca^{2+} and GTP, even after delaying the stimulus by more than 20 min. A peptide based on the N-terminus of hARF1 (2-17; 25 μM) blocked the exocytotic response triggered by a mixture of Ca^{2+} (800 nM) and GTP (1 mM). This peptide also inhibited the secretory response elicited by flash photolysis of caged Ca^{2+} (with 1 mM GTP) or caged GTPγS. These results implicate ARF or a target protein of ARF as a component of the exocytotic fusion pore scaffold.

MECHANOENZYMES

Th-PM-Sym-1

ACTIN POLYMERIZATION AND THE PROPULSION OF *LISTERIA MONOCYTOGENES* ((J. A. Theriot)) Whitehead Institute, Cambridge, MA 02142.

Listeria monocytogenes, a Gram-positive bacterium, is a facultative intracellular parasite that grows directly in the cytoplasm of host cells and uses a form of actin-based motility for intra- and intercellular spread. Moving intracellular *L. monocytogenes* are associated with a polarized "comet tail" made up of short crosslinked actin filaments. Fluorescence photoactivation of labeled actin filaments in the tail indicates that the actin filaments remain stationary in the cytoplasm as the bacterium moves. The bacterium induces assembly of actin filaments near its surface, which are then released and crosslinked into the stationary tail structure. The bacterial surface protein ActA is required for actin assembly. In infected cells, the host actin monomer-binding protein profilin is localized to the surface of motile *L. monocytogenes*, and profilin binding is correlated with rapid and efficient movement. This localization depends on a proline-rich domain of ActA. A distinct domain of ActA appears to be involved in actin filament nucleation. Actin-based *L. monocytogenes* motility can be faithfully reconstituted in cytoplasmic extracts of *Xenopus laevis* eggs. Extracts from which profilin has been depleted do not support bacterial motility. There is no evidence for the involvement of myosin in the propulsion of *L. monocytogenes*. Actin polymerization itself may produce propulsive force through a "Brownian ratchet" mechanism.

Th-PM-Sym-2

KINESIN CAN SUPPORT MINUS-END DIRECTED, DEPOLYMERIZATION-DRIVEN MOTILITY BY COUPLING OBJECTS TO SHORTENING MICROTUBULES. ((J.R. McIntosh, V.A. Lombillo, C. Nislow, V.I. Gelfand*, T.J. Yen#, and R.J. Stewart&)) Dept. of Molecular, Cellular, and Developmental Biology, Univ. Colorado, Boulder, 80309; * Univ. Illinois, Urbana, 61801; # Fox Chase Cancer Center, Philadelphia, PA 19111; & Rowland Inst. Cambridge, MA 02142.

Chromosomes and vesicles will bind to microtubules (MTs) growing from detergent-extracted pellicles of Tetrahymena and move in (toward the minus MT end) when the MTs depolymerize, even without ATP (Coue et al., 1991. JCB 112:1165). We have investigated this motility with antibodies (Abs) to known kinetochore proteins. Function blocking Abs to cytoplasmic dynein have no effect, but motility is stopped by Abs to the heavy chain of kinesin that bind a broad range of kinesin-like proteins (KLPs) (Rodionov et al., 1991. PNAS 88: 4956). Chromosome speed is slowed >3-fold by Abs against the kinetochore KLP, CENP-E (Yen et al., Nature 359: 536). Abs to other kinetochore antigens had no effect. Depolymerization-driven motility has been recon-stituted using latex micro-spheres, coated with HeLa kinesin. Without ATP, these move in at 23 ± 6 um/min (N=26), about the speed of chromosomes in our assay. With ATP and tubulin they move out at ca. 50 um/min. With ATP but w/o tubulin, they come in with the MT ends at ca. 50 um/min. Beads coated with a nonmotile chimeric KLP move with MT disassembly at 186 ± 84 um/min (N=45). Our data suggest that KLPs can bind MTs in the absence of ATP by associations that permit motility driven by the free energy released with tubulin depolymerization.

Th-PM-Sym-3

DIRECT OBSERVATION OF KINESIN STEPS. ((K. Svoboda[1,2], C.F. Schmidt[2], B.J. Schnapp[3], & S.M. Block[2])) [1]Committee on Biophysics, Harvard University, Cambridge, MA 02138; [2]Rowland Institute for Science, Cambridge, MA 02142; [3]Department of Cellular & Molecular Physiology, Harvard Medical School, Boston, MA 02115.

Do molecular motors make characteristic steps? That is, are there well-defined dwell times interspersed with relatively rapid periods of forward motion? To address this question, we constructed instrumentation with the spatial and temporal sensitivity needed to resolve movement on a molecular scale. We observed the motion of small glass beads carrying single kinesin molecules by interferometry while exerting calibrated loads with optical tweezers. The compliance of the bead-to-motor linkage was nonlinear, which led to a decrease in noise with increasing load: this made the observation of steps possible under three different conditions. At 10 μM ATP and low load (velocity unaffected), a statistical analysis of trajectories revealed steps. When the motion was slowed mechanically (high load) or chemically (low ATP), it was possible to see steps directly. Steps measured 8 nm, corresponding to the distance between α-β tubulin dimers along the microtubule protofilament. From our low ATP data we argue that "ATP fractionation" (several cyclical mechanical events per ATP hydrolyzed) does not occur for kinesin. In the diffusion limit, a motor that fractionates energy spends most of its time waiting for an ATP arrival before making a rapid cluster of steps. Such clusters were not observed. By measuring the time it takes the optical trap to pull a bead back by 8 nm after it releases from the microtubule, we placed a short, rigorous upper limit on the putative cycle off-time (< 50 μs). Finally, we show that single kinesin molecules can move against tensions up to 5 pN.

Th-PM-Sym-4

SINGLE MYOSIN STEPS AND FORCES MEASURED WITH OPTICAL TRAPS. ((Jeffrey T. Finer[1], Robert M. Simmons[2], and James A. Spudich[1])) [1]Department of Biochemistry, Stanford University, Stanford, CA 94305. [2]MRC Muscle and Cell Motility Unit, Kings College London, London WC2B 5RL, U.K.

We have developed a new *in vitro* assay for myosin function which can measure piconewton forces and nanometer displacements produced by single myosin molecules. A single actin filament was held in solution under a microscope coverslip by two optical traps (optical tweezers) via latex beads attached near each end. The actin filament was then allowed to interact with single myosin molecules which were attached to a platform above the microscope coverslip surface. When the movement of a trapped bead was monitored with nanometer precision at millisecond rates by a quadrant photodiode detector, discrete displacements were seen in the direction parallel to the long axis of the actin filament. At low load, the single displacements averaged 11 nm. In order to measure force under conditions approaching those of isometric muscle contraction, the stiffness of the optical trap was increased by feedback control in which the laser beam was deflected by acousto-optic modulators, and the movement of the laser beam was proportional to the external force applied to the trapped bead. Under these conditions single force transients averaging 4 pN were measured. When the ATP concentration was reduced well below the K_m for *in vitro* movement, the average duration of the single displacements and single forces increased, as one might predict, due to a decrease in the rate of the ATP dependent detachment of actin from myosin. These single myosin molecule measurements are consistent with conventional models of muscle contraction.

MODEL CHANNELS

Th-PM-A1

A NEW APPROACH TO MODELING THE K$^+$ CHANNEL PORE.

((K.V. Soman, J. A. McCammon[a], and A.M. Brown)) Dept. of Molecular Physiology & Biophysics, Baylor College of Medicine & [a]Dept. of Chemistry, University of Houston.

A molecular model for the pore of the voltage-activated K$^+$ channel *Kv2.1* (*drk1*) has been built combining secondary structure predictions and hydrophobic moment profiles with experimental results. The modeled segment is the "H5-P" region (361:PASFWWATITMTTVGYGDIYP:381) between the transmembrane α helices S5 and S6. Secondary structure prediction and hydrophobic moment analysis of the sequence favored mainly β-strand conformation. Accordingly, the model is an eight-stranded, antiparallel β barrel built out of a β-hairpin from each of the four subunits, forming a symmetric tetramer. The β sheet that constitutes the barrel has the favored right-handed twist, and the polypeptide chain has a right-handed tilt about the axis of the cylinder. Both the backbone and side chain conformations of the model are heavily dependent on experimental results on *Kv2.1* and related channels. Some features of the model follow: *Ile*-369 is located just inside the external mouth of the pore. *Thr*-372 and *Val*-374 are close to the cytoplasmic entrance of the pore. The side chains of residues 369 and 374 project into the pore and may interact both within and across subunits. *Tyr*-380 is located in the external vestibule, just outside the narrow region of the pore, allowing for *aromatic-aromatic* interaction with residue 369 in some mutants. Recent experimental results have shown that the pore is spread over a larger region of the molecule including residues outside H5. Progress in building a more complete model for the *Kv2.1* channel, consistent with these results, will also be reported. *Supported by: American Heart Association Texas 93G-1186, Texas ATP # 0032, and the W.M. Keck Center for Computational Biology.*

Th-PM-A2

STRUCTURE OF A SYNTHETIC PEPTIDE CORRESPONDING TO THE S4 SEGMENT OF A VOLTAGE-GATED POTASSIUM CHANNEL

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The S4 segment in voltage-gated ion channels has been proposed to function as a voltage-sensor. We have synthesised a peptide corresponding to the S4 region of the Drosophila Shaker voltage-gated K$^+$ channel. Structural studies on this peptide were conducted using FTIR and CD spectroscopy. The peptide in aqueous solution is in a disordered conformation at pH 7.4. However, in 90% TFE it adopts an α-helical structure. A highly helical structure is also observed for the S4 peptide in lysophosphatidyl choline micelles as well as in DMPC and DMPG bilayers. The coil to helix transition observed for the S4 peptide upon its transfer from an aqueous solution to a membrane environment indicates that it has a high degree of conformational flexibility and can undergo large structural changes in response to changes in the surrounding environment. This finding is consistent with its role in the voltgae activation process during which the S4 segment has been postulated to move from a lipid bilayer to an aqueous extracellular media.

Th-PM-A3

NMR DETERMINATION OF CONFORMATION OF ION-TRANSPORT-COMPETENT GRAMICIDIN IN DPC MICELLES: A RIGHT-HANDED, HEAD-TO-HEAD DIMERIZED, SINGLE-STRANDED $\beta^{6.3}$-HELIX.

(Naijie Jing, Kari U. Prasad and Dan W. Urry*)) Laboratory of Molecular Biophysics, The University of Alabama at Birmingham, VH300, Birmingham, Alabama 35294-0019.

The conformation of malonyl gramicidin molecules in dodecyl phosphocholine micelles and the characteristics of ion interaction with the peptide channel were studied by means of ^{1}H, ^{23}Na, and ^{31}P NMR. First, malonyl-bis-desformyl gramicidin, CH$_2$(CO-L-Val^1-Gly^2-L-Ala^3-D-Leu^4-L-Ala^5-D-Val^6-L-Val^7-D-Val^8-L-Trp^9-D-Leu^{10}-L-Trp^{11}-D-Leu^{12}-L-Trp^{13}-D-Leu^{14}-L-Trp^{15}-NHCH$_2$CH$_2$OH)$_2$, was used to form the putative channel, and dodecyl phosphocholine (DPC), CH$_3$(CH$_2$)$_{11}$PO$_4$(CH$_2$)$_2$N(CH$_3$)$_3$, was used to package malonyl gramicidin molecules into micelles as a model lipid environment. The quality of the incorporation into DPC micelles was assessed by CD spectra and relaxation studies of ^{31}P-NMR, in which the relaxation times T_1 and T_2 were distinctly decreased when malonyl gramicidin molecules were incorporated into DPC micelles. The information on ion interactions with malonyl gramicidin molecules was obtained from Na$^+$ ion titration studies using ^{23}Na-NMR relaxation and spin-echo methods. The results suggest that the malonyl gramicidin molecules form an ion transport competent state in DPC micelles. Two-dimensional proton NMR was employed to determine the conformation of the ion interacting state. The COSY and NOESY spectra of malonyl gramicidin molecules incorporated in DPC (d_{38}) micelles in D_2O and in H_2O (90%) were obtained at 70°C, pH4.4 for 20h accumulations. Assignments of the chemical shifts and the peptide sequence were determined based on the proton spin systems and the NOE spatial connectivities (d_1 and d_3). Analyses of nuclear Overhauser effects and spin-spin couplings in the NOESY spectrum show that 17 NOE connectivities match the structure of a right-handed helix rather than a left-handed helix. It is clear that the conformation of ion-transport-competent gramicidin in the DPC lipid micelle system is an amino end-to-end dimerized, single-stranded, right-handed β-helix with approximately 6.3 residues per turn.

Th-PM-A4

THE STRUCTURE OF THE GRAMICIDIN/KSCN COMPLEX.

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The linear hydrophobic antibiotic gramicidin was crystallised from a methanol solution containing the salt potassium thiocyanate using a variation of the crystallisation method of Koeppe et al (Nature, 1979, Vol 279:723-725). A data set to 1.9Å resolution was collected that included 5746 unique reflections. A difference map was calculated using the coordinates of the gramicidin/caesium chloride complex (Wallace & Ravikumar, Science, 1988, Vol 241:182-187) with all the ions removed. New ion positions appeared both for the potassium and thiocyanate ions within the central pore; in addition alterations are seen in the backbone conformation. At present the R-factor at 2.5Å resolution is 20%. These potassium complexes, along with other gramicidin/ion complexes, are providing detailed information on ion/protein interactions as an ion passes through a membrane channel.

Th-PM-A5

Na^+ IN GRAMICIDIN: THE PROTOTYPE PERMION

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The reaction path and free energy profile of Na^+ in gramicidin were calculated using the program *MOIL*. Gramicidin was represented in atomic detail, but surrounding water and lipid molecules were not included: only short-range interactions were investigated. The permeation path of the ion was an irregular spiral, far from a straight line.

Permeation cannot be described by motions of a single Na^+ ion: the minimum energy path includes significant motion of water and channel atoms as well. We think of permeation as motion of a ***permion***, a quasi-particle that includes the "many body" character of the permeation process, comparable to quasi-particles of solid-state physics: holes, electrons, and phonons.

The mass, free energy, and memory kernel (time-dependent friction) of short-range interactions were calculated. The mass of the permion (properly normalized) is much less than Na^+. Friction varies substantially along the path. The free energy profile has two deep minima and several maxima. Na^+ is accompanied by a plug of water molecules, and motions of water, Na^+, and the atoms of gramicidin are highly correlated. In certain regions, the dominant motions along the reaction path are those of the channel protein, not the permeating ion: there, the ion "waits" while the other atoms move. At these waiting sites, the permion's motion along the reaction path is a displacement of the atoms of gramicidin that prepare the way for the Na^+ ion.

Th-PM-A6

STOCHASTIC THEORY OF THE OPEN CHANNEL.

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The description of trajectories of individual ions in an open channel requires a stochastic theory that distinguishes incoming and outgoing ions. These trajectories have velocities of opposite sign and so velocity is needed as a state variable (in the Langevin and Fokker-Planck equations) even when friction dominates movement of ions, as in classical diffusion theory.

Analytical expressions, valid for potential barriers of any shape or size, have been derived for conditional probabilities, first-passage times, flux, and its *cis* $\{L|L\}$ & $\{R|R\}$, and *trans* components $\{R|L\}$ & $\{L|R\}$.

$$J_{net} = J_f - J_b = \left(\sqrt{\sqrt{2\pi\varepsilon}}\right)\left(C_L \,\mathrm{Prob}\{R|L\} - C_R \,\mathrm{Prob}\{L|R\}\right)$$

The *trans* components J_f & J_b are the unidirectional fluxes measured with radioactive tracers in classical experiments; ε is a dimensionless temperature.

Permeation is a reaction moving ions on the *Left* to the *Right*:

$$L \underset{k_b}{\overset{k_f}{\rightleftharpoons}} R; \qquad \text{where } k_f = \mathrm{Prob}\{R|L\};\ k_b = \mathrm{Prob}\{L|R\}$$

This generalization of the law of mass action is exact ***for any shape barrier*** if concentrations C_L & C_R in the baths are maintained constant.

Conditional probabilities can be evaluated analytically if ions follow simple dynamics. The probabilities can be estimated by simulations if dynamics are complex, for example, if ions in the channel interact with each other or significantly modify the shape of the electric field.

Th-PM-A7

PERSISTENCE—A NEW STATISTIC FOR CHARACTERIZING ION CHANNEL ACTIVITY

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We explain how the *persistence*, defined as

$$\rho(I,t) = P(\hat{I}(t_0+t) = I \mid \hat{I}(t_0) = I),$$

where $\hat{I}(t)$ is the current through the channel at time t, can be used as a robust statistic for the discovery of channel conductance levels and as a measure of their lifetime. Several quantities derived from $\rho(I,t)$ are useful: A plot of $\rho(I,t)$ vs I for t small but positive reveals conductance levels which may be difficult to discover in a histogram. The decay of $\rho(I,t)$ with increasing t, for fixed I, measures the longevity of the current level I. The integrated persistence $\rho(I) = \int_0^\infty (\rho(I,t) - \pi(I))\,dt$ (where $\pi(I) = \lim_{t\to\infty} \rho(I,t)$ is the steady state probability $P(\hat{I}(t) = I)$), evaluated for I at a peak of $\rho(I,t)$ vs I, measures the lifetime of a conductance level and is more stable against the effects of noise than is the usual mean dwell time. These quantities can be computed for a limited portion of a data set, and, by using a sliding window, insight into the time variation of the signal, including baseline variations, can be obtained. We shall briefly discuss the mathematical concepts, explain how they are applied to real single channel patch clamp data, and illustrate them by application to data obtained for the NMDA receptor in rat brain by D. Colquhoun and A. Gibb.

Th-PM-A8

A STOCHASTIC MARKOVIAN MODEL TO DESCRIBE COOPERATIVE BEHAVIOR IN ION CHANNEL GATING

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Single-channel recordings from membrane patches frequently exhibit multiple conductance levels. In some preparations, the steady state probabilities of observing these levels do not follow a binomial distribution. This behavior has been reported in gap junction channels and various other channels. A non-binomial distribution suggests interaction of the channels, or the presence of channels with different open probabilities. However, current recordings from some membranes exhibit single transitions spanning several levels. Since the probability of simultaneous transitions of independent channels is infinitesimally small, such observations strongly suggest a cooperative gating behavior. We present a model to describe the cooperative gating of channels using only the current amplitude histograms for the probability of observing the various conductance levels. We investigate the steady state properties of a system of N channels and provide a scheme to express all the probabilities in terms of just two parameters. The main feature of our model is that lateral interaction of channels gives rise to cooperative gating. Another useful feature is the introduction of the language of graph theory which can potentially provide a different avenue to study ion channel kinetics. We write down explicit expression for systems of two, three and four channels and provide a prescription to describe the system of N channels.

VISUAL PHOTORECEPTORS

Th-PM-B1

INOSITOL PENTAPHOSPHATE INDUCED MEMBRANE POTENTIAL OSCILLATIONS IN *LIMULUS* VENTRAL PHOTORECEPTORS.

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Although inositol 1,3,4,5,6- pentaphosphate ($Ins(1,3,4,5,6)P_5$) represents a large fraction of the inositol phosphate content of mamallian cells, it's function is unknown. To test for the possible function of $Ins(1,3,4,5,6)P_5$ in cells I injected *Limulus* ventral photoreceptors with $Ins(1,3,4,5,6)P_5$ dissolved in an injection buffer consisting of 100 mM K-asp, 10 mM Hepes at pH 7.0. Injection of 1mM $Ins(1,3,4,5,6)P_5$ into the light-sensitive region of the photoreceptor results in an immediate depolarization of greater than 40 mV followed by a series of repeating smaller depolarizations. The repeating responses appear to be an $Ins(1,3,4,5,6)P_5$ induced membrane oscillation (10-20 mV in amplitude, 1-2 s in duration, with a frequency of 0.05-0.5 Hz) which can last for at least one hour, the maximum time I have so far recorded for. Oscillations were induced by a series of small injections from a pipette containing 100 μM $Ins(1,3,4,5,6)P_5$ giving an estimated final cellular concentration of 10 μM $Ins(1,3,4,5,6)P_5$. Bright light stimulation , of a cell oscillating from a previous $Ins(1,3,4,5,6)P_5$ injection, reversibly inhibited the oscillation, suggesting that $Ins(1,3,4,5,6)P_5$ activates the pathway normally activated by light. Our previous work has shown that *Limulus* photoreceptors contain a calcium store that releases calcium in response to $Ins(1,4,5)P_3$ and a calcium activated conductance that depolarizes the cell. Thus, I suggest that both the immediate response and the maintained oscillation, induced by $Ins(1,3,4,5,6)P_5$ injection, result from an underlying immediate rise in intracellular free calcium (Ca_i) and a subsequent Ca_i oscillation.

Th-PM-B2

THE LIGHT-ACTIVATED K CONDUCTANCE OF INVERTEBRATE PHOTORECEPTORS. RECTIFICATION BLOCK AND SINGLE CHANNEL RECORDINGS.

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The mollusks *Pecten irradians* and *Lima scabra* possess two classes of photoreceptors, rhabdomeric and ciliary, whose receptor potentials are depolarizing and hyperpolarizing, respectively. We had previously described the basic electrical properties of enzymatically isolated ciliary cells. Here we further examine their light-sensitive conductance using the patch-clamp method. The sensitivity of ciliary cells is lower (by > 2 log units) than that of rhabdomeric cells, and single-photon responses cannot be resolved. The activation kinetics is very fast, with latencies approaching 15 ms. The reversal of the outward photocurrent is near -80 mV in standard solutions, and shifts in a Nernstian way (≈40 mV) in the positive direction when external K is increased five-fold. There is a pronounced rectification in the outward direction largely due to a voltage-dependent block of the light sensitive conductance by divalent cations, a situation similar to vertebrate photoreceptors. The K-selective photocurrent is only weakly blocked by TEA at concentration up to 50 mM, but is extraordinarily sensitive to 4-aminopyridine (4-AP), with half-maximal suppression in the sub-micromolar range. 4-AP does not appear to impair early links of the transduction cascade; for example: prolonged aftercurrents are still obtained and the ERP is unaffected. In *Pecten* we also obtained recordings of single channels specifically activated by light with properties that parallel those of the macroscopic photocurrent. Their unitary conductance is ≈ of 28 pS. The nature of their gating mechanism is under study. This system may provide information about the divergence of the two major evolutionary lines of photoreceptors. Supported by NIH grant RO1-EY07559.

Th-PM-B3

ELECTRON CRYOMICROSCOPY OF RETINAL ROD cGMP PHOSPHODIESTERASE. ((Justine A. Malinski & Theodore G. Wensel)) The Verna & Marrs McLean Department of Biochemistry, Baylor College of Medicine, Houston, TX 77030-3498.

In vertebrate photoreceptor cells, light initiates a cascade of protein conformational changes that activate a cGMP-specific phosphodiesterase (PDE). While the biochemistry and physiology of activation are well characterized, it's structural context is virtually unexplored. We have begun to address this question using high resolution electron cryomicroscopy and image processing to visualize single molecules of bovine rod PDE in vitreous ice.

Images of holo-PDE ($PDE_{\alpha\beta\gamma\gamma}$) and trypsinized PDE ($PDE_{\alpha\beta}$) are being analyzed by multivariate statistical analysis to determine molecular size, shape, and subunit organization. To facilitate subunit localization, the inhibitory PDEγ subunit has been covalently labeled at cys68 with a 1.4 nm gold particle.

This work is supported by NIH grants RR02250 to the National 3-D Electron Microscopy Resource Center, Dr. Wah Chiu, Baylor College of Medicine and EY07981.

Th-PM-B4

MODULATION OF THE ROD cGMP-GATED ION CHANNEL BY CALMODULIN AND AN ENDOGENOUS FACTOR DISTINCT FROM CALMODULIN. ((Sharona E. Gordon and Anita L. Zimmerman)) Section of Physiology, Brown University, Providence, RI 02912.

Cyclic GMP-gated cation channels mediate the electrical response to light in retinal rods. Recently, calmodulin has been found to inhibit these channels in vesicles prepared from bovine rod outer segments. Here we report similar effects of calmodulin on the channels in excised patches from frog rod outer segments, as well as evidence for a patch-associated endogenous factor, distinct from calmodulin, that similarly inhibited the channels and appeared to act at the same site. Calmodulin was found to bind more tightly to the patches at low than at high [cGMP], inhibiting the channels by decreasing cGMP affinity or altering channel gating. Unlike calmodulin, the endogenous inhibitory factor remained stably associated with the patch unless exposed to solutions low in Ca^{2+} (less than about 20 nM). Trypsin prevented the response to calmodulin and to low Ca^{2+}, but also reduced the total cGMP-activated patch current. Our results raise the question of whether an endogenous Ca^{2+}-binding protein and/or calmodulin modulate channel function in visual transduction.

Supported by NIH EY07774 & NSF RCD-9054722.

Th-PM-B5

ASYMMETRY OF THE PERMEATION PATHWAY IN CATFISH CONE cGMP-GATED CHANNELS. ((L.W. Haynes)) Department of Medical Physiology and Neuroscience Research Group, University of Calgary, 3330 Hospital Dr. N.W., Calgary, Alberta, T2N 4N1, Canada.

The cGMP-gated channels of cone photoreceptors are non-specific cation channels that conduct both mono- and divalent cations. Previously, permeability and conductance measurements have been obtained under biionic conditions using extracellular Na as the reference ion and changing the ion at the cytoplasmic side of the patch. In order to determine the degree of symmetry in the cGMP-gated channels, permeability and conductance ratios have now been determined under biionic conditions using intracellular sodium as the reference. Inside-out patches were excised from channel catfish (*Ictalurus punctatus*) cone outer segments and current-voltage relations under biionic conditions between ±80 mV were obtained by applying paired 120 mV s^{-1} ramps. The resulting currents at each voltage were averaged to reduce capacitive artifacts. Net currents were obtained by subtracting the averaged currents before and after cGMP from the currents with cGMP. The pipet contained (in mM): 120 XxCl, 0.1 XxEGTA, 0.1 XxEDTA, 5.0 XxHEPES (pH 7.6), where Xx is one of Li, Na, K, Rb, Cs or NH_4. The bath contained either an identical solution (symmetrical ionic conditions) or the Na solution. 1 mM cGMP free acid was added to the bath solution when required. The permeability ratios with respect to sodium on the cytoplasmic side of the channel followed the sequence NH_4 > Li > K > Na > Rb > Cs (2.3 : 1.2 : 1.05 : 1 : 0.94 : 0.8) while the conductance ratios at +50 mV followed the sequence NH_4 > Na = K > Rb > Li > Cs (2.3 : 1 : 0.82 : 0.66 : 0.46). Under symmetric ionic conditions, the single channel conductances ranked in the order NH_4 > Na > K > Rb > Cs (59, 48, 43, 24, 15 pS). The single channel conductance for Li could not be determined. For comparison, permeability and conductance ratios with respect to external Na are NH_4 > K > Li > Rb = Na > Cs (2.31 : 1.19 : 1.10 : 1.01 : 1 : 0.79) and Na = NH_4 > K > Rb > Li = Cs (1 : 0.89 : 0.83 : 0.63 : 0.39 : 0.38), respectively. Thus, the channel shows a slight degree of asymmetry. Block by extracellular divalent cations is currently being investigated. [Supported by the MRC of Canada and Alberta Heritage Foundation for Medical Research].

Th-PM-B6

ENTHALPIC AND ENTROPIC CONTRIBUTION TO THE IONIC SELECTIVITY OF THE CYCLIC GMP ACTIVATED CHANNEL IN VERTEBRATE PHOTORECEPTORS

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The selectivity sequence of the cyclic GMP activated channel in rods of the tiger salamander, based on the reversal potential is NH_4 > Li > Na = K > Rb > Cs. In order to establish the enthalpic and entropic contribution to the ionic selectivity we have investigated the effect of the temperature on the reversal potential under biionic conditions in inside out membrane patches excised from outer segments of tiger salamander rods. The current was activated by 100μM cyclic GMP and measured under voltage clamp conditions. When Li was inside the patch pipette and the other ions were in the bathing medium the reversal potential at 26°C was -14, +4, +4, +12 and +18 mV for NH_4, Na, K, Rb and Cs respectively. When the temperature was decreased to 5°C the observed reversal potential for the same ions was -12, 0, 0, 8 and 15 mV respectively. Changing the temperature between 35° and 5°C caused continuous changes of the reversal potential and of I-V relations measured between -100 and +100 mV. These results indicate a significant entropic contribution to the ionic selectivity of the cyclic GMP activated channel and do not support the view of a selectivity caused primarily by a high energy field.

Th-PM-B7

LIGHT ADAPTATION OF FLASH-INDUCED PHOSPHODIESTERASE ACTIVITY IN SALAMANDER RETINAL RODS. ((G.J. Jones)) Dept Physiol., Boston Univ. Sch. Med., Boston MA 02118 (Spon. by M.C. Cornwall)

The flash sensitivity of isolated salamander rod photoreceptors is reduced by background light. In the dark, flash-induced phosphodiesterase (PDE) activity, deduced from the flash photocurrent, rises as a delayed ramp (Lamb & Pugh, *J. Physiol.* (1992), **449**, 719). The influence of steady backgrounds on this initial rise in PDE activity has been investigated, with the aim of elucidating a change in phototransduction gain in light adaptation. Background adaptation does produce a decrease in the deduced rate of rise of PDE activity after a flash. The rise still appears as a delayed ramp, over the same range of light-sensitive current and time. However, the reduction is only prominent (3- to 4-fold) for weak flashes and intense backgrounds, and is far from sufficient to explain the flash desensitization (more than 100-fold) produced by these backgrounds. At low backgrounds no effect is seen. At intermediate and strong backgrounds, the reduction in initial flash-induced PDE activity depends on flash intensity, being stronger for weaker flashes. This last observation suggests that background-dependent effects are not exerted at the level of the light-sensitive channel. These results indicate that a reduction in gain of the early stages of phototransduction makes a small contribution to the process of light adaptation in rods. Supported by NIH EY01157.

Th-PM-B8

MECHANISM OF TRANSDUCIN GTPase REGULATION BY THE γ-SUBUNIT OF cGMP PHOSPHODIESTERASE FROM BOVINE ROD OUTER SEGMENTS. **((V.Y.Arshavsky[*], V.Z.Slepak[&], Y.Zhu[*], M.I.Simon[&] and M.D.Bownds[*#])) [*]Laboratory of Molecular Biology, [#]Department of Zoology and [#]Neuroscience Training program, University of Wisconsin, Madison, WI 53706; [&]Division of Biology, California Institute of Technology, Pasadena, CA 91125. (Spon. by Y.Saimi)**

Our previous studies have shown that the γ-subunit of rod cGMP phosphodiesterase (PDE_γ) is a GTPase activating protein for retinal G-protein, transducin, (Arshavsky and Bownds, *Nature*, 357, 416, 1992), and that the GTPase activating epitope is located within the C-terminal third of PDE_γ (Arshavsky et al., *Inv.Oph.Vis.Sci.*, 34, 1068, 1993). Now we demonstrate that this function is conferred through several hydrophobic amino acid residues of the PDE_γ C-terminal. The ability of recombinant PDE_γ to stimulate transducin GTPase is significantly suppressed when Val^{66}, Trp^{70}, Phe^{73} or Leu^{76} are substituted by alanine. Another group of experiments indicate that PDE_γ is capable of accelerating transducin GTPase only in the presence of another factor from photoreceptor membranes: the accelerating effect of PDE_γ is dramatically increased with the increase of photoreceptor membranes concentration. The nature of this factor will be discussed.

Th-PM-B9

MECHANISMS OF TRANSDUCIN INACTIVATION BY THE INHIBITORY GAMMA SUBUNIT OF cGMP PHOSPHODIESTERASE

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Inactivation of the photoreceptor G protein transducin ($G_{t\alpha}$) and its effector cGMP phosphodiesterase (PDE) occurs slowly (rate constant of 0.03 s^{-1}) in dilute suspensions of bovine rod outer segments (ROS). Addition of the inhibitory gamma subunit of PDE (PDEγ) to dilute ROS (4 μM rhodopsin) increased the rate of PDE inactivation without affecting the rate of GTP hydrolysis by $G_{t\alpha}$. This effect of PDEγ on PDE inactivation was observed when either GTP or GTPγS was used to activate $G_{t\alpha}$, and resulted in long-term inhibition of $G_{t\alpha}$. Inhibition of $G_{t\alpha}$-GTPγS activated PDE by PDEγ occurred with a rate constant of 2 x 10^6 M^{-1} s^{-1}. $G_{t\alpha}$ GTPase activity was increased to 0.12 s^{-1} by a ROS membrane-associated protein distinct from PDE or PDEγ. When PDEγ was added along with membranes containing this activity, $G_{t\alpha}$ GTPase was further accelerated to at least 0.7 s^{-1}. PDEγ did not greatly accelerate $G_{t\alpha}$ GTPase when added with protein-free vesicles, or with trypsinized ROS membranes lacking the GTPase accelerating protein activity.

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Na CHANNELS: STRUCTURE-FUNCTION

Th-PM-C1

THE SAXITOXIN / TETRODOTOXIN BINDING SITE ON CLONED RAT BRAIN IIA NA CHANNELS IS IN THE ELECTRIC FIELD.

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The rat brain IIa (BrIIa) Na channel α-subunit and the brain ß1 subunit were coexpressed in *Xenopus* oocytes, and peak whole-oocyte Na current (I_{Na}) was measured at a test potential of -10 mV. Hyperpolarization of the holding potential resulted in an increased affinity of STX and TTX block of BrIIa Na channels. The apparent ½-block concentration (ED_{50}) for STX of BrIIa current decreased with hyperpolarizing holding potentials (V_{hold}). At V_{hold} of -100 mV the ED_{50} was 2.1±0.4 nM and the affinity increased to a ED_{50} of 1.2±0.2 nM with V_{hold} of -140 mV. In the absence of toxin the peak current amplitude was the same for all potentials negative to -90 mV, demonstrating that channels were maximally available to open in this range of holding potentials as if they were all in the closed conformation. The increase of the STX ED_{50} as a function of holding potential was fit to the Woodhull model (1973). The equivalent electrical distance of block (δ) by STX was 0.18 from the extracellular milieu when the valence of STX was fixed to +2. Analysis of the holding potential dependence of TTX block yielded a similar δ when the valence of TTX was fixed to +1. We conclude that the guanidinium toxin site is located partially within the transmembrane electric field. Previous site-directed mutagenesis studies demonstrated that an isoform-specific phenylalanine in the BrIIa channel is critical for high affinity toxin block. Therefore, we propose that amino acids at positions corresponding to this Phe in the BrIIa channel, which lie in the outer vestibule of the channel adjacent to the pore entrance, are partially in the membrane field.

Th-PM-C2

THE OPEN SODIUM CHANNEL AS A METALLOPROTEIN.

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Transitions in proteins frequently involve metal ions, such as Fe^{2+} in the transition between the R and T states of hemoglobin. Monovalent cations appear to play a similar role in the transition between open and closed states of the Na channel (and presumably also of other ion channels). It was proposed last year (HRL, BJ 64: A85, 1993; Bull. Am. Phys. Soc. 38:1668, 1993; see also BJ 62:22-24, 1992) that the positively charged S4 segments are α helical in the closed state; that, because of Coulomb repulsion, these α helices are unstable without forces induced by the resting potential; and that a depolarization thus leads to a transition in which the interloop hydrogen bonds are broken. This helix-coil transition opens a membrane-spanning pathway of potential wells at backbone carbonyl oxygen sites, which can become occupied by unhydrated Na^+s via thermally activated hopping from the aqueous media. In this way the channel becomes a metalloprotein, in which amide carbonyl oxygen sites on the four S4 segments are coordinated by mobile Na^+s. Such a pathway can explain the high conductance of the open channel, as in superionic Na^+ conductors (e.g. β alumina and Nasicon).

Th-PM-C3

DIRECT INTERACTION BETWEEN INTERNAL TETRA-ALKYLAMMONIUMS AND THE INACTIVATION GATE OF CARDIAC NA CHANNELS.

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The effects of internal tetrapentylammonium (TPeA) and tetrabutylammonium (TBA) were studied on human cardiac Na channels (hH1) expressed in a mammalian cell line. TPeA and TBA cause concentration-dependent increases in the apparent rate of inactivation at positive voltages. The blocking and unblocking rate constants of TPeA were estimated from these data and found to be voltage dependent. The inhibition constant (K_i) at 0 mV is 9.8 μM, and the blocking site is located δ=0.42 of the way into the membrane field from the cytoplasmic face of the channel. K_i and δ for TBA, measured by reduction of amplitude of single channel currents, are 0.46 mM and 0.48. Raising $[Na]_o$ reduces the TPeA-modified inactivation rates, suggesting that external Na ions displace TPeA from its binding site within the pore. TPeA or TBA induce a use-dependent block; 1-Hz trains of depolarizing pulses cause a progressive decrease in Na current amplitude. Tetrapropylammonium (TPrA) blocks open Na currents by a fast-block mechanism (K_i=4.7 mM, δ=0.5), but does not alter inactivation or show use dependence. Internal TPrA antagonizes both the TPeA-induced effect on the apparent inactivation rate and the use dependence, suggesting that all 3 compounds compete for a common binding site. We postulate that when TBA is in its blocking site, the central nitrogen is located ~half-way through the membrane electric field, and that one of its 7-Angstrom arms can just reach a cytoplasmic inactivation gate, inhibiting the normal recovery from inactivation.

Th-PM-C4

NA CHANNEL MUTATIONS IN PARAMYOTONIA CONGENITA UNCOUPLE INACTIVATION FROM ACTIVATION.

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Mutations in the adult human skeletal muscle Na channel α-subunit are responsible for the disease paramyotonia congenita (PC). Two PC mutations, R1448H and R1448C, substitute histidine and cysteine for the outermost arginine in the S4 segment of domain 4 (S4/D4). These mutations, expressed in a cell line, have only small effects on the activation of whole cell Na currents, but mutant channels inactivate more slowly than wild-type (WT) channels, and exhibit an enhanced rate of recovery from inactivation, showing that PC mutations destabilize inactivation. In a voltage range where WT channels are steeply voltage dependent (-60 to 0 mV), mutants show little or no voltage dependence in the rate of inactivation. The inactivation rate of R1448C displays greater temperature sensitivity than that of WT. R1448C has greater effects on inactivation than R1448H. Increase of extracellular pH makes the inactivation of R1448H similar to that of R1448C, suggesting that this residue has access to the extracellular solution, and that its charge is important for normal inactivation. Analysis of single channel data show no enhancement in modal gating in mutant channels, and reveal that they inactivate normally from closed states, but poorly from the open state. The data suggest a critical role for the S4/D4 helix in the coupling between activation and inactivation.

Th-PM-C5

CHARACTERIZATION OF AN "INACTIVATION-DEFECTIVE" VOLTAGE-GATED SODIUM CHANNEL.

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Previous studies have shown the importance of a hydrophobic triplet of amino acids (IFM) in the III-IV linker region of the Na channel (West *et. al.*, 1992). We altered a single amino acid, F1304Q, in this region of μ1, the rat skeletal muscle Na channel. Expressed in *Xenopus* oocytes, μ1 wild-type and μ1-F1304Q (FQ) whole-cell currents activate and peak at similar voltages. However, FQ has a markedly slowed decay of the macroscopic current producing a "plateau current" not seen in μ1. Steady-state inactivation curves of peak current obtained using a 5 sec. prepulse protocol show a +21 mV rightward shift for FQ. In addition, repriming protocols demonstrate a faster return from inactivation in FQ. Single-channel experiments demonstrate similar single channel conductance (μ1 27.0 pS, N=4 vs. FQ 29.7 pS, N=3). While FQ open times were unchanged for depolarizations to -60 mV (μ1 .27$\pm$.05 msec vs. FQ .35$\pm$.08 msec), a significant difference was observed for steps to 0 mV (μ1 .97$\pm$.07 msec vs. FQ 2.66$\pm$.24 msec, N=3 for both). Convolution analysis supports the finding that the "plateau current" is due primarily to reopenings of the channel from the inactivated state rather than changes in open times or first latencies. FQ specifically slows the O $\rightarrow$ I transition rate thus prolonging open times; it also destabilizes the inactivated state such that it is no longer absorbing. Chemicals such as batrachotoxin and fenvalerate alter inactivation to prolong open times; however they also affect permeation and/or activation. FQ appears to have a specific effect on inactivation allowing the channel to reopen multiple times during a single depolarization without significantly affecting permeation or activation. Rather than being inactivation deficient, the inactivated state is present but "defective" in this μ1 mutant. These characteristics make μ1-F1304Q a useful channel background to study permeation, channel block, gating and local anesthetic action.

Th-PM-C6

LIDOCAINE BLOCK OF MUTANT NA$^+$ CHANNELS WITH DEFECTIVE INACTIVATION: IMPLICATIONS FOR THE STRUCTURE OF THE LIDOCAINE ANESTHETIC RECEPTOR.

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The III-IV linker in mammalian sodium channels is proposed to serve as the inactivation gate (West *et al.*, 1992). A single polar amino acid substitution (F1304Q) within a hydrophobic triplet of amino acids (IFM) in this region of the μ1 rat skeletal muscle Na channel rendered the channel inactivation-defective and slowed decay of whole-cell current expressed in *Xenopus* oocytes. Lidocaine (3-1000 μM) produced dose-dependent blockage of the peak currents for μ1 and μ1-F1304Q with half-blocking concentrations (IC_{50}) of 397$\pm$16 μM (N=8) and 584$\pm$10 μM (N=5), respectively. Unexpectedly, the decay of μ1-F1304Q current was accelerated by lidocaine and the non-inactivating component of this current was almost an order of magnitude more sensitive to lidocaine (IC_{50}= 82$\pm$12 μM) than the peak. The mechanism of the effect on the non-inactivating current was explored at the single-channel level using cell-attached patches and 300 μM lidocaine at potentials from -60 to +10 mV; relative to drug-free conditions: [1] reopenings were less frequent; [2] mean open times were essentially unchanged; and [3] unitary current amplitudes were slightly reduced. These findings are consistent with a model in which lidocaine stabilizes a non-absorbing inactivated state of the mutant channel. We hypothesize that lidocaine binding to its receptor enhances the compromised hydrophobic interaction of the mutant inactivation gate with its docking site and thereby restores an inactivating phenotype.

Th-PM-C7

THE EFFECTS OF INACTIVATION-DIRECTED MUTATIONS ON HUMAN HEART Na$^+$ CHANNEL GATING.

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In rat brain type IIa (rB2a) Na$^+$ channels a sequence of three hydrophobic residues (Ile, Phe, Met) in the cytoplasmic linker between domains III and IV regulates fast inactivation (West *et al.*, PNAS 89, 10910, 1992). In particular, a Phe$\rightarrow$Gln substitution (F1489Q) caused the removal of >85% of fast inactivation. Since this residue is strictly conserved in voltage-gated Na$^+$ channels, we tested whether F1485Q, the analogous mutation in human heart (hH1) Na$^+$ channels, has a similar functional effect. In wild type (WT) channels expressed in *Xenopus* oocytes, fast inactivation was complete within 15 ms at a test potential of 0 mV and its time course was biexponential with time constants of 0.4 ms and 2 ms. In contrast to rB2a, the FQ mutation in hH1 removed less than half of the normal fast inactivation process, and increased mean single channel open time by only two-fold. Residual fast inactivation was monoexponential with a time constant similar to that of the slower phase of normal inactivation (2 ms). In the mutant channels, unlike WT, the voltage range of steady-state inactivation coincided exactly with that of activation, suggesting that residual inactivation was tightly coupled to the open state. As in rB2a, complete removal of fast inactivation was achieved by simultaneous mutations of I1484Q and M1486Q, in addition to F1485Q. Our results suggest that in heart Na$^+$ channels, the IFM cluster controls the stability of both open and closed channel inactivation in a manner qualitatively similar to that in brain. Quantitative differences may explain the distinct gating patterns in channel subtypes. (Supported by NIH grant H36930).

Th-PM-C8

RESTORATION OF INACTIVATION AND BLOCK OF SODIUM CHANNELS BY AN INACTIVATION GATE PEPTIDE.

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Mutations of the hydrophobic sequence at positions 1488-1490 containing the amino acids IFM in the intracellular loop between domains III and IV of the rat Type IIA sodium channel prevent fast inactivation. To test whether the IFM motif acts as the inactivation particle, the effects of pentapeptides containing IFM on the inactivation time course of slowly inactivating mutant and wild-type sodium channels were examined. Using whole-cell voltage clamp techniques, we find that the IFM peptides restore fast inactivation to non-inactivating sodium channels with mutation F1489Q and to wild-type channels with inactivation slowed by α-scorpion toxin. Similar peptides without an intact IFM motif are unable to restore inactivation. Electrophysiological recordings of tail currents show that peptides containing IFM compete with the intrinsic inactivation particle of wild-type sodium channels for a common receptor site and prevent channel closure and inactivation. Pentapeptides containing IFM also cause open channel block of wild-type sodium channels that is both voltage- and frequency-dependent. We conclude that the IFM motif of the sodium channel at positions 1488-1490 is the inactivation particle and enters and occludes the intracellular mouth of the pore.

Th-PM-C9

P LOOPS DO NOT PARTICIPATE EQUIVALENTLY IN THE FORMATION OF THE SODIUM CHANNEL PORE

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High affinity blockade of the cardiac isoform of the Na channel by Cd^{2+} and Zn^{2+} and insensitivity to tetrodotoxin (TTX) is mediated by a cysteine residue in the P loop of repeat I. Substitution of the tyrosine at the equivalent position in the skeletal muscle isoform is sufficient to confer the cardiac phenotype to the skeletal muscle channel (Backx *et al.*, 1992). We hypothesize that the topology of the channel pore can be mapped by substitution of residues in this region by cysteine and determination of the Cd^{2+}/Zn^{2+} sensitivity and the voltage- dependence of binding of these cations. Replacement of the neighboring tryptophan in repeat I with cysteine produces a Na channel with intermediate sensitivity to Cd^{2+}/Zn^{2+} and TTX. In contrast, cysteine substitutions in the P- loops of other repeats of the skeletal muscle isoform at the position equivalent to that producing the phenotypic change in the first repeat of the channel, do not alter divalent cation or TTX sensitivity. Our data indicate that these substitutions do not equally modify the permeation, divalent cation, and guanidinium toxin blocking properties of the channel. These data suggest that the Na channel pore is formed by an asymmetrical arrangement of the P-loops of each repeat.

Th-PM-D1

THREE-DIMENSIONAL STRUCTURES OF HIV-1 REVERSE TRANSCRIPTASE COMPLEXED WITH A dsDNA TEMPLATE-PRIMER AND A NONNUCLEOSIDE INHIBITOR

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The crystal structure of HIV-1 RT complexed with a dsDNA template-primer and an Fab fragment (HIV-1 RT/dsDNA/Fab) has been solved at 3 Å resolution (Jacobo-Molina *et al, PNAS* 90, 6320-6324, 1993) and is being refined using XPLOR (R=0.25 at 2.8 Å resolution). The crystal structure of HIV-1 RT complexed with a nonnucleoside inhibitor, α-anilinophenylacetamide (α-APA; Pauwels *et al., PNAS* 90, 1711-1715, 1993), has also been solved at 3.0 Å resolution (Ding *et al.*, in preparation). In both structures, the p66/p51 heterodimer of RT contains four subdomains in the polymerase domains [named fingers, palm, thumb, and connection] (Kohlstaedt *et al., Science* 256, 1783-1790, 1992), which have similar folding but different spatial arrangement in the two subunits (p66 and p51). The bound DNA template-primer has both A- and B-form regions separated by a significant bend (40-45°) and its interactions with RT involve primarily the sugar-phosphate backbone of the DNA and amino acid residues of the palm, thumb, and fingers of p66. The α-APA inhibitor is located in the core of the highly hydrophobic nonnucleoside inhibitor binding pocket and most of the nonnucleoside resistance mutations that have been reported map to amino acid residues which are close to the inhibitor. The current resolution of the structures has enabled us to formulate detailed hypothesis about possible mechanisms of RT drug resistance. The proximity of the inhibitor to Glu138 of the p51 subunit is experimental evidence supporting the prediction that resistance to the TSAO compounds caused by Glu138Lys mutation is manifested through a change in p51 (Nanni *et al., Persp. Drug Discovery and Design*, 1, 129-150, 1993). The structural results and biochemical studies both suggest that the effects of many nucleoside resistant mutations may be exerted through direct interactions with the template-primer substrate, which may affect the geometry of the dNTP binding site (Boyer *et al.*, submitted).

Th-PM-D2

PROTEIN-PROTEIN INTERACTIONS BETWEEN *trp* REPRESSOR DIMERS UNDER VARYING SOLUTION CONDITIONS

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Identifying and understanding factors which are energetically linked to the function of genetic regulatory proteins provides insight into the physical mechanisms involved in the modulation of transcription. The *trp* repressor (TR) of *E. Coli* is a transcription regulating protein that is small (dimer of 25 kD) and for which extensive structural information is available. These attributes make TR a good model system to study energetics in conjunction with structure to gain deeper insight into the modulation of transcription. The interactions between TR dimers are studied using fluorescence methods. Large oligomers of the *trp* aporepressor (ApoR) exist under various solution conditions and are destabilized by the addition of corepressor, L-tryptophan. Increasing salt concentration and pH (range 6.0 to 8.5) destabilize high order oligomers of ApoR and HoloR (aporepressor with two corepressor molecules bound) toward dimers, indicating an electrostatic component to the interaction between ApoR and HoloR dimers. Single site functional mutants of TR that repress transcription at lower corepressor concentrations have been identified. One such "superrepressor" (EK18) is the result of mutation at the 18th amino acid in the monomer chain: negatively charged glutamic acid is replaced with positively charged lysine. EK18 was studied under the solution conditions of interest (pH 6.0, 7.6, 8.5; 0-200 mM KCl), and the oligomerization properties were found to be distinct from those of wild-type TR. The effect of charge changes on the interactions between dimers suggests the superrepressor properties of EK18 result from the effect of added positive charge on the protein, altering protein-protein, protein-ligand, and protein-DNA interactions.

Th-PM-D3

DNA BINDING OF ETS1 PROTEIN AND PEPTIDES. ((R. J. Fisher, J. R. Casas-Finet, J. W. Erickson, M. J. Fivash, S. V. Bladen and T. S. Papas)) PRI/DynCorp and NCI, FCRDC, Frederick, MD 21702 (Spon. by D. Tsao)

The human ETS1 oncoprotein binds to DNA through an 85-amino acid C-terminal domain lacking homology to known DNA binding motifs. We have used surface plasmon resonance technology and spectroscopic methods to monitor the binding of ETS1 (p42 and p51 isoforms) and of peptides spanning the conserved sequence (I66N, residues 335-400; K37N, res. 364-400) to various DNA sequences. Double-stranded DNA oligos with the specific ETS1 binding motif (wt: GCCGAAGT) or a single base mutation (M4: GCCAGAAGT) were attached to the biosensor surface, and ligand binding to DNA measured using surface plasmon resonance. Analysis of the progress curves of the real-time biospecific interaction shows that binding of both peptides to the canonical DNA sequence contains specific and non-specific components, whereas it is purely non-specific with mutated M4 site. ETS1 p42 and p51 showed avid binding to the specific DNA sequence, whereas no binding was detected to mutated oligos. Binding to sequences carrying single point mutations (M1-M9) through the canonical site indicated different pattern of specifity for the peptides and full-length ETS1; p42 and p51 had identical pattern. Lattice binding was shown by the poly(U)-induced I66N Trp quenching (55%) or the enhancement of poly(ethenoA) fluorescence. In contrast, CCGGAAGT binding induced a 25% *enhancement* of I66N Trp fluorescence, suggesting a different interaction with this sequence. While K37N showed β-structure in aqueous buffers, EtOH induced α-helix formation. Our results suggest that these small peptides are conformationally flexible and, while capable of DNA binding, require additional structural information to allow high affinity site-specific binding.

Th-PM-D4

BINDING STUDIES OF HIV-1 REV PROTEIN AND PEPTIDES TO RRE SEQUENCES. ((José R. Casas-Finet, Elena Afonina, Sergei Gulnik, Yoshifumi Adachi, George N. Pavlakis, and John W. Erickson)) PRI/DynCorp and ABL-BRP, FCRDC, Frederick, MD 21702 (Spon. by J. R. Casas-Finet)

Rev protein plays an important role in the regulation of proviral HIV-1 genome expression. Characterization of Rev and its interaction with its specific RNA sequence, the Rev responsive element (RRE), has been hampered by the tendency of both free and RRE-bound Rev to oligomerize. We have investigated the binding of recombinant rev protein to RRE sequences of various lengths (330, 220, and 70 nucleotides) containing a high-affinity nucleation site by spectrofluorimetry, UV photocrosslinking and gel mobility shift assays (GMSA). Rev fluorescence peaks at 341 nm and is dominated by a Trp contribution typical of a partially solvent-exposed chromophore. Rev bound avidly to the 220-mer RRE sequence, inducing a *ca.* 40% quenching and a 7 nm blue-shift of its emission maximum. The single site (70-mer) RRE induced a 30% Trp quenching but resulted in a significantly more blue-shifted band, indicating that Trp solvent shielding increased drastically. This strongly suggests that the single Trp residue is located in the RNA binding site. Large Trp quenching was also seen with a basic peptide spanning the putative Rev RNA-binding domain. We measured a dissociation constant of 2.5 nM for the interaction of Rev with 220-mer RRE. GMSA suggests that Rev bound to all RRE sequences containing the high affinity site with similar affinity. Up to six bands were detected above free 220-mer RRE, whereas only one band migrated as a Rev/70-mer RRE complex. High [NaCl] reversed complex formation, indicating an electrostatic component to the binding process.

Th-PM-D5

OLIGOMERIZATION OF THE REV PROTEIN OF HIV-1: IMPLICATIONS FOR FUNCTION.

((James L. Cole, John D. Gehman, Jules A. Shafer and Lawrence C. Kuo)) Department of Biological Chemistry, Merck Research Laboratories, West Point, PA 19486.

Rev is an RNA-binding protein of HIV-1 and is required for expression of incompletely spliced viral transcripts. Oligomerization of rev is thought to be associated with RNA binding and rev function. Here, we have characterized the oligomerization of rev using equilibrium analytical centrifugation. Rev is predominantly monomeric at low concentrations but reversibly polymerizes to produce large aggregates at higher concentrations. The data fit well to an unlimited isodesmic self-association model in which the association constants for addition of a monomer to each aggregate are equal [K = 1.08 x 10^6 M^{-1} at 4°C]. Thermodynamic parameters derived from the temperature dependence of the association constant over the range of 0 °C to 30 °C reveal that the primary contribution to the free energy of oligomerization is a large negative enthalpy. Binding of rev to the rev responsive element RNA (RRE) was characterized using a nitrocellulose filter assay and by equilibrium analytical centrifugation. Rev binds to the RRE at a protein concentration where rev is predominately monomeric, suggesting that solution multimerization of rev is not required for rev function.

Th-PM-D6

STUDY OF A BURIED SALT-LINKED TRIAD IN ARC PROTEIN.((C.D. Waldburger and R.T. Sauer)) MIT, Cambridge, MA 02139. (spon. by J. King)

In the structure of Arc, a dimeric DNA binding protein encoded by bacteriophage P22, there is a triad of charged residues (Arg31, Glu36, and Arg40) that interact by forming two partially buried salt-bridges. We have measured the stabilities of wild-type Arc and mutant variants containing single, double, and triple alanine substitutions at positions 31, 36, and 40 by urea denaturation experiments and have determined the interaction free energies for the Arg31-Glu36 salt-bridge (~2 kcal/mol/dimer) and the Glu36-Arg40 salt-bridge (~5 kcal/mol/dimer).

We have randomized the three salt-bridge residues and isolated mutants that retain activity. The spectrum of residues in the mutant proteins indicated that comparable stability had been achieved by replacing the salt-bridge interactions with hydrophobic interactions. Six variants were purified (R31E36R40→M31Y36L40, M31W36L40, V31Y36I40, I31Y36V40, L31M36I40, and Q31Y36V40, respectively) and were found to be 2-5 kcal/mol more stable than wild-type.

Arc binds to DNA as a tetramer and the binding reaction is highly cooperative, in part due an interaction across the tetramer interface between the Arg31 side-chain and the protein backbone. The six purified active mutants were defective for cooperative DNA binding in vitro. These results are consistent with a model in which the cooperativity defect of these mutants is compensated by increased protein stability conferred by hydrophobic interactions at residues 31, 36, and 40.

Th-PM-D7

A CALORIMETRIC STUDY OF LINKAGE IN SPECIFIC PROTEIN-DNA INTERACTIONS. ((Knut Langsetmo and Robert T. Sauer)) Dept. of Biology, MIT, Cambridge, MA 02139. (Spon. by Peter Kim)

Two aspects of linkage are addressed. The energetic contribution of a hydrogen bond network in λ repressor N-terminal domain to binding of O_L1 was examined using thermodynamic 'mutant cycles'. The contribution of N-terminal domain dimerization to binding was also assessed.

Binding of λ repressor N-terminal domain was examined by calorimetric titration with the synthetic O_L1 operator. At 28 °C the total binding reaction is enthalpy driven, with a slightly unfavorable entropy. The co-crystal structure of N-terminal domain and O_L1 shows a hydrogen bonding network between residues Q33, Q44, and the phosphate and base of adenine 2 of the operator. Mutation of either Q33 or Q44 to alanine results in a less favorable binding enthalpy. Changes in binding energetics resulting from mutation of both of these residues are not additive, but rather linked, indicating an interaction between these residues

The overall reaction includes a dimerization component. At 28 °C dimerization is entropy driven. The thermodynamic parameters for the binding component of the reaction were determined by subtracting the values obtained in the dimerization experiments from the results for the total binding reaction.

Both the total reaction and dimerization are strongly temperature dependent. This indicates hydrophobic interactions are important in both dimerization and specific protein-DNA binding.

Th-PM-D8

CHROMATIN NUCLEOPROTEIN POLYMERASE COMPLEXES CONTAINING ONCOGENES AND SUPPRESSER GENES: THEIR POTENTIAL REGULATORY CONTRIBUTION TO METASTASIS ((Nancy L. Rosenberg-Nicolson and Garth L. Nicolson)) Rhodon Foundation for Biomedical Research and The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030. (Spons. by G. Nicolson)

Intact nuclei from metastatic variants of the murine RAW117 large-cell lymphoma cell line were subjected to direct digestion with *Msp*-I by a previously described method designed to fractionate the nucleus into ~6 Nucleoprotein (NP) fractions containing specific polymerase-containing NP complexes. Individual NP constituents of the *Msp*-I-derived subnuclear complexes were purified by two-dimensional isofocusing reducing SDS-polyacrylamide electrophoresis followed by gel excision, electroelution, and removal of SDS by extractigel chromotography. The purified NPs were either dot- or slot-blotted onto a solid support and screened for the presence of a panel of genes (*p53*, *abl*, β-casein, μ chain immunoglobulin, etc.) by hybridization. Analyses were performed on NPs obtained from metastatic variant lines of RAW117. Reproducible differences in NP distribution in the SDS gels and presence of tightly bound genes were noted amongst the metastatic variants, particularly in the presence of *p53* and *abl* genes. We speculate that a specific chromatin NP-gene organization exists in metastatic RAW117 cells. Supported by NCI grant CA44352 to G.L.N.

Th-PM-D9

ZUOTIN, A YEAST Z-DNA BINDING PROTEIN, MAY BE INVOLVED IN DNA REPAIR. ((A. Yu, S. Zhang, Y. Xing, A. Rich, and M. Mitas)) Dept. of Biochemistry and Molecular Biology, 246 Noble Research Center, Oklahoma State University, Stillwater, OK. 74078. Dept. of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139. (Spon. by A. Rich).

ZUO1 encodes a putative yeast Z-DNA binding protein, named Zuotin. In order to understand better the function of Zuotin, and the function of the DNA to which it binds, we constructed a yeast cell line (LNZ14) containing a deletion in the Zuotin gene. Absorbance studies indicated that the wild-type yeast cells grew twice as fast as LNZ14 in rich liquid medium. The ratios of single cells:budding cells in wild-type and LNZ14 were 1:1.1 and 1:1.9, respectively, suggesting that LNZ14 remained longer at the unsegregated phase. These observations suggest that *ZUO1* might be important in maintaining some of the normal events in the yeast cell cycle. To determine whether a mechanism involving DNA repair might be responsible for the differences observed between wild-type yeast and LNZ14 cells, both were subjected to varying doses of mutagenic agents, and the numbers of surviving colonies were determined. The number of LNZ14 cells that survived exposure to low concentrations of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), *N*-methyl-*N*-nitrosourea (NMU), 4-nitro N-oxide (4NQO), or dimethyl sulfate (DMS), were 1.3 to 13-fold reduced with respect to wild-type cells. However, no differences in survival between the two cell lines were observed in response to various doses of UV irradiation. These data indicate that the yeast Z-DNA binding protein Zoutin may be involved in DNA repair, possibly in DNA base excision repair.

Th-PM-D10

A DIRECT MEASURE OF THE CONTRIBUTION OF SOLVATION TO THE THERMODYNAMICS OF BINDING. (M. C. Chervenak and E. J. Toone*, Department of Chemistry, Duke University, Durham, NC 27708-0346)

The nature of the forces contributing to ligand binding in aqueous solution is still obscure. We have calorimetrically evaluated the thermodynamics of binding of a variety of systems, including protein-carbohydrate, protein-nucleoside, and small molecule-small molecule interactions in both light and heavy water. In each case, the enthalpy of binding was less negative in D_2O than in H_2O, with a compensating change in entropy. For the systems evaluated, the change in molar heat capacity on binding was not affected by solvent isotopic substitution. A plot of the difference in enthalpy between light and heavy water processes, $\Delta\Delta H$, against ΔC_p gives a straight line that passes through the origin with a slope of 5°. We interpret these results in terms of solvent reorganization during binding. We have used a thermodynamic cycle to estimate the magnitude of the contribution of solvent reorganization to the net ethalpy of binding. Our analysis shows 4-18 kcal mol^{-1} of binding enthalpy, representing 25-100% of the total, can be accounted for by the return of water surrounding solutes to bulk solvent during binding.

HEME PROTEINS II

Th-Pos1

PRESSURE MODULATION PERTURBATION OF PROTEIN MOTIONS ((R.M. Ernst, R. Philipp, G.U. Nienhaus, R. Noble, R.D. Young, H. Frauenfelder)) Department of Physics, University of Illinois, 1110 W. Green St., Urbana, IL 61801.

We present results which show that it is possible to couple to functionally important motions of proteins with the application of time-dependent pressure. This pressure modulation technique consists of monitoring the transient visible absorption of the protein while applying isotropic pressure with amplitudes of up to 10 atm at frequencies from 2 Hz to 2 kHz. We have studied sperm whale myoglobin and carp hemoglobin in both the R and T states, and find that pressure modulation dramatically increases the rate at which the CO ligand passes from the protein interior to the solvent for both the myoglobin and T state samples. These results suggest that the myoglobin and T state proteins are more susceptible than the R state hemoglobin to motions induced by the oscillating pressure; this is consistent with a relaxation model of rebinding in these proteins. The pressure modulation technique presents a new method of probing the correlation between protein motions and function, which is key to understanding the structure/function relationship in proteins. (This work supported by the NIH and the ONR.)

Th-Pos2

HEME RELAXATION MODEL OF PROTEIN REBINDING ((Y. Abadan, K. Chu, R.M. Ernst, R. Philipp, G.U. Nienhaus, R. Noble, R.D. Young, H. Frauenfelder)) Department of Physics, University of Illinois 1110 W. Green St., Urbana, IL 61801.

We discuss a model of ligand binding to heme proteins in which the reaction rate is controlled by the heme out-of-plane distance. To support this model, we present IR absorption and visible flash photolysis data from carp R and T state hemoglobin and sperm whale myoglobin. The IR measurements probe the structure of the heme group, while flash photolysis reveals the rebinding function of the protein; the two techniques yield results consistent with a model in which the heme relaxes in discrete steps following ligand dissociation. Ligand concentration studies using flash photolysis, and a novel pressure modulation perturbation method further indicate that the T state matrix is more susceptible than the R state to motions that enable the heme to relax. (This work supported by the NIH.)