W-Pos354

[^{14}C]-METHYLAMINE-UPTAKE STUDIES DEMONSTRATE A H^+-ATPASE IN *PARAMECIUM* DENSE CORE SECRETORY VESICLES.

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Dense core secretory vesicles are isolated from the tam-8 secretory mutant of *Paramecium tetraurelia* by means of homogenization and differential centrifugation followed by Percoll gradient separation of organellar constituents. These preparations produce two bands of dense core proteins: one which has membranes and one which does not. The band with membranes (vesicles) is essentially pure by thin-section electron microscopy. The fact that vesicles accumulate acridine orange only in the presence of ATP lends evidence to the presence of a functioning H^+-ATPase within the vesicles. The further use of proton pump inhibitors allows us to classify this vesicular H^+-ATPase as a vacuolar-type. Incubation of fresh preparations of vesicles in [^{14}C]-methylamine resulted in rapid uptake in the presence of 5 mM Mg^{++}-ATP (energized vesicles) but not in its absence. Dense core proteins without surrounding membranes did not accumulate methylamine in the presence or absence of Mg^{++}-ATP. The addition of 20 μM CCCP to energized vesicles resulted in dissipation of the methylamine to the level of non-energized vesicles. In addition, western-blots using polyclonal antibodies directed against the 57 kD protein of the chromaffin granule H^+-ATPase (from Dr. N. Nelson) showed reactivity to one band at M_r 57,000 in preparations of intact dense core secretory vesicle proteins.

W-Pos355

NUMERICAL SIMULATION OF A CALMODULIN ACTIVATED CALCIUM PUMP AND LEAK SYSTEM: NORMAL HUMAN RED BLOOD CELLS IN VITRO

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Few quantitatively explicit models for regulation of ion transport are established. The red blood cell (RBC) offers a simple system in which the transport of ions across the plasma membrane can be studied without the complications of other membranes. Using fluo-3, an intracellular Ca^{2+} sensitive fluorescent probe (Minta et al., JBC 264: 8171-8178, 1989), we recently found that normal human RBCs in vitro exhibit an intracellular free Ca^{2+} of 195 nM (Vincenzi et al., in Calcium Transport and Intracellular Calcium Homeostasis, D. Pansu & F. Bronner, eds., Springer Verlag, Berlin, pp. 185-195, 1990). We prepared a steady state model of the Ca^{2+} pump/leak system of normal human RBCs using STELLA (HIgh Performance Systems, Inc., Hanover NH) operating on a Macintosh computer. It was assumed that all influx of Ca^{2+} is passive and that all efflux is active and is equal to the sum of pumping carried out by the Ca^{2+} pump ATPase in its basal and calmodulin (CaM) activated forms. Na^+/Ca^{2+} exchange was not included in the model because human RBCs lack this process (Ferreira and Lew in Membrane Transport in Red Cells, J.C. Ellory & V.L. Lew, eds., Academic Press, New York, 1977, pp. 53-91). In the basal state the Ca^{2+} pump ATPase was assumed to exhibit Kd Ca of 14 μM, Vmax of 100 μmol/min/l RBC and Ca cooperativity of 1.5. In the CaM activated state these were assumed to be 1 μM, 300 μmol/min/l RBC and 1.5, respectively (Niggli et al., JBC 256:395-401, 1981). The Kd Ca of CaM was assumed to be 1 μM, with a cooperativity of 4 (Dedman et al., JBC 252:8415-8422, 1977) for activation of the Ca^{2+} pump ATPase. The model predicted intracellular free Ca^{2+} of 178 nM under 'normal' in vitro conditions (at which the pump operated at approximately 0.05% of CaM activated Vmax). The model also predicted regulation of intracellular free Ca^{2+} over wide ranges of assumed permeabilities and Vmax values. Regulation was accomplished mainly by 'switching' of the pump from a basal to CaM activated state. Predicted intracellular Ca^{2+} was sensitive to the assumed Kd Ca values, but not very sensitive to assumed Vmax, CaM cooperativity or permeability. Supported in part by a grant from the Washington Affiliate of the American Heart Association.

W-Pos356

IDENTIFICATION OF MITOCHONDRIAL PROTEINS IN MEMBRANE PREPARATIONS FROM *CHLAMYDOMONAS REINHARDTII*

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Preparations enriched in *Chlamydomonas reinhardtii* thylakoid membranes (1) have proven useful in combined biophysical, biochemical and genetic studies of photosynthesis. Many of their polypeptides however remain unidentified, among them hemoproteins h_1 and h_2 (2) and protein P_3 ("70 kD protein" in ref.3). We report that h_1, h_2 and P_3 belong to mitochondrial membranes that copurify with the thylakoids.

h_1, h_2 and P_3 are present in all tested mutants of *C. reinhardtii* lacking either one or several of the complexes of the photosynthetic chain (PSI, PSII, b_6/f, ATP synthase). h_2 is a nuclear-encoded soluble protein with a MW of 9-10 kD by SDS-PAGE and 14 kD by gel filtration. It is a *c*-type cytochrome reducible by ascorbate with absorbance peaks at 549.5 nm (α), 520 nm (β) and 514 nm (Soret). h_2 cross-reacts immunologically with cytochromes *c* from Horse heart and yeast. It contains the peptide FAEAPA, found at the N-terminus of *C. reinhardtii* cytochrome *c* cDNA sequence (4). P_3 is a nuclear-encoded extrinsic protein that cross-reacts with *C. reinhardtii* chloroplast ATP synthase β subunit (3,5). Its N-terminal sequence is 38 % identical to that of the yeast β subunit. h_1 belongs to an intrinsic protein complex that is absent in the mitochondrial mutant DUM-1 lacking functional bc_1 complex (6).

Because *C. reinhardtii* mitochondria have not been successfully purified, none of their proteins has been biochemically characterized yet. Our results indicate that h_2 is cytochrome *c*, P_3 ATP synthase β subunit and h_1 part of the bc_1 complex. Such identifications are particularly interesting in *C. reinhardtii*, whose mitochondria encode a very restricted set of proteins (see ref.7), for studies of mitochondrial protein synthesis and import and for the examination of chloroplast/mitochondrion interactions.

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W-Pos357

EVIDENCE FOR THE DEVELOPMENT OF AN INTERMONOMERIC ASYMMETRY IN THE COVALENT BINDING OF DIDS TO BAND 3 TETRAMERS.

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Covalent binding of DIDS (4,4'-diisothiocyanostilbene-2,2'-disulfonate) was used to assay the conformational state of band 3 monomers intermonomerically crosslinked by BS^3 (bis(sulfosuccinimidyl)suberate) in the presence of DNDS (4,4'-dinitrostilbene-2,2'-disulfonate). DNDS binds reversibly to band 3 with a 1:1 monomeric stoichiometry and is expected to protect the stilbene disulfonate site of all monomers from intramonomeric BS^3 crosslinking. However, we have found an intermonomeric asymmetry which was manifest as a fraction of band 3 monomers active in transport, but refractory towards covalent binding of DIDS, despite being capable of binding DIDS reversibly. The appearance of these DIDS-refractory monomers correlates with the $DNDS/BS^3$-generated formation of a unique oligomeric structure composed of noncovalent tetramers of two covalent band 3 dimers [Salhany, J.M., Sloan, R.L. & Cordes, K.A. (1990) J. Biol. Chem. 265, (in press)]. DIDS pretreatment of all monomers prior to BS^3 crosslinking, predominantly yields an alternate structure containing covalent band 3 dimers. We conclude that these two observable forms are alternate in situ quaternary conformational states of band 3. The significance of these two quaternary states to band 3 function is unknown.

W-Pos358

CHARACTERIZATION OF CROSS-LINKED PRODUCTS FORMED ON IRRADIATING THE BOVINE HEART F_1-ATPASE DERIVATIZED WITH 5'-P-FLUOROSULFONYLBENZOYL-8N_3-2[^{3}H]ADENOSINE

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After inactivating the bovine heart F_1-ATPase (MF_1) in the dark with 5'-p-fluorosulfonylbenzoyl-8N_3-adenosine (FSB-8N_3-A), removing excess reagent, and irradiating the derivatized enzyme, cross-linked species were observed when the photolyzed enzyme was submitted to SDS-PAGE. The major cross-linked species migrated as a doublet with an M_r of about 120,000. Minor, higher order cross-linked species were also observed. Treatment of the component of the doublet of higher M_r with alkali followed by SDS-PAGE liberated equal amounts α and β subunits, indicating that it is an $\alpha-\beta$ dimer. Only the β subunit was liberated when the component of the doublet of lower M_r was submitted to the same treatment. Two major radioactive fragments were resolved when a tryptic digest of MF_1, inactivated with FSB-8N_3-[^{3}H]A in the dark at pH 8.0, was submitted to reversed-phase HPLC. Sequence analysis showed that one peptide contained Tyr-β368 and the other contained His-β427. These two residues were identified earlier to be present at or near noncatalytic sites when the enzyme was inactivated with FSBA (D.A. Bullough and W.S. Allison (1986) *J. Biol. Chem.* **261**, 5722). Irradiation of MF_1 inactivated with FSB-8N_3-2[^{3}H]A led to disappearance of the peptides containing Tyr-β368 and His-β427 with concomitant appearance of new radioactive peptides. The analysis completed so far suggests that the $\alpha-\beta$ and $\beta-\beta$ dimers observed arise from cross-links involving Tyr-β368 and His-β427. Isolation and sequence analysis of the radioactive, cross-linked tryptic peptides will establish the amino acid residues involved in connecting the $\alpha-\beta$ and $\beta-\beta$ dimers.

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W-Pos359

VANADATE CATALYZED PHOTOCLEAVAGE OF ADENYLATE KINASE IN THE GLYCINE RICH LOOP- Direct Chemical Evidence for Amino Acids Involved in Polyphosphate Binding

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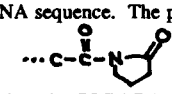
It has been previously shown by us that chicken muscle adenylate kinase is specifically oxidized at the active site by irradiation with UV light in the presence of vanadate (Hatlelid & Cremo (1990) *Biophysical J.* **57**, 423a). Oxidation of the enzyme (21 kDa) results in inactivation and cleavage of the polypeptide backbone to yield a small fragment and an 18.5 kDa peptide. The small fragment has been purified by HPLC and identified as a 17 amino acid peptide containing the N-terminus. Amino acid analysis and mass spectral analysis have localized the cleavage site to between proline-18 and glycine-19 in the glycine-rich loop. The analyses also shows that the N-terminal residue of this peptide is an acetylated serine, not the methionine predicted from the DNA sequence. The proline on the "C" terminus has been oxidized to



The 18.5 kDa cleavage peptide was sequencable, giving predominantly GSGKGT... and a small amount of SGKGT... in agreement with the position of cleavage indicated by the 17 amino acid peptide. This is the first evidence for involvement of proline in vanadate catalyzed photocleavage reactions. Because we have previously shown that the photocleavage reaction is inhibited by ATP, AMP, and AP5A, this is direct chemical evidence that this region of the enzyme is involved in polyphosphate binding.

W-Pos360

FACTORS AFFECTING THE SPECIES-HOMOLOGOUS AND SPECIES-HETEROLOGOUS BINDING OF MITOCHONDRIAL ATPase INHIBITOR, IF_1, TO THE MITOCHONDRIAL ATPase OF SLOW AND FAST HEART-RATE HEARTS. William Rouslin and Charles W. Broge, Department of Pharmacology and Cell Biophysics, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267-0575.

Slow heart-rate hearts have heart rates of approx. 200 beats/min or less and contain predominantly the slow myosin ATPase isoform. Fast heart-rate hearts are capable of heart rates of approx. 300 beats/min or more and contain predominantly the fast myosin ATPase isoform . Earlier studies by us (Rouslin, W. Am. J. Physiol. 252, H622-H627, 1987) showed that, while slow heart-rate hearts exhibit a marked IF_1-mediated inhibition of their mitochondrial ATPase during ischemia, fast heart-rate hearts, including those that contain a full complement of IF_1, do not.

In the present study we used rabbit as the slow heart-rate species and pigeon as the fast heart-rate species; pigeon is a fast heart-rate species that contains a full complement of IF_1. We examined the effects of ionic strength upon the binding of rabbit heart IF_1 (present in crude IF_1-containing extracts) to both the rabbit and pigeon heart ATPase (present in IF_1-depleted, sonicated mitochondria) as well as the effects of ionic strength upon the binding of pigeon heart IF_1 (also present in crude extracts) to the rabbit and pigeon heart ATPase. While the binding of rabbit heart IF_1 to both the rabbit and pigeon heart ATPase was relatively unaffected by increasing the ionic strength of the binding medium, the binding of pigeon heart IF_1 to both the rabbit and pigeon heart ATPase was markedly suppressed by increasing the ionic strength of the medium. The results suggest that the lack of IF_1 binding during ischemia in fast heart-rate hearts is not due to species-related differences in the ATPase, but, rather, appears to be related to the source of the IF_1-containing extract.

Conclusion: Either fast heart-rate hearts lack a factor (e.g., an IF_1-F_1-ATPase complex stabilizing peptide) required for IF_1 binding at ionic strengths prevailing in the matrix space of intact mitochondria, or the fast heart-rate heart IF_1 has a lower affinity for the ATPase than the slow heart-rate inhibitor. (Supported by NIH grant HL30926)

W-Pos361

PURIFICATION OF A VACUOLAR ATPASE FROM BARLEY ROOTS. F.M. DuPont and P.J. Morrissey, USDA Agricultural Research Service, Albany, CA.

Proton transport by a vacuolar type ATPase was characterized using a tonoplast-enriched membrane fraction from roots of barley (Hordeum vulgare L. cv CM72). The ATPase creates a proton gradient that drives the uptake of Na^+ into the vacuole, via a Na^+/H^+ antiporter which is activated by exposing roots to NaCl. Therefore, we investigated whether ATPase activity was upregulated by salt stress. Transport was assayed using acridine orange, quinacrine, or methylamine. Rates of proton transport increased 2- to 4-fold when the seedlings were grown in 100 mM NaCl, but the rate of ATP hydrolysis was not affected by the salt treatment. Membranes were solubilized in Triton X-100 and the nitrate-inhibited ATPase activity was purified by chromatography on a Sephacryl S-400 column followed by chromatography by FPLC on a Mono Q column. Purity of the enzyme was tested by centrifugation on a glycerol gradient. The purified ATPase preparation contained polypeptide bands of 116, 66, 56, 45, 42, 32, 30 16, 13 and 12 Kd when separated by SDS-PAGE. ATPase which was purified from control and salt-treated roots had identical subunit composition when analyzed by SDS-PAGE. The increase in proton transport when roots were grown in salt could not be accounted for by a gross change in subunit composition of the vacuolar ATPase. Although Ca^{2+} did not support proton transport in the native membrane vesicles, ATP hydrolysis by the purified enzyme was stimulated by Ca^{2+} or Mg^{2+}. Activity in the presence of 1 mM Ca^{2+} was 80% of the activity in the presence of 1 mM Mg^{2+}. A separate peak of Ca^{2+}-stimulated ATPase was separated from the nitrate-inhibited vacuolar ATPase during purification on the Sephacryl S-400 column. However, the remaining Ca^{2+}-stimulated ATPase activity that copurified with the vacuolar ATPase seems to be due to the vacuolar ATPase itself.

W-Pos362

REGULATION OF THE ERYTHROCYTE Ca^{2+}-ATPase BY MUTANT CALMODULINS WITH AMINO ACID SUBSTITUTIONS IN THE Ca^{2+} BINDING DOMAINS. Danuta Kosk-Kosicka* and Tomasz Bzdega, University of Maryland, School of Medicine, Baltimore, MD 21201, *Presently at The Johns Hopkins University, School of Medicine, Baltimore, MD 21205.

Mutant calmodulins (CaM) with site-specific amino acid substitutions in the four Ca^{2+} binding domains have been selected to study activation of the human erythrocyte Ca^{2+}-ATPase by the calmodulin dependent pathway. To assess the role of some highly conserved amino acid residues in the Ca^{2+} binding domains of CaM in this activation process two kinds of amino acid substitutions were introduced in CaM: Ala for Glu sequentially in position 12 in each of the four Ca^{2+} binding domain, and Ala, Gly, Phe or Tyr for Ser 101 in the third Ca^{2+} binding domain. All mutant calmodulins were generated in M. Watterson's laboratory (Craig et al., 1987). Our studies were performed on two kinds of enzyme preparations, purified monomeric Ca^{2+}-ATPase (Kosk-Kosicka & Bzdega, 1988) and erythrocyte ghost membranes with comparable results. The Ca^{2+}-ATPase activity was measured as a function of Ca^{2+} and CaM concentrations. The study revealed dramatic differences in the functional importance of Glu in position 12 in each of the four Ca^{2+} binding domains: the Glu located in the carboxyl-terminal half of calmodulin is crucial for activation of the Ca^{2+}-ATPase at physiologically significant Ca^{2+} concentrations. Single substitutions for Ser 101 in the third Ca^{2+}-binding domain showed diverse effects strongly dependent on the introduced amino acid, most significant with Tyr which totally eliminated enzyme activation by CaM at pCa 7. The activity could be recovered by increasing Ca^{2+} concentration to pCa above 6. The results suggest that the size and the chemical character of the side chain at position 101 are strongly related to the ability of calmodulin to activate the Ca^{2+}-ATPase.

W-Pos363

THE CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR: SYNTHETIC PEPTIDES CORRESPONDING TO A CRUCIAL FUNCTIONAL REGION OF THE PROTEIN. Philip J. Thomas, P. Shenbagamurthi, Xavier Ysern, and Peter L. Pedersen, Department of Biological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, MD.

Recently identified mutations in the gene responsible for cystic fibrosis are located within a predicted nucleotide binding fold in the amino terminal half of the cystic fibrosis transmembrane conductance regulator (CFTR) protein. A 67 amino acid peptide (P-67), corresponding to the central region of this putative nucleotide binding site, was chemically synthesized and purified. In the first direct demonstration of a function for the CFTR gene product, this peptide bound adenine nucleotides. The apparent binding constants (K_ds) for the trinitrophenyl adenine nucleotides TNP-ATP, TNP-ADP, and TNP-AMP are 300 nM, 200 nM, and greater than 1 μM respectively. The K_d for ATP is 300 μM. CD spectroscopy indicates that the peptide assumes a predominantly β-sheet structure in solution, a finding consistent with secondary structure predictions. Based on this information the crucial phenylalanine at position 508 (which is deleted in 70 % of all cystic fibrosis patients) resides in an important β-strand within the nucleotide binding fold. Therefore, deletion of this residue is predicted to induce a significant change in the β-strand leading to altered nucleotide binding. We are currently synthesizing a second peptide, lacking the phenylalanine residue, in order to directly test the effect of the deletion on nucleotide binding and peptide structure.

W-Pos364

THE MITOCHONDRIAL ATP SYNTHASE: MUTATIONAL ANALYSIS OF THE CONSENSUS NUCLEOTIDE BINDING SEQUENCES IN THE β-SUBUNIT. Philip J. Thomas, David N. Garboczi, and Peter L Pedersen, Department of Biological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, MD.

The β-subunit of the mitochondrial ATP synthase, thought to contain the catalytic nucleotide binding site, contains several sequences homologous to others found in many ATP utilizing enzymes. The consensus of these sequences in conjunction with the common nucleotide binding function of the proteins in which they are found argue for their involvement in nucleotide binding. Four such regions of homology have been identified to date in the β-subunit: the A consensus sequence (G x_4 G K T), or glycine flexible loop; the B type consensus sequence (R x_{6-8} h_4 D); the C region; and a region homologous to the phosphorylation domain of the P-type ATPases. In order to assess the contribution of each of these domains to nucleotide binding, and to assess if they alone are sufficient to explain binding to the isolated β-subunit, plasmids coding for deletion mutants which lacked each consensus sequence were constructed and induced to overexpress the mutant proteins in *E. coli*. Furthermore, several single residues within the putative nucleotide binding sequences in the β-subunit were altered using site directed and random mutagenesis. Recombinant mutant proteins were purified from *E. coli* and assessed for function. The results indicate that the A consensus sequence provides the bulk of the interactions necessary for nucleotide binding to the isolated β-subunit while the B consensus sequence contributes more weakly to nucleotide binding. (Supported by NIH grant CA10951 to P.L. Pedersen)

W-Pos365

Molecular Biology of Vacuolar H^+-ATPase.
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The vacuolar ATPase is the main source of energy for the vacuolar system of eukaryotic cells. Our studies have elucidated the identity and subunit composition of the enzyme complex and unraveled certain aspects of its mechanism of action. Previously, cold inactivation studies of V-ATPases determined the subunit structure of the catalytic sector of these enzymes. It is now apparent that the catalytic sector is composed of five different polypeptides denoted as subunits A to E in order of decreasing molecular weights. Yeast mutants in which genes encoding subunits of the vacuolar H^+-ATPase were interrupted, were assayed for their vacuolar ATPase and proton uptake activities. The vacuoles from the mutants laking subunits A(72 kDa), B(57 kDa) or c(16 kDa) were completely inactive in these reactions. Immunological studies revealed that in the absence of each one of those subunits the catalytic sector was not assembled. 14C-N,N'-dicyclohexylcarbodiimide (DCCD) labeling showed the presence of the c subunit in vacuoles of mutants in which genes encoding subunits of the catalytic sectors were interrupted. No DCCD labeling was detected in the mutant in which the gene encoding the proteolipid (subunit c) was interrupted. It was concluded that out of all the ATPase subunits only the proteolipid is assembled independently and it serves as a template for the assembly of the other subunits. Site-specific mutants in the gene encoding the proteolipid were generated. All of the drastic changes and replacements were inactive. About half of the single amino acid replacements were active. Changing glutamic acid 137 by several amino acids, except for aspartic acid, abolished the activity of the enzyme. Other amino acids that may function in proton conductance were changed. It was found that glycine residues may replace amino acids with exchangeable protons.

W-Pos366

60 Hz MAGNETIC FIELDS AND CALCIUM METABOLISM IN RAT THYMOCYTES. Robert P. Liburdy, Jan Walleczek, and Darlene J. de Manincor, Bioelectromagnetics Research Facility, Lawrence Berkeley Laboratory, UC Berkeley, CA 94720

We have recently observed that a 60 Hz sinusoidal magnetic field of nonthermal intensity increases $^{45}Ca^{2+}$ uptake in rat thymocytes if the cells are exposed in the presence of the mitogen Concanavalin A (20ug/ml)(Con A)(Walleczek & Liburdy, accepted in *FEBS Letters*). Cells were exposed at 37° C for 60 minutes in an annular petri dish that established an induced electric field intensity of 1 mV/cm and this produced an average 2.7-fold increase in Con A-dependent calcium uptake compared to isothermal control cells. Significantly, non-mitogen treated thymocytes did not respond to the applied magnetic field. In this regard, cell activation appears to be important for field coupling to this cellular system.

To further characterize this field response we have employed three methods to quantitate Ca^{2+} transport: membrane filtration, centrifugation through oil, and cell pellet washing. In addition, multi-well annular ring petri dishes are being employed in studies to manipulate exposure dosimetry so that a magnetic field vs. an electric field effect can be identified. A further characterization of Con A dose response indicates that 1 ug/ml corresponds to an optimal dose for $^{45}Ca^{2+}$ uptake over the range 0 - 20 ug/ml, and this corresponds to the concentration most effective for mitogenesis.

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W-Pos367

THE MEASUREMENT OF INTRACELLULAR PH IN SACCHAROMYCES CEREVISIAE USING FLUORESCENT DYES. R. Haworth, B. Lemire, & L. Fliegel. Dept. of Pediatrics and Biochemistry, U. of AB, Edmonton, AB, Canada.

Intracellular pH in yeast is regulated by a plasma membrane H+-ATPase. Our experiments are aimed at developing a method for the measurement of intracellular pH in yeast using fluorescent compounds, and establishing the presence or absence of a native Na+/H+ exchanger, which is widely believed to be responsible for internal pH regulation in mammalian cells. We tested the efficacy of three fluorescent probes for the measurement of intracellular pH in *Saccharomyces cerevisiae* DAU-1. (1) 2',7'-Bis-(carboxyethyl)-5(6')-carboxyfluorescein pentaacetoxymethyl ester was not easily taken up by the yeast, and was de-esterified very slowly. (2) Fluorecein diacetate was readily loaded into yeast and de-esterified, but was rapidly lost to the external medium. (3) Carboxy-semi-naphthorhodafluor-1-acetoxymethyl ester was found to be most suitable of the dyes studied. The dye was readily loaded into the cell, where the fluorescent compound (SNARF) was released by intracellular esterases. The quantity of intracellular dye did not decrease during experimentation. The fluorescence intensity of SNARF was influenced by changes in the external medium. Addition of ammonium chloride caused an initial, rapid decrease in fluorescence intensity, indicating a rise in cellular pH. This was followed by slow acidification. In contrast to similar experiments with mammalian cells, removal of ammonium chloride from the medium did not result in further intracellular acidification. The addition of glucose resulted in intracellular alkalinisation. Addition of nigericin to low-pH media, followed by potassium chloride, resulted in decreased intracellular pH, possibly caused by the equilibration of internal and external pH. Fluorescence microscopy showed that SNARF had a heterogeneous distribution. Most cells showed a concentration of SNARF in one particular area of the cell, close to the cell membrane. This area may correspond to vacuoles. Some cells showed a more even distribution of the compound, although longer incubations of the cells with SNARF resulted in greater localisation of the dye. Future experiments will concentrate on finding conditions and cell strains which result in a more even distribution of SNARF within the cell. Supported by MRC and AHFMR.

W-Pos368

REGULATION OF pH IN THE GARFISH OLFACTORY NERVE IN THE ABSENCE OF BICARBONATE. D.J. Mills, R.K. Drake, and G.R. Kracke, Department of Anesthesiology, University of Missouri, Columbia, MO 65212.

The olfactory nerve of the garfish contains a uniform population of small diameter, unmyelinated axons. Intracellular pH was measured in the nerve with the fluorescent indicator, BCECF, using the ratio method: excitation at 490 and 439 nm, emission at 530 nm, and calibration with high K solutions and 20 uM nigericin. Resting pH_i was 7.42 ± 0.06 (SEM, n = 7). The nerve was acid-loaded by the addition and removal of 20 mM NH_4Cl in a nominally bicarbonate-free medium containing, in mM, 131 NaCl + NH_4Cl, 3.5 KCl, 1 $MgCl_2$, 4 $CaCl_2$, 24 glucose, 10 Tris-Cl, pH 7.4, 295 mosm/kg, at 27°C. Intracellular buffering power, calculated from the decrease in pH_i immediately after removal of NH_4Cl, was 9.0 ± 1.5 mM (SEM, n = 8). The pH_i recovery after an NH_4Cl acid load was relatively linear for about 100 s and the rate of recovery was determined by a linear fit. The rate of recovery was a hyperbolic function of the extracellular Na concentration (N-methyl-d-glucamine was Na substitute) with a Km = 21 ± 5 mM Na and V_{max} = 5.3 ± 0.5 pH units/h (SEM, n = 3). The rate of recovery in 131 mM NaCl was inhibited 86% by 10 uM 5-(N,N-hexamethylene)-amiloride, a selective inhibitor of Na/H exchange, with EC_{50} = 0.16 ± 0.05 uM (Range/2, n = 2). It is concluded that the regulation of intracellular pH after an acid load in the olfactory nerve is due in part to an amiloride-sensitive Na/H exchange mechanism. (Supported by the American Heart Association, Misssouri Affiliate.)

W-Pos369

INTERACTION OF LIPOPHILIC CATIONS WITH MITOCHONDRIA. B.C. Pressman, Y-F Shi, and C. Calderon-Higginson. Dept. of Molecular and Cellular Pharmacology, University of Miami, Miami, FL 33136 (Intro. by B. Rose).

Accumulation of the Parkinson's disease-inducing 1-methyl-4-phenylpyridinium (MPP^+) by mitochondria (M) with concomitant Site 1 selective inhibition of ε transport parallels the properties of guanidinium derivatives reported previously and suggests that these properties may be generic for lipophilic cations. Other properties shared by lipophilic cations are: potency rises proportionally with octanol : H_2O partition; slow onset of inhibition, greater in State 4 than State 3; slow incomplete reversal of inhibition by uncouplers; concomitant uptake of phosphate along with cation potentiation by μM ϕ_4B^- and φundecadiborane$^-$. Partitions are carried out with perchlorate in H_2Oas gegenion to facilitate phase transfer of cation. Some of the esoteric cations belonging to this category include ϕ_2I^+, ϕ_3 pyrilium$^+$, tropylium$^+$,N-methylquinolinium,N-methylacridiniumandcarbocyanine, rhodamine and saffranine dyes. We have synthesized several homologs of N-alkyl pyridiniums in order to dissect lipophilicity from intrinsic cation potency; N-alkyl pyridiniums are 3x, and N-methyl-4-alkyl 15x, more potent than guanidiniums of equivalent lipophilicity. If Em is calculated from apparent gradients by naively assigning all accumulated cation to M matrix, paradoxically Em appears to rise with lipophilicity (guanidine, -62 mV; hexylguanidine -121 mV; octylguanidine -159 mV); by selecting an appropriately lipophilic guanidine, any predesired Em can be obtained. Osmotic swelling experiments indicate that guanidinium is indeed a permeant cation which freely enters the M matrix. ϕ_4P^+, ϕ_4As^+, ϕ_4Sb^+ all behave as typical lipophilic cations. $\phi_4P^+$and $\phi_3CH_3P^+$, much used as Em probes, correspond in lipophilicity to hexylguanidine implying they overestimate Em by ~ -60 mV, due to accumulation within or upon the energized M membrane, while other more polar cations (biguanidines, pyridiniums) also yield Em in the -60 to -70 mV range, presumably the true transmembrane Em. Implications of these data for M bioenergetics will be discussed. Supported by NIH GM 38920.

W-Pos370

BACTERIORHODOPSIN IN ICE: ACCELERATED PROTON TRANSFER FROM THE PURPLE MEMBRANE SURFACE

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Only pH-indicator dyes covalently linked to the extracellular surface of bacteriorhodopsin (BR) or, slightly less efficiently, to the surrounding lipid headgroups, allow exact determination of the H^+-release kinetics. Fluorescein bound to BR monitors the released proton about 10 times faster than indicators such as pyranine or fluorescein residing in the aqueous bulk phase distant to the purple membrane (Heberle and Dencher, Biophys.J. 57, 183a, 1990). The rate of the diffusion controlled transfer of protons from the surface to the bulk is a function of the surface properties. To gain insight into the molecular mechanism of the surface/bulk transfer as well as to correlate H^+-release and photocycle kinetics, BR and pH-indicators quickly frozen in ice were studied by time-resolved flash-spectroscopy. Under these conditions, pyranine in the aqueous bulk phase responds to the ejected proton as fast as fluorescein attached to BR, demonstrating that the surface/bulk transfer in ice is no longer rate limiting. The pumped H^+ appears at the extracellular surface during the L - M transition, characterized by the fast phase of the 410 nm absorbance increase. The Arrhenius plot of the M-formation rates does not reveal a discontinuity at the freezing point. This indicates that protons in BR are translocated via an ice-like structure. Experiments like these might clarify, if it is really the H^+ which is pumped, or the OH^-.

W-Pos371

CHARGE SUBSTITUTIONS AT THE BACTERIORHODOPSIN PROTON CHANNEL RIM. K. McMillan and R. Renthal, U. of Texas at San Antonio, San Antonio, TX 78285

The atomic resolution model of bacteriorhodopsin (bR) shows an apparent proton transport channel traversing the purple membrane (PM). The rim of the channel contains numerous charged amino acids, none of which is essential for proton pumping. We now report conditions for conversion of negatively charged groups to positively charged groups at the rim of the proton channel. Purple membrane was deionized and then reacted with the carboxyl group modifying reagent 1-ethyl-3-[^{14}C]trimethylaminopropyl carbodiimide (ETC). Approximately 3 moles of ETC were covalently bound per mole of bR. The modified groups were identified as follows. After 30 min. proteolysis with proteinase K in 0.2% dodecyl sulfate, nearly all of the ^{14}C was found in small fragments. Reverse phase HPLC separated the fragments into two main fractions. Amino acid analysis and gas phase sequence analysis showed that the major modified peptides contained Glu 74 and Glu 194. Smaller amounts of labeling were found on fragments containing Asp 38, Glu 9 and the carboxyl terminal tail. All of these groups are placed at the surface in the bR model. Glu 9, 74, and 194 are at the rim of the proton release channel. A striking characteristic of ETC-modified PM is a shift in the pK of the low pH purple-to-blue transition from 3.2 to 2.4, and a change of the slope of the titration curve from 2 to 1. Identification of the modified carboxyl groups suggests two possible explanations of the altered purple-to-blue transition. Blue membrane forms when Asp 85, inside the proton release channel, becomes protonated. An increase of the net charge by 4 at the rim of the proton release channel would increase the local surface potential, thereby increasing the local pH and decreasing the apparent pK of Asp 85. Alternatively, the ETC group is long enough to span the distance from the surface Glu groups (9, 74, 194) to form ion pairs with the Asp groups inside the channel (85, 212). Ion pair formation would lower the pK of Asp 85. (Supported by NIH)

W-Pos372

APPARENT UP REGULATION OF Na^+ PUMPS PRODUCED BY HYPO-OSMOLARITY IN FROG MUSCLE. R.A. Venosa, Department of Physiology & Biophysics, School of Medicine, University of La Plata, La Plata, Argentina.

The purpose of this work was to determine if hypotonicity in addition to the stimulation of active Na^+ transport (Venosa, BBA 510: 378, 1978) promoted changes in: a) active K^+ influx, b) passive Na^+ and K^+ fluxes, and c) the number of ^{3}H-ouabain binding sites. Experiments were performed on sartorius muscles from L. ocellatus. The results indicate that a reduction of external osmotic pressure (π) to one half of its normal value (π= 0.5) produced the following effects: 1) an increase in active K^+ influx of the order of 160 %, 2) a 20 % reduction in Na^+ influx and K^+ permeability (PK), 3) a 40 % increase in the apparent density of ouabain (OUA) binding sites. OUA binding was reasonably well fitted by a Michaelian hyperbolic function of [OUA]. In π= 1, maximum binding (B_m) was 3500 ± 310 molec.μm^{-2} with $K_{0.5}$= 5.4 ± 1.8 µM; in paired muscles equilibrated in π= 0.5, it was B_m= 4860 ± 160 molec.μm^{-2} and $K_{0.5}$= 1.1 ± 0.2 µM. The data suggest that the hypotonic stimulation of the Na^+ pump is not caused by an increased leak of either Na^+ (inward) or K^+ (outward). It is unlikely that the stimulation of active Na^+ extrusion and the rise in the apparent number of pump sites produced by hypotonicity were due to a reduction of the intracellular ionic strength. It appears that, at least in part, the stimulation of active Na^+ transport takes place whenever muscles are transferred from one medium to another of lower tonicity even if neither one was hypotonic (for instance π= 2 to π= 1 transfer).

Comparison of the present results with those previously reported (Venosa, 1978) indicates that in addition to the number of pump sites, the cycling rate of the pump is increased. Active Na^+ and K^+ fluxes were not significantly altered by hypertonicity (π= 2).

W-Pos373

METABOLIC SUBSTRATE EFFECTS, FORCE PRODUCTION AND RESTING PHOSPHATE METABOLITE LEVELS IN ISOLATED MOUSE SKELETAL MUSCLES. R.W.Wiseman, S.K.Phillips+, R.C.Woledge+, and M.J.Kushmerick. Depts. of Radiology and Physiology and Biophysics, University of Washington Medical Center, Seattle, WA,91895 and +Dept. of Physiology, University College London, Gower Street, London, WC1E 6BT.

Isometric twitch force increased in slow twitch (soleus) muscles but not in fast twitch (extensor digitorum longus, EDL) muscles, when substrate was changed from 11 mM glucose to 20 mM pyruvate (Phillips and Woledge, J. Physiol., 426, 33P,1990). It has been hypothesized that these substrate effects were due to a decrease in the levels of inorganic phosphate (Pi) when pyruvate was the substrate provided. To test this hypothesis, we studied the effects of replacing glucose with pyruvate on force production while making parallel measurements of the resting phosphorus metabolite levels by ^{31}P-NMR spectroscopy. In soleus, when 11 mM glucose was provided as a substrate, phosphorus NMR spectra contained a substantial Pi peak, with a Pi/PCr ratio of 0.27+/-0.05 (mean+/-SEM, n=6). However, after 1 hr incubation in 20 mM pyruvate, the Pi peak decreased, reducing the Pi/PCr ratio to 0.12+/-0.06, n=6. Concurrent force measurements under these same conditions revealed ~20% increase in force when normalized to force production in glucose alone. These effects are fully reversible when pyruvate is removed. In EDL, when glucose is the substrate, ^{31}P-NMR spectra contain a Pi peak which is barely visible above the baseline noise, and as in the soleus, when incubated in 20 mM pyruvate, the Pi peak decreases. However, force did not increase. From these observations we conclude that the resting phosphate metabolite levels in the soleus and EDL are different, with the much higher Pi/PCr ratios being present in the soleus. Further, treatment with pyruvate decreases the Pi levels in slow twitch muscle and this correlates with an increase in force in this muscle. This effect is absent in EDL, presumably due to the smaller amount of Pi in these muscles. The mechanism of this effect is not known, but may be related to similar effects in cardiac muscle. Supported by NIH F32AR08105-02 to RWW, Wellcome Trust to SKP, and NIH AR36281 to MJK.

W-Pos374

REDOX CHANGES WITH WORK IN RAT HEART. K. E. Conley, C. H. Barlow, J. J. Kelly, D. A. Rorvik and M. J. Kushmerick. Depts. of Radiology and of Physiology and Biophysics, University of Washington Medical Center, Seattle, WA 98195 and Barlow Scientific, Inc., Olympia, WA 98502.

Surface fluorescence of the heart was monitored to evaluate NADH dependent kinetic limitations to respiration during work jumps. Isolated hearts were perfused with Ringers supplemented with 15 mM glucose at 37 and 25 °C. Digital images of the heart surface fluorescence excited by a flash at 330-380 nm were obtained at 450nm, the wavelength for maximum NADH fluorescence emission. Data were normalized to the fluorescence from pentobarbital treated hearts (100% reduction). Surface fluorescence was 77.5±1.2% [mean±SE] at 37 °C and 49.5±0.5% at 25 °C under Langendorff perfusion. Parallel ^{31}P nuclear magnetic resonance experiments showed that ADP was not limiting to respiration since its level was above the K_m for mitochondria (~25 μM) at both temperatures (37 °C: 38±11 μM; 25 °C: 35±6 μM). Significant decreases in surface fluorescence (70.2±1.7% at 37 °C and 47.2±0.6% at 25 °C) were observed with Langendorff to working-mode transitions where the rate-pressure product increased 3-fold. No change in ADP occurred at 37 °C (43.6±7.6 μM) but ADP increased to 56.9±3.3 μM at 25 °C. An increased surface fluorescence with a work jump is required if NADH level limits flux into the electron transport chain. Thus, NADH level did not kinetically limit respiration since surface fluorescence decreased in work jumps in rat heart. Supported by NIH grants AM 36281, HL 37798, HL 43473 and UW Dept. of Radiology.

W-Pos375

CA^{++} CONTROL OF MITOCHONDRIAL METABOLISM IN INTACT CARDIAC TISSUES. Bang Wan, Deborah A. Berkich, Chris Doumen, Kathryn F. LaNoue. Pennsylvania State University College of Medicine, Hershey, PA.

Previous studies of isolated mitochondria suggest that control of α-ketoglutarate may be more stringent and more sensitive to Ca^{++} levels than the other two Ca^{++} sensitive mitochondrial dehydrogenase, isocitrate dehydrogenase and pyruvate dehydrogenase. Since α-ketoglutarate is a potent competitive inhibitor of flux through mitochondrial glutamate oxalacetate transaminase in intact mitochondria, we have proposed that cytosolic free Ca (Ca_f) may control the malate aspartate cycle and oxidation of cytosolic reducing equivalents. To test this hypothesis, rat myocytes were plated on glass cover slips coated with laminin and (Ca_f) was measured in fura 2 loaded cells using a Shimadzu dual wavelength spectrofluorometer in the presence of 6 mM and 40 mM K^+. $Ca^{++}{}_f$ doubled due to K^+ depolarization. The experiment was repeated with cells not loaded with fura 2 and the effect of K^+ depolarization on mitochondrial NADH fluorescence, and flavoprotein fluorescence was measured to quantitate mitochondrial redox state as a measure of dehydrogenase activity. Both determinations suggest significant increases in dehydrogenase activity on K^+ depolarization when lactate but not pyruvate was the perfusate substrate. Since lactate oxidation is dependent on the malate aspartate cycle, but pyruvate is not, the data imply Ca^{++} control of the malate aspartate cycle. Further experiments measured NADH fluorescence and free Ca^{++} levels in single cardiac cells in primary culture. The $Ca^{++}{}_f$ transients following electrical depolarization were followed and NADH was also quantitated. The effect of various rates of electrical depolarization (beating) on NADH were assessed. As opposed to the effect of K^+ depolarization, pulsatile electrical depolarization caused an oxidation of NADH when lactate was the substrate. This may be due to the opposing effect of increased heart work on redox. To further explore the putative Ca^{++} control of the malate aspartate cycle in perfused rat hearts the effect of work, and substrate supply on the mitochondrial membrane potential was assessed using ^{3}H-tetraphenyl phosphonium. Preliminary experiments indicate that it is possible to measure the mitochondrial membrane potential in perfused hearts with 5 nM H3TPP. The lipophillic cation at 5 nM and 50 nM does not alter heart work or creatine phosphate levels. Supported by HL18708.

W-Pos376

THE BEHAVIOR OF A FAST-RESPONDING BARBITURIC ACID POTENTIAL-SENSITIVE MOLECULAR PROBE IN SUBMITOCHONDRIAL PARTICLES AND DIMYRISTOYLPHOSPHATIDYL CHOLINE MULTILAMELLAR SUSPENSIONS J. C. Smith, D. Gill, D. D. Hamilton, H. P. Hopkins, and T. Tran, Dept. Chemistry and LMBS, Georgia State Univ., Atlanta, GA -The barbituric acid molecular probe diBa-C_2-(5) responds to the formation of a membrane potential (ΔΨ) in bovine heart submitochondrial particles (SMP) by a CCCP-sensitive 5-7 nm red shift of the probe absorption spectrum. The spectral shift is enhanced by nigericin + K^+; the probe is thus specifically sensitive to ΔΨ. Binding analyses indicate that the origin of the potential-dependent shift is enhanced association of the probe with the SMP membrane. The time course of the signal is complete in less than 0.2 second and obeys a single exponential rate equation. The pseudo first order rate constants obtained by the latter analyses, however, were a linear function of the probe concentration at fixed membrane protein concentration. The resulting second order rate constant of 1.5 x 10^7 $M(dye)^{-1}$ s^{-1} is two to three orders of magnitude larger than that of other well established indicators of ΔΨ in SMP. The invariance of the rate of oxidation of cytochrome c_1 by ATP-driven reversed electron transport in the presence and absence of diBa-C_2-(5) indicates that the probe does not cross the SMP membrane on a time scale of milliseconds to minutes when ΔΨ is generated.- DiBa-C_2-(5) caused a 0.2° C shift in the Tm and an approximately 15 % reduction of the ΔH of the gel to liquid crystal phase transition in multilamellar DMPC preparations at 5 mol percent. The cooperativity of the latter transition is reduced as the mol fraction of diBa-C_2-(5) is increased. The Tm of the pretransition is shifted from 14° to 9° C over a probe mol % range of 0 to 5, and the experimentally determined ΔH is lowered; the latter decrease causes an apparent increase in the cooperativity of the pretransition as the mol fraction of diBa-C_2-(5) is increased. The latter observations suggest a location of the probe near the DMPC head groups. Support NIH GM30552; diBa-C_2-(5) was generously supplied by Prof. A. S. Waggoner.

W-Pos377

EFFECTS OF CALCIUM ON THE RATE OF OXYGEN UPTAKE OF ISOLATED YEAST (S. cerevisiae) MITOCHONDRIA. Salvador Uribe and Pablo Rangel (Intro. by Antonio Peña) Instituto de Fisiología Celular, Univ. Nal. Autónoma de México, Apdo. Postal 70-600, 04510 México D. F., México.

Free matrix calcium (Ca_m) controls diverse enzyme activities in mitochondria from many sources. In yeast mitochondria, however, calcium is not taken up against a gradient. Using the fluorescent dye fluo3, the Ca_m in yeast mitochondria was measured. Depletion of external calcium (Ca_{ext}) resulted in a decrease in Ca_m which was less pronounced in the presence of phosphate than with acetate. Addition of Ca_{ext} resulted in a rise in Ca_m to the original value but not higher. When the uptake of ^{45}Ca was measured, a small uptake of calcium was observed which was inhibited by the addition of antimycin and was higher in the presence of phosphate than in the presence of acetate. To examine the effect of Ca_m on mitochondrial metabolism oxygen consumption was measured in the presence of different substrates. With ethanol, oxygen consumption was inhibited by the addition of EGTA while succinate supported oxygen consumption was not affected. As alcohol dehydrogenase (ADH) is a matrix enzyme, calcium must be inside the matrix to control its activity. Direct measurements on NAD reduction by ADH, however, showed no effects of calcium. In contrast, when the activity of both ADH and NADH dehydrogenase were assayed together by using ethanol to feed mitochondria and DCPIP to receive the protons liberated by NADH dehydrogenase the calcium withdrawal effect was observed. This suggested that Ca_m is controling the interaction between ADH and NADH dehydrogenase. In addition, these results suggest that Ca_m controls some enzymatic activities in yeast mitochondria as it does in mitochondria from most other sources.

W-Pos378

Germination and Respiration of Bacillus cereus Spore in Organic Solvents.
Contreras, M. and Escamilla, E. Intro. by Peña, A. Instituto de Fisiología Celular, UNAM, México.

Enzymology in colloidal solutions of water in apolar organic solvents stabilized with amphiphilic compounds has become an attractive research area. Catalytic activity in reverse micellar systems has been demonstrated for isolated enzymes and for reaction sequences performed by a set of enzymes individually trapped in separate micelles (Hilhorts et al. Eur. J. Biochem. 144: 459-466. 1984). Catalytic activity has been shown also in the case of enzyme complex in which protein to protein interactions are instrumental for catalysis (Escamilla et al. Arch. Biochem. and Biophys. 272: 332-343. 1989). Outstanding, whole bacterial cells transferred into an organic apolar medium, seems to display some of its metabolic capabilities (Haering et al. B.B.R. C. 127: 911-915. 1986). Here we demonstrate that a complex metabolic response, like the germination of the bacterial dormant spore, can take place in an organic apolar medium composed of isoctane, azolectin and limited amounts of water. The germinative response in the apolar medium was specifically triggered by alanine plus adenosine. Electron microscopy and the ampherometric recording of the cell respiration were used as structural and metabolic criteria of germination.

W-Pos379

OXIDATION OF EXOGENOUS NADH ($NADH_{ex}$) BY ISOLATED CARDIAC MITOCHONDRIA. Patricia M. Sokolove, Department of Pharmacology & Experimental Therapeutics, University of Maryland Medical School, Baltimore, MD 21201.

Mitochondria are generally thought to oxidize internal NADH *via* a rotenone-sensitive inner membrane (i.m.) NADH dehydrogenase (DH). Nohl [Eur. J. Biochem. (1987) 169: 585] has proposed that an NADH DH on the outer face of the i.m. of heart mitochondria, with rotenone-sensitivity determined by electron (e^-) acceptor, participates in the reduction of redox active drugs. A third, rotenone-insensitive, NADH DH, localized in/on the outer membrane (o.m.) has been identified in liver and plant mitochondria. The latter two enzymes are capable of oxidizing $NADH_{ex}$. Using dual wavelength spectroscopy (340 - 370 nm), we have examined the oxidation of $NADH_{ex}$ by isolated, tightly coupled, rat heart mitochondria with the following results. (1) $NADH_{ex}$ is oxidized at a substantial rate (90 nmol·min·mg protein) in a reaction which is largely (> 97%) rotenone-sensitive. (2) In the presence of cyt c, two oxidation processes can be identified: one rotenone-sensitive, with V_{max} 445 and K_m (cyt c) = 0.17μM, the other rotenone-insensitive and mersalyl-sensitive, with V_{max} = 80 and K_m(cyt c) = 1μM. (3) All $NADH_{ex}$ oxidation is stoichiometrically linked to O_2 consumption and inhibited by KCN. (4) All $NADH_{ex}$ oxidation is uncoupled; no increase in electron transport is elicited by 200 nM F-CCP. (5) In the same mitochondria, oxidation of *internal* NADH is coupled. These observations indicate participation of the mersalyl-sensitive o.m. DH in a low capacity $NADH_{ex}$ oxidation pathway which depends completely on added cyt c. A second, high capacity, rotenone-sensitive $NADH_{ex}$ oxidation pathway is stimulated by added cyt c. This pathway is notable for its combined sensitivity to rotenone and lack of coupling. Cyt c concentrations in the intermembrane space of mitochondria *in vivo* are sufficient to support both processes. [Supported by NIH (HL-32615) and the Graduate School, University of Maryland, Baltimore]

W-Pos380

PRODUCTION OF ATP AND PPi IN R. RUBRUM CHROMATOPHORES USING FERROCYANIDE ILLUMINATION TO PRODUCE CHEMIOSMOTIC PROTON GRADIENTS, E.A. Harang, H. Baltscheffsky* and D.W. Deamer. Department of Zoology, University of California at Davis, Davis, CA 95616, *Department of Biochemistry, Arrhenius Laboratory, Stockholm University, Stockholm, Sweden S106 91.

Recent investigations have established that ferrocyanide solutions undergo large pH shifts upon illumination. Because complex ions of ferrous iron were likely to be a relatively abundant species in the prebiotic environment, it seemed worthwhile to explore the possibility that the pH shift could be used as a chemiosmotic energy source for pyrophosphate bond formation. For this purpose, we developed a model system consisting of isolated R. rubrum chromatophores in which the normal electron transport systems were inhibited. Illumination of ferrocyanide in the external medium produced pH gradients across the chromatophore membrane, ranging up to 3.5 pH units. Because the pH increase is external, hydrogen ions were expected to move outward through the FoF1 ATPase and PPase, thereby providing energy sufficient for production of ATP (adenosine triphosphate) or PPi (pyrophosphate). When ATP production was measured, NaF was present to inhibit the PPase. When 0.1 M ferrocyanide was present to provide gradients of approximately 3 pH units, ATP synthesis was twice the control value after two minutes illumination. When gradients of two pH units were produced by 10 mM ferrocyanide, PPi synthesis, but not ATP synthesis, could be detected after 2 minutes. This system provides a relatively simple source of photochemical energy, in which plausible components of the prebiotic environment produce a transmembrane pH gradient with sufficient energy to drive pyrophosphate bond formation by chemiosmotic mechanisms. (Supported by NASA grant NAGW-1119.)

W-Pos381

THE VOLUME AND PH OF THE MITOCHONDRIAL INTERMEMBRANE SPACE DOES NOT AFFECT INTERMEMBRANE IONIC STRENGTH. J.D. Cortese, A.L.Voglino, and C.R.Hackenbrock, Dept. Cell Biol. and Anat., U. North Carolina at Chapel Hill, Chapel Hill, NC 27599-7090. The ionic strength (*I*) of the intermembrane space (IMS) of intact mitochondria (IMS-*I*) is known to affect the electron transport activity of cytochrome *c*. It has been hypothesized that IMS-*I* is similar to cytosolic *I*, given that the mitochondrial outer membrane is highly permeable to ions and small molecules, or very low, given that highly charged and closely opposed inner and outer membranes will exclude ions from the IMS. We previously reported (Cortese et al., 1990, Biophys. J. 57, 559a) that IMS-*I* in intact mitochondria is within 20% of the external, bulk *I*, over a wide range of bulk *I*. We now consider the possible effects of IMS-pH and IMS-volume on IMS-*I*. IMS-pH and IMS-*I* were measured with pH- and *I*-sensitive fluorescent probes (highly fluorescent FITC-dextrans for IMS-pH and FITC-BSA for IMS-*I*). These probes were delivered into the IMS of intact mitochondria via probe encapsulation into asolectin liposomes, followed by low pH-induced fusion of the liposomes with outer membranes of intact mitochondria. The effects of altering external bulk *I* and pH on the fluorescence of IMS-entrapped probes were followed by fluorescence excitation ratios [468/450 nm]. IMS-pH was 0.5 units lower than bulk pH over the pH range of 6.0-8.5 for mitochondria with a large IMS-volume (condensed configuration), and 0.2 units lower for mitochondria with a small IMS-volume (orthodox configuration). This small pH difference between IMS-pH and bulk pH did not modify the similarity of IMS-*I* and bulk *I*. When the IMS-volume is decreased using 120-150 mOsm media, IMS-*I* shifts with bulk *I* above 10 mM but does not respond to changes in bulk *I* below 10 mM. The lack of response of the IMS-*I* to changes in the bulk *I* below 10 mM indicates that the close proximity of the two mitochondrial membranes can exclude ions only at low, nonphysiological *I*. Thus the similarity of IMS-*I* and bulk *I* is unaffected by either IMS-pH or IMS-volume above a bulk *I* above 10 mM, and at cytosolic physiological *I* (100-150 mM) cytochrome *c* can be expected to be a free, 3-dimensional diffusant in the IMS irrespective of the pH or volume of the IMS. Supported by NIH GM-27804.

W-Pos382

THERMODYNAMIC AND KINETIC LINKAGES BETWEEN $\Delta\Psi$, ΔpH AND ATP SYNTHESIS AND HYDROLYSIS IN SUBMITOCHONDRIAL PARTICLES AND EVERTED E. COLI VESICLES. A. Zolkiewska and R. W. Hendler (Intro. by B. Reynafarje), NHLBI, NIH, Bethesda, MD 20892.

The chemiosmotic theory gives equal weight to the voltage and chemical components of $\Delta\tilde{\mu}_{H^+}$. Several studies have shown that there is an apparent threshold of -100 mV, followed by an exponential relationship in the kinetics of ATP synthesis powered by $\Delta\Psi$. We have confirmed this finding and have found that the kinetics of ATP hydrolysis as a function of $\Delta\Psi$ shows an inverse relationship compared to that of synthesis, but with a much lower influence of $\Delta\Psi$. Studies underway are intended to define the quantitative relationships between ΔpH and the forward and reverse kinetics of ATP synthesis, as well as the individual relationships of each component of the $\Delta\tilde{\mu}_{H^+}$ with the ΔG of ATP synthesis. An approach will be described for the establishment of defined relationships between $\Delta\Psi$ and ΔpH and the activation energies for ATP synthesis and degradation.

W-Pos383

QUINONE REDOX POTENTIALS: The Medium is the Message, or, The Variation of E° with Solvent Dielectric.
Jason R. Rose and Todd P. Silverstein, Chemistry Department, Willamette University, Salem, OR 97301

Quinones are critical membrane soluble electron transfer chains that shuttle electrons between large oxidoreductase complexes in bioenergetic membranes. Since the free energy liberated by these electron transfer steps is utilized to pump protons and ultimately to synthesize ATP, it is important to measure quinone redox potentials. Since quinones are generally water insoluble, investigators often make their measurements in aqueous ethanol solutions. This however is too polar a medium to accurately reflect behavior in biological membranes, the site of biological quinone activity.

Accordingly, we have studied the influence of solvent dielectric constant on quinone reduction potential and on reduction pathway as well. Half-wave potentials were measured polarographically and solvent dielectric was varied by addition of water miscible hydrocarbon solvents, both protic and aprotic. Results were successfully modeled down to $\varepsilon \approx 50$-60, using the Born charging equation and the Nernst equation. All quinones tested showed significant increases in redox potential with decreasing ε, as predicted by the Born/Nernst model.

Ionic radii for the solvated Cu^{2+} and H^+ ions were larger in protic than in aprotic solvents, probably due to hydrogen bonding interactions. This effect was not observed for the protonated semiquinone cation ($^{\cdot}QH_2^+$), which as a large hydrophobic ion may not hydrogen bond effectively. Below $\varepsilon \approx 50$-60, specific non-electrostatic solvation effects not included in the Born equation predominate. These results show that for biological quinone redox reactions, both bulk solvent dielectric and specific non-electrostatic solvation interactions (with protein) are important to consider.

W-Pos384

THE GOUY-CHAPMAN MODEL OF THE DIFFUSABLE DOUBLE LAYER IS ADEQUATE TO DESCRIBE THE ELECTROSTATIC PROPERTIES OF THE SOLUTION-MEMBRANE INTERFACE IN PHOTOSYNTHETIC SYSTEMS. A.Corazza, A.B.Melandri, G.Venturoli and R.Casadio; Dept.of Biology, University of Bologna, Bologna, Italy.

Following Electrostatics, the Nernst oxido-reduction potential of electroactive centers of membrane proteins as detected in the bulk (E') differs from the true value at the redox site, embedded in the membrane, (E_m), by a factor equal to the electrostatic potential difference at the solution-membrane interface. With this notion, determinations of E'_m of known redox centers in the photosynthetic membranes of Rdb.capsulatus were performed (by utilizing fast kinetic spectroscopy in combination with redox potentiometry) at different ionic strengths so that to modulate the extent of the surface potential. It was found that for the reaction center, the Q_A acceptor, and the cytochrome b_{561}, located at different depths within the membrane region, the dependence of E'_m on the surface potentials is linear and with the expected unitary slope, when the relation of the surface potential to the surface charge density is modeled by the Grahame equation of the double layer. The value of the surface charge density, from all the redox centers investigated, is evaluated to be 1.25 $\times 10^{-3}$ e.A^{-2} and similar on both sides of the membrane.

In spite of the roughness of the membrane surface, according to our results, the Gouy-Chapman model of the diffusable double layer is sufficient to describe electrostatic properties at the interfaces also in biological systems.

W-Pos385

RESONANCE RAMAN SPECTROSCOPY OF PHOTOINDUCED ELECTRON TRANSFER REACTIONS IN A RUTHENIUM BIS-BIPYRIDINE DICARBOXYBIPYRIDINE CYTOCHROME C (LYS 72) DERIVATIVE.

J. D. Hobbs [1], D. J. Nunez [1], R. W. Larsen [1], L. P. Pan [2], F. Millet [2] and M. R. Ondrias [1].
1. University of New Mexico, Department of Chemistry, Albuquerque, NM 87131.
3. University of Arkansas, Department of Chemistry and Biochemistry, Fayetteville, AR 72701.

We have studied the photoinduced electron transfer reactions of equine cytochrome c labeled at a single lysine residue (Lys 72) with ruthenium bis-bipyridine dicarboxybipyridine using transient and time-resolved resonance Raman spectrocopy. The Raman spectra of this derivative obtained at low laser fluences, using either Soret or Q band excitations, is identical to that reported for equine cytochrome c. At higher laser fluences, the 10 ns high frequency Raman spectra display bands indicative of photoinduced reduction of the heme iron. This effect is found to be power dependent and reversible at neutral pH. Absorption spectra obtained imediately following the Raman experiment show no evidense for irreversible heme reduction or protein degradation. In the presence of EDTA, the transient and time-resolved Raman spectra suggest irreversible heme reduction. Preliminary time-resolved Raman experiments indicate that the photo-excited Ru(II)* can be pumped using an 8 ns, 355 nm laser pulse and that subsequent heme reduction occurs within 50 ns of the excitation pulse. This work is supported by the NIH (GM33300 to MRO and GM20488 to FM).

W-Pos386

Structure of Photosystem I Reaction Center.

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Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ 07110.

Photosystem I (PS I) catalyzes the photoreduction of ferredoxin using plastocyanin as an electron donor. The complex contains two large molecular weight subunits (approximatley 80 kDa) which bind all the pigments of the complex and catalyze the primary photochemical reaction. There are also several lower molecular weight subunits (8 to 22 kDa), whose functions are only now being identified. We have embarked on a program of cloning and characterizing genes encoding these subunits from *Synechocystis* sp. PCC 6803 and then generating subunit-specific mutants. We recently cloned and sequenced the genes encoding four of the subunits of PS I. We have generated mutants lacking two of these subunits. Lack of subunit IV in the PS I of the *psaE*^{k} mutant does not affect photosynthetic activities and growth of the mutant drastically. In contrast, the absence of subunit II severely reduces photoautotrophic growth of the mutant cells. Photosystem I is assembled in both mutants. Recently we obtained several types of crystals of photosystem I. Our approach is to eliminate the lower molecular weight subunits and to crystallize the core complex. The characterization of targeted mutants and crystals of photosystem I will be presented.

W-Pos387

THE TAMPER EFFECT: ENVIRONMENTAL INFLUENCE ON ELECTRONIC INTERACTIONS IN A MODEL FOR THE PHOTOSYNTHETIC REACTION CENTER.

J.K. Delaney, M. Evans, D.C. Mauzerall; The Rockefeller University

Weak electronic interactions have been measured by fluorescence lifetime in a tetra-bridged co-planer zinc porphyrin-quinone (ZnPQ) molecule. The lifetimes were 2.4 times shorter in solvents that were both higher in electron density and constrained by their volume to remain external to the ZnPQ cage. All the solvents had similar static dielectric constants. These results are interpreted as the effect of the external solvent on the electronic overlap term, i.e. the Tamper effect. This effect can be theoretically modeled by calculating the energy splitting due to tunnelling between two potential wells. The potential barrier between the donor and acceptor is determined by the internal material. The potential barrier outside the double well is determined by the external solvent. Calculations using this model show that for a donor and acceptor with an edge to edge separation of 5 A and with a fixed internal barrier potential of 2 eV a five fold increase in rate of tunnelling can occur if the external barrier (solvent) is varied between 1 and 4 eV's. The solvent barrier may be below the internal barrier because it is of infinite extent. The range of solvent potentials considered is consistent with the range of tunnelling parameters, α, determined experimentally. NSFDMB 87-18028

W-Pos388

ELECTRON TUNNELING PATHWAYS IN PROTEINS.

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Electron transfer rates in chemical and biological systems are controlled by the electronic coupling between donor and acceptor sites, T_{DA}, and by the coupling of the tunneling electron to nuclear modes. Biological systems have tuned these rates to optimize particular tasks, such as energy conversion in photosynthesis. Recently, we developed a method to calculate the tunneling matrix elements in proteins [1]. The matrix element is dominated by interactions along a few sequences of covalent bonds, hydrogen bonds, and van der Waals contacts. Each specific sequence is called a pathway and contributes to the tunneling matrix element. Neglecting interactions between pathways, the tunneling matrix element is the sum of interactions on each of these paths. The challenge in proteins, then, is to identify chains of orbitals that define dominant pathways. We will describe three aspects of the problem: i) with the above strategy, we predict the dominant tunneling pathways in several proteins and use the results to interpret experimentally measured rates; ii) a new theoretical formalism will be described based on Green's functions techniques that permits determination of the precise role played by multiple pathways and interference between paths; iii) noncovalent contacts on the tunneling pathways can introduce temperature dependence to the tunneling matrix element. We will present preliminary results about the possibility of detecting such temperature dependences.

[1] D.N. Beratan, J.N. Onuchic, J.N. Betts, B.E. Bowler, H.B. Gray, J. Am. Chem. Soc., in press

W-Pos389

THE DISTANCE-RATE RELATIONSHIP OF INTRAMOLECULAR ELECTRON TRANSFER

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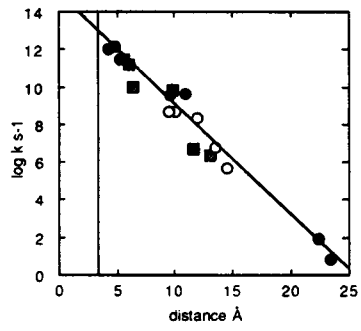
The figure shows, in a variety of intramolecular electron transfer reactions, how distance between donor and acceptor molecules relates to the rate of electron transfer. Plotted is the center to center distance between the closest atoms on donor and acceptor molecules incorporating π orbitals involved with oxidation-reduction; vanderWaals contact is at about 3.6Å. Rates are presented as the log of first order rate constant from light pulse activation. The symbol ● represents in Rp. viridis and Rb. sphaeroides reaction center protein electron transfers whose rates are measured at ΔG^0 close to reorganization energy λ. ○ represents reactions which have been adjusted for comparison to $\Delta G^0 = \lambda$ using rates obtained at other ΔG^0 values fitted to a Marcus plot incorporating multiple vibrational modes and a λ value of 600 meV (Gunner, Dutton JACS 111, 3400, 1989). ■ represents reactions in semi- or fully-synthetic systems at $\Delta G^0 = \lambda$. The figure demonstrates that the intervening medium of reaction centers is electronically relatively homogeneous for all reactions and is biologically unremarkable. The slope indicates a 10 fold rate change for a 1.7Å change in distance. The Marcus ΔG^0/rate plots demonstrate that the dominating dependence of rate on distance can be modulated up to only 2-3 orders of magnitude by ΔG^0. The extrapolated rate at vanderWaals contact of 13.1 s-1 matches nuclear vibration frequencies. This extraordinarily general description provides a ruler with which to predict intramolecular electron transfer distances in proteins of unknown structure. For example, the ruler applied to the extensive kinetic data from the PSI reaction center constructs a consistent spatial model of its redox centers and predicts there to be an intermediary redox cofactor between P700 and Chl687 (Ao).



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W-Pos390

PHOTOINDUCED ELECTRON TRANSFER IN COMPLEXES BETWEEN RUTHENIUM-CYTOCHROME c AND CYTOCHROME OXIDASE.

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A new technique has been developed to measure intra-complex electron transfer between cytochrome c and cytochrome oxidase. Ten different cytochrome c derivatives labeled at single lysine amino groups with ruthenium bis-bipyridine dicarboxybipyridine were prepared as previously described (Durham et al., Biochemistry 28 8659, 1989). Excitation of Ru(II) with a laser light pulse resulted in the formation of the excited state Ru(II*), which transferred an electron to the ferric heme group to form Fe(II) with rate constants ranging from 1×10^5 to 30×10^6 s^{-1}. Intra-complex electron transfer from the Fe(II) in cytochrome c to cytochrome oxidase could then be detected on a microsecond time scale. All of the ruthenium cytochrome c derivatives formed tight 1:1 complexes with cytochrome oxidase at low ionic strength. The rate constants for electron transfer from cytochrome c to cyt a in cytochrome oxidase were 600 s^{-1}, 600 s^{-1}, and 3,000, and 6,000 s^{-1} for the derivatives modified at lysines 13, 72, 27, and 25 at the top, left side, and right side of the heme crevice, respectively. The derivatives modified at lysines further removed from the heme crevice domain all had rate constants of 6,000 s^{-1} or greater. The rate constants for all the derivatives decreased as the ionic strength was increased and the 1:1 complex dissociated.

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W-Pos391

CONTROL OF CYTOCHROME OXIDASE ACTIVITY

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The electron transfer and proton pumping functions of cytochrome oxidase are under the control of the transmembrane electrochemical gradient. Brunori et al (1) proposed that the electrical component of the gradient controls the rate of electron transfer and the efficiency of coupling to proton translocation via a shift in the conformational state of the enzyme. We have carried out new experiments to further substantiate our hypothesis. Pre steady-state and steady-state experiments by optical spectroscopy allow to quantitate the effect of the gradient on the steady-state levels of the different cytochromes, and thus to explore the redox states of the enzyme populated during turnover. Direct experiments allow to demostrate the effect of the transmembrane gradient on the internal electron transfer from cytochrome a-CuA to the binuclear center (which we have shown to be the rate-limiting step during turnover, 2). The data are discussed with reference to a 'cubic scheme' which formally illustrates the linkage between electron transfer and proton translocation in cytochrome oxidase.

1. Brunori et al. EMBO J., 4 2365 (1985)
2. Malatesta et al. Proc. Natl. Acad. Sci. USA, 87 (1990) In press.

W-Pos392

pH-DEPENDENT AND ENERGY-LINKED CO BINDING TO CYTOCHROME OXIDASE IN INTACT MITOCHONDRIA.

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The rate of recombination of CO with fully reduced cytochrome oxidase in intact beef heart mitochondria was measured at 445nm following flash photolysis at temperatures between 180 and 230 K. A single Arrhenius slope corresponding to an apparent energy of activation (Ea) of approximately 10.5 kcal/mole is observed at pH 7.4 in the presence/absence of the uncoupler CCCP; in the absence but not the presence of CCCP, recombination rates in the presence of 100% CO are twice those in 1% CO. At pH 5.5, Ea's of 11.3 and 7.1 kcal/mole are observed above and below 210 K in 100% CO, while Ea's of 7.4 and 11.1 kcal/mole are observed above and below 210 K in 1% CO. At pH 9, Ea's of approx. 9.2 and 10.8 kcal/mole are observed in the presence of 1% and 100% CO, respectively; at warmer temperatures the rates in 100% CO are twice the rates in 1% CO. In the presence of CCCP, an Ea of approximately 10.3 kcal/mole is observed with 1% and 100% CO; the rates are the same with 1% and 100% CO. These findings suggest models where up to 3 energy barriers are encountered in the migration of CO from the suspending medium to the heme iron and where the intermediate regions can hold 1 or 2 CO, depending on pH and energy state. The data implicate a role for the undetectable copper center in ligand binding to the oxidase.

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W-Pos393

A TOPOGRAPHICAL EXAMINATION OF CYTOCHROME B IN THE INNER MEMBRANE OF YEAST MITOCHONDRIA.

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Cytochrome b is a 42kD membrane-spanning hemoprotein which functions in electron transfer from ubiquinol to cytochrome c, and in proton translocation across the inner membrane in the Complex III segment of the respiratory chain of mitochondria. Hydropathic amino acid analysis using protein sequences deduced from the coding sequences from yeast and bovine genes has permitted the proposal of two models for cytochrome b structure. Differences between the two models make indeterminate the specific histidine residues which coordinate cytochrome b-hemes, as well as the orientation of the N- and C-termini and the proposed site modified by DCCD which blocks proton translocation. The conclusive determination of cytochrome b structure and its orientation with respect to the native inner mitochondrial membrane is essential for understanding the role it plays in electron transfer and proton translocation.

Using mild digestion of intact Complex III purified from yeast, we have identified several proteolytic fragments of cytochrome b. Preliminary analysis of the molecular weights of these fragments suggests that the N-terminal region, the region bound by the first and second predicted transmembrane segments, and the region preceding the last three predicted transmembrane segments are exposed in the intact complex, and may likely be so in the liposome-reconstituted complex. Efforts are currently underway to examine the incorporation of DCCD into these fragments, so that the site of DCCD-modification can be identified. Using protease-susceptibilities of core proteins and cytochrome c1 as markers for Complex III orientation, the location of proteolytic sites in cytochrome b will be determined in a population of proteoliposomes of defined orientation.

W-Pos394

RESPIRATION OF YEAST CYTOCHROME bc_1 COMPLEX MUTANTS

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Ubiquinol cytochrome $\underline{c}$ oxidoreductase, the cytochrome $\underline{bc_1}$ complex is a mitochondrial respiratory enzyme complex common to eukaryotic energy tranducing system. This enzyme complex transfers electrons from ubiquinol to cytochrome $\underline{c}$ and is coupled with proton translocation, thus generating ATP. The yeast mitochondrial $\underline{bc_1}$ complex consists of 8 - 10 subunits and is homologous to that of the beef heart mitochondria. Recently, the sequence of the yeast gene encoding the 17 kDa protein, subunit VI of the $\underline{bc_1}$ complex, has been reported and predicted amino acid sequence of the 17 kDa protein shows extensive homology (36 %) of that of the beef heart hinge protein. The deletion of the gene encoding the 17 kDa protein resulted in reducing the ubiquinol cytochrome $\underline{c}$ reductase activity to 50 % of that of wild-type. In order to elucidate the role of the 17 kDa protein by the genetic studies, we have established a phenotype for the 17 kDa protein gene mutation, a double mutant which has a glycerol$^-$ phenotype and shows the respiratory capacity well below. It is found that efficient electron transport becomes dependent on the 17 kDa protein when the cytochrome $\underline{c}$ level is limiting, implying that the 17 kDa protein plays a role in the interaction of the $\underline{bc_1}$ complex with cytochrome $\underline{c}$. Thus, the 17 kDa protein becomes essential for cell respiration when the cytochrome $\underline{c}$ level is limiting. We have also established several double mutants in which the cytochrome $\underline{c}$ level is varying and their cell respirations are comparable. We have also deleted N-terminal acidic cluster of the 17 kDa protein, using the oligomutagenesis technique and reconstituted it into the $\underline{bc_1}$ complex lacking the 17 kDa protein to understand the structural importance of the unique acidic cluster of the 17 kDa protein (AHA and NIH supported).

W-Pos395

BINDING OF ANTIMYCIN A, MYXOTHIAZOL AND STIGMATELLIN TO THE BC_1 COMPLEX IN CHROMATOPHORES OF RHODOBACTER SPHAEROIDES GA.

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The binding stoichiometry and Kd of these inhibitors have been studied at controlled redox potentials through titrations of a) the spectral signal changes induced on the b type cytochromes and b) the inhibition of the light induced redox changes of the electron flow chain and the slow phase of the carotenoid band shift.

Antimycin (inhibitor of the Qc site) and Stigmatellin (inhibitor of the Qz site) showed saturating effects at 0.45±0.05 mole/mole cyt b high potential with a Kd in the nM range. On the contrary, Myxothiazol (inhibitor of the Qz site) bounded with a 0.9±0.1 mole/mole stoichiometry and a Kd 10-20 fold higher than the former.

These results are discussed in terms of a possible dimeric structure of the bc1 complex in situ.

W-Pos396

COMPARISON OF ENERGY-TRANSDUCING CAPABILITIES OF THE TWO-SUBUNIT CYTOCHROME $\underline{aa_3}$ FROM PARACOCCUS DENITRIFICANS AND THE 13-SUBUNIT BEEF HEART ENZYME.

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Mammalian cytochrome $\underline{aa_3}$ displays a complex redox behavior which indicates a high degree of cooperativity. Since all of the redox centers are on 2 subunits but 13 are present, the possibility exists that the complex behavior is the result of interactions with the "extra" peptides. However, we have found that the same redox behavior is present in the 2-subunit bacterial enzyme. It is possible that the redox characteristics in common for both enzymes are needed for energy transduction. Is there a correlation between the commonality of redox behavior and functionality of energy conversion? The present work shows that the 2-subunit enzyme is as fully capable as the structurally more complex enzyme in pumping protons and converting respiratory energy into a ΔpH and a $\Delta\Psi$. A correlation does exists for cooperative redox interactions and energy transduction in the minimal two subunit and the 13-subunit forms of cytochrome $\underline{aa_3}$.

W-Pos397

INTERMEMBRANE IONIC STRENGTH OF INTACT MITOCHONDRIA AFFECTS CYTOCHROME c PROXIMITY TO MEMBRANES: A RESONANCE ENERGY TRANSFER STUDY. J.D. Cortese, A.L. Voglino, and C.R. Hackenbrock, Department of Cell Biology and Anatomy, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7090
(Intro. by B.N. Chazotte).

The ionic environment of the intermembrane space (IMS) of mitochondria is important for understanding cytochrome *c* activity in electron transport and its binding to IMS-membrane proteins and lipids. Although it is known that cytochrome *c* binding to mitoplasts (outer membrane-free mitochondria) is ionic strength (*I*)-dependent, the characteristics of cytochrome *c* binding within the IMS of intact mitochondria are unknown. To answer this question, we incorporated highly functional, FITC-monoderivatized cytochrome *c* into the IMS of intact mitochondria through encapsulation of cytochrome *c* into asolectin liposomes followed by controlled low pH-induced fusion of the liposomes with the outer membranes of mitochondria. A cytochrome *c* enrichment of up to 40% (7,000 molecules per mitochondrion) was verified measuring the amount of cytochrome *c* incorporated (by SDS-PAGE) and by measurements of individual electron transfer reactions (polarographically). This enrichment makes possible the measurement of resonance energy transfer (RET) from IMS-entrapped FITC-cytochrome *c* to octadecylrhodamine B (R_{18}) incorporated in both mitochondrial membranes. Spectroscopic parameters of the RET couple were measured, and the proximity of cytochrome *c* to IMS-membranes was calculated using various values for the orientation factor (κ^2). As a standard for RET studies on intact mitochondria, we measured the proximity of FITC-cytochrome *c* to isolated inner and outer mitochondrial membranes. RET measurements in intact condensed mitochondria showed that the average distance between functional cytochrome *c* and the two IMS-membrane surfaces increased with increasing IMS-*I*. The data reveals that cytochrome *c* diffuses in 3 dimensions in the IMS of intact mitochondria at physiological *I* (i.e., 100-150 mM).
Supported by NIH GM-28704.

W-Pos398

REDOX POTENTIAL ASSIGNMENT FOR THE FOUR HEME GROUPS OF CYTOCHROME c_3 FROM DESULFOVIBRIO VULGARIS. Hideo Akutsu, Jo H. Hazzard, Robert G. Bartsch and Michael A. Cusanovich. Dept. of Biochemistry, University of Arizona, Tucson, AZ 85721

The reduction of the tetraheme cytochrome c_3 from Dv. Miyazaki and Dv. Hildenborough by reduced flavins generated by laser flash photolysis follows a monophasic kinetic profile although the hemes do not have equivalent redox potentials. Rate constants for reduction of the individual hemes are obtained upon incremental heme reduction by phototitration. The heme which is most exposed to solvent is assigned the lowest redox potential. The ionic strength dependence of the apparent second-order rate constant in the fully oxidized state for the electron transfer from reduced methylviologen does not exhibit saturation at high ionic strength (1 M), unlike all previously examined cytochromes. This unusual behavior is confirmed in the ionic strength dependence for reduction by a neutral flavin, which shows a decrease in reactivity of the high potential heme and an increase in reactivity of the low potential heme at 500 mM ionic strength. Possible changes in the environment of the low potential heme at high ionic strength are suggested with reference to the low ionic strength crystal structure.

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W-Pos399

COMPARISON OF THE REACTIVITIES OF c-TYPE CYTOCHROMES FROM YEAST, HORSE, AND RHODOBACTER CAPSULATUS WITH CYTOCHROME c PEROXIDASE COMPOUND I.

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The effect of ionic strength on the kinetics of reduction of yeast cytochrome c peroxidase Compound I (CcP) with c-type cytochromes from yeast (iso-1), horse, and R. capsulatus has been determined. Although the maximum values for the limiting first order rate constant for intracomplex electron transfer from the cytochromes to CcP were comparable, marked differences in the ionic strength dependencies were observed. Thus, maximal values of k_{et} for the R. capsulatus, horse and yeast cytochromes occurred at I = 30, 70, and 260 mM, respectively. In all cases, k_{et} values decreased at ionic strengths above and below the optimum. The data are consistent with a model in which electrostatic forces act as a "coarse adjustment" which allows the reacting proteins to assume an approximately correct mutual orientation for electron transfer. Optimization is achieved by subsequent dynamic motions of the proteins relative to one another, which are inhibited by the stronger electrostatic interactions at low ionic strengths. The different ionic strength dependencies probably reflect differences in the relative contributions of electrostatics, hydrophobic interactions, hydrogen bonding, steric effects, etc. to the achievement of an optimum orientation between the various cytochromes and CcP within the intermediate complex. Work supported by NIH grant DK15057 (to G.T.) and USPHS grant GM21277 (to M.A.C.).

W-Pos400

CRYSTALLIZATION OF MITOCHONDRIAL UBIQUINOL—CYTOCHROME *C* REDUCTASE. Chang—An Yu, Wen—Hai Yue, and Linda Yu, Department of Biochemistry, OAES, Oklahoma State University, Stillwater, OK 74078

Ubiquinol-cytochrome c reductase of beef heart mitochondria was crystallized in the presence of decanoyl—N—methyl—glucamide, heptanetriol, and sodium chloride using polyethylene glycol as precipitant. Crystallization was carried out by vapor diffusion technique using Pastuer pipets or test tubes. The crystallizing apparatus was placed in a constant temperature, shock—free environment. Crystals form within two to four weeks. In Pastuer pipets, after incubation most of crystals first appear as thin octagonal plates that grow into an ellipsoid—shape (Fig. 1a). A crystal as large as 4 x 4 x 1 mm. was obtained. Crystals grown in the test tube appear as red thin plates (Fig. 1b). The dimensions of the thin square crystals are approximately 0.4 x 0.4 x 0.1 mm. The crystalline enzyme is composed of ten subunits. The smallest protein (11th) subunit observed in the purified complex is apparently not a part of this complex. The crytalline complex contains 2.5 nmol ubiquinone and 140 nmol phospholipid per mg protein. Of the latter 36 % is with diphosphatidyl—glycerol. The crystals are very stable in the cold and show full enzymatic activity when redissolved in aqueous solution. Absorption spectra of the redissolved crystals show a Soret to UV ratio of 0.88 and 1.01 in the oxidized and the reduced forms, respectively. The ratio of cytochromes *b* to c_1 is 2. Supported in part by a grant from NIH (GM 30721).

(a) (b)

Fig.1. Crystals of ubiquinol—cytochrome *c* reductase—— (a). Crystals formed in a Pastuer pipet. (b). Crystals formed in a test tube.

W-Pos401

CRYSTALLIZATION OF THE LUMEN-SIDE DOMAIN OF TURNIP CHLOROPLAST CYTOCHROME *f*. S.E. Martinez, A. Szczepaniak, J.L. Smith, and W.A. Cramer, Dept. of Biological Sciences, Purdue University, West Lafayette, IN 47907

Cytochrome *f* was purified from turnip leaf chloroplasts as a monodisperse non-aggregating preparation, with an M_r on SDS-PAGE approximately 2,800 smaller than in the thylakoid membrane. In this respect, the cytochrome resembled those purified by Gray from turnip and other *Cruciferae* [Eur. J. Biochem. *82*, 133-141 (1978)]. The N-terminus (6 residues) was identical to the spinach cytochrome *f*. Preliminary C-terminal sequencing data indicate that the turnip cytochrome was cleaved after residue 251 or 252 (spinach sequence) near the lumen-side interface. The initial sequence data indicate that the lability of the turnip cytochrome *f* might be ascribed to an altered residue at position 252 after a conserved Leu-Arg-Val sequence at positions 249-251. Two crystal forms have been obtained. The first (space group $P4_222$, a = b = 143 Å, c = 253 Å) is of poor diffracting quality. The second crystal form diffracts to at least 2.2 Å and appears suitable for a high resolution structure determination. The space group is orthorhombic $P2_12_12_1$, with a = 81.9 Å, b = 79.4 Å, and c = 46.2 Å. There is one monomer in the asymmetric unit. [Supported by a grant from the Lucille P. Markey Charitable Trust (JLS) and NIH GM-38323 (WAC)]

W-Pos402

TRANSMEMBRANE ΔpH HETEROGENEITY IN CYTOCHROME-OXIDASE PROTEOLIPOSOMES MEASURED WITH PAIRS OF OPTICAL PROBES. P. Butko and P. Nicholls, Dept. Biol. Sci., Brock University, St. Catharines, Ontario L2S 3A1, Canada.

The turnover of cytochrome oxidase reconstituted in vesicles (COV) results in a pH gradient (ΔpH) across the lipid membrane, alkaline inside when the cytochrome c binding side faces out. ΔpH in turn exerts respiratory control over the enzyme turnover. The average bulk ΔpH in a COV population is not necessarily the local ΔpH close to an enzyme molecule in a single vesicle. We devised two types of system employing pairs of optical probes to reveal inhomogeneities in ΔpH, if they exist: (a) hydrophilic pyranine to monitor bulk pH inside COV, plus hydrophobic 4-heptadecyl-7-hydroxycoumarin (HOC) to monitor pH at the internal surface of the COV membrane. Although a surface potential dependent pH gradient from internal surface to the bulk interior phase exists, no flux-dependent steady-state gradient could be detected. Spatial pH heterogeneity inside COV is probably purely thermodynamic in nature; (b) two probes with different pK values (pyranine + phenol red, pyranine + carboxyfluorescein, or pyranine + HOC) to detect heterogeneity in transmembrane pH values among different COV. Although a few vesicles without inlaid oxidase show no active ΔpH, and the average such ΔpH is thus slightly higher than the calculated from passive gradients, substantial ΔpH heterogeneity among active COV seems not to occur. The significance of these results for our understanding of cytochrome oxidase performance will be discussed.
Supported by Canada NSERC grant #A-0421 to PN.

W-Pos402A

THE RHODOSPIRILLUM RUBRUM CYTOCHROME bc_1 COMPLEX: REDOX PROPERTIES, INHIBITOR SENSITIVITY AND PROTON PUMPING

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A detergent-solubilized, three subunit-containing cytochrome bc_1 complex, isolated from the photosynthetic bacterium R. rubrum has been shown to be highly sensitive to stigmatellin, myxothiazol, antimycin A , UHDBT and the concentration dependence of inhibition has been determined. Antimycin A band shift in the α- band maximum of reduced cytochrome b_H has been observed with the detergent-solubilized R. rubrum complex and stigmatellin has been shown to alter both the E_m values for all the electron-carrying prosthetic groups determined by oxidation-reduction titrations and EPR g-values of the Rieske iron-sulfur protein in the complex. The solubilized cytochrome bc_1 complex has been shown to be capable of coupling electron flow to the translocation of protons across a membrane after incorporation into liposomes.

W-Pos403

IMMUNOHISTOCHEMICAL LOCALIZATION OF INOSITOL TRISPHOSPHATE RECEPTOR IN BRAIN AND SPINAL CORD. A.H. Sharp, C.A. Ross, T.M. Dawson and S.H. Snyder. Department of Neuroscience, The Johns Hopkins University School of Medicine, Baltimore, MD 21205

The inositol trisphosphate receptor (IP3 receptor) is a 260 kDa membrane protein that functions to release Ca2+ from intracellular stores when phosphoinositde hydrolysis is stimulated. IP3 receptors are highly enriched in cerebellum and were originally purified from this source. Previous studies in our laboratory have localized the IP3 receptor to the endoplasmic reticulum of cerebellar Purkinje cells. We have recently developed affinty purified polyclonal antibodies against the purified rat cerebellar IP3 receptor that react with a 260 kDa protein in forebrain and a variety of peripheral tissues. Using these antibodies we have now localized the IP3 receptor in the rat forebrain and spinal cord by an immunoperoxidase method. Labeling in the brain was most intense in cerebellar Purkinje cells consistent with previous studies. In the forebrain, staining was observed in the hippocampus, cortex, and striatum as well as in various nuclei of the basal forebrain, thalamus, hypothalamus and brainstem and the gray matter of the spinal cord. Interestingly, we observed intense staining in the subcommisural organ and the area postrema. These results and their comparison with results of in situ hybridization studies are allowing us to determine neuronal pathways which may utilize IP3 as a second messenger.

W-Pos404

PURIFIED IP_3 RECEPTOR FROM SMOOTH MUSCLE FORMS AN IP_3 GATED AND HEPARIN SENSITIVE Ca^{2+} CHANNEL IN PLANAR BILAYERS

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The IP_3 receptor of aortic smooth muscle, purified to near homogeneity, was incorporated into vesicle derived planar bilayers at about 230 receptors (homotetramers) per bilayer. The receptor forms channels which are gated by IP_3 (0.5 μM) and are permeable to Ca^{2+}. The channel open probability increases with applied voltage with an onset at 50 mV driving force. At higher voltages, IP_3 elicited multi-channel currents, which were stable with time and could be completely blocked by heparin (25 μg/ml). In the absence of ATP, IP_3 induced currents were 2 to 5 times lower than in the presence of ATP (50 μM). At driving forces less than 50 mV, IP_3 induced single current events, which consistently exhibited several subconductance levels, are characterized by voltage independent conductance values with the same reversal potential. Conductance values of the main population levels are 32 ± 3, 16 ± 2, and 8 ± 2 pS. This multiplicity of conductances in single events is likely to reflect the interaction of the four subunits of the IP_3 receptor. Recovery of activity was estimated to be 28% based on a conservative estimate for the unknown open probability of the channel. Thus, the observed functional properties are a characteristic of the IP_3 receptor homotetramer and channel function has been conserved during purification and reconstitution. [Supported by Austrian Research Fonds S45-07 (H.S.) and NIH HL 32711 (S.F.)]

W-Pos405

CHARACTERIZATION OF AN INOSITOL (1,3,4,5)-TETRAKISPHOSPHATE RECEPTOR IN BRAIN MEMBRANES

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Activation of the phosphoinositide hydrolysis pathway leads to the formation of inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3], which mediates intracellular Ca^{2+} release. Ins(1,4,5)P_3 can be phosphorylated to inositol 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)P_4], but the role of the latter molecule is not yet known, although it is hypothesized that Ins(1,3,4,5)P_4 also has a role in cellular calcium handling. We have identified the presence of an Ins(1,3,4,5)P_4 receptor in rabbit and bovine brain membranes. [^{3}H]Ins(1,3,4,5)P_4 binding was routinely assayed for 30 min at 0°C in the following solution: 4 nM [^{3}H]Ins(1,3,4,5)P_4, 1 mM EDTA, 25 mM KH_2PO_4, 25 mM Na-acetate (pH 5.1). Bound ligand was measured after separation from unbound ligand by filtration or ultracentrifugation. Non-specific Ins(1,3,4,5)P_4 binding was measured in the presence of a 1000-fold excess of unlabeled Ins(1,3,4,5)P_4. Ins(1,3,4,5)P_4 binding is maximal when assayed at acid pH (5.0-6.0). In crude membrane preparations, 60 to 75% of the total activity represents specific binding. Specific [^{3}H]Ins(1,3,4,5)P_4 binding is enriched in membranes isolated from hippocampus, neocortex and cerebellum (20-30 fmol/mg protein), as compared to other brain parts or spinal cord. This pattern indicates a clearly different tissue localization than that of the Ins(1,4,5)P_3 receptor. [^{3}H]Ins(1,3,4,5)P_4 binding to bovine cerebellar membranes is inhibited 50% by 20 nM unlabeled Ins(1,3,4,5)P_4. The IC_{50} for Ins(1,3,4,6)P_4, Ins(1,4,5)P_3 and Ins(1,2,3,4,5,6)P_6 are 2 μM, 3 μM and > 10 μM, respectively. Two different affinity sites for Ins(1,3,4,5)P_4 are determined, a high-affinity site (K_d 3.5 nM, B_{max} 27 fmol/mg) and a low-affinity site (K_d 93 nM, B_{max} 377 fmol/mg). Approximately 60% of the binding activity can be solubilized with 2.5% CHAPS in the presence of 10 mg/ml phosphatidylcholine. Partial purification was obtained using heparin-agarose column chromatography. The partially purified Ins(1,3,4,5)P_4 receptor can be specifically precipitated using lectin-coupled beads (*e.g.* wheat germ agglutinin Sepharose) suggesting that the receptor is a glycoprotein. The solubilized Ins(1,3,4,5)P_4 receptor migrates slower in a sucrose density gradient than the solubilized Ins(1,4,5)P_3 receptor, again indicating that both receptors are separate functional entities. Purification of the Ins(1,3,4,5)P_4 receptor, which is currently underway in our laboratory, should help ascertain the functional role of the Ins(1,3,4,5)P_4 receptor in neuronal tissue.

K.P. Campbell is an Investigator of the Howard Hughes Medical Institute.

W-Pos406

PURIFICATION AND CHARACTERIZATION OF A RECEPTOR FROM CEREBELLUM WHICH BINDS INOSITOL 1,4,5-TRIPHOSPHATE (IP_3) AND INOSITOL 1,3,4,5-TETRAPHOSPHATE (IP_4).

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Cerebellum microsomes were extracted using CHAPS and the receptor purified by column chromatography. The purified receptor, which we refer to as the IP_3/IP_4 receptor, is capable of binding IP_3 (Kd = 0.7 μM, B_{max} = 1.8 nmol per mg of protein) and IP_4 (Kd = 0.9 μM, B_{max} = 2.8 nmol per mg of protein). As determined by SDS PAGE, the purified receptor consists of three polypeptides with M_r ~ 116,000, 106,000, and 50,000, which are present in the approximate ratio of 1:4:4. Electron microscopy reveals a particle of about 12 nm diameter. IP_3 inhibits IP_4 binding (K_i = 1 μM), and IP_4 inhibits IP_3 binding (K_i = 1 μM). Inositol 1-phosphate and inositol 1,4-diphosphate do not effectively compete for IP_4 binding. Binding of both IP_3 and IP_4 is inhibited by ATP (K_i ~ 100 μM), GTP (K_i ~ 100 μM), and heparin (K_i ~ 0.5 μg/ml). Micromolar concentrations of calcium do not inhibit binding of either IP_3 or IP_4. The role of the IP_3/IP_4 receptor in intracellular signalling is being investigated.

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W-Pos407

CHARACTERIZATION OF AN INOSITOL-1,4,5-TRISPHOSPHATE (IP_3) RECEPTOR IN ISOLATED OLFACTORY CILIA. D. Lynn Kalinoski, Scott Aldinger, Ardithanne Boyle, Taufiqul Huque & Diego Restrepo (Monell Chemical Senses Center, Phila., PA).

A previous study has demonstrated that olfactory stimuli (amino acids) elicit G-protein-linked stimulation of phosphoinositide turnover resulting in the elevation of intracellular IP_3 in olfactory cilia from the channel catfish (Ictalurus punctatus) (Huque & Bruch, BBRC 137:36,1986). Additionally, we have recently identified an IP_3-gated calcium channel, which can mediate plasma membrane depolarization, in catfish olfactory cilia (Restrepo et al Science 249:1166, 1990). Studies of specific IP_3 binding to microsomal membranes have characterized a related channel which mediates the release of calcium from internal stores closely associated with the endoplasmic reticulum (Ferris et al Nature 342:87, 1989; Spat et al Nature 319:514, 1986; & Worley et al JBC 262:12132). In studies with ^3H-IP_3, we find that IP_3 binding to ciliary membranes is specific and saturable with a K_d of 1.29 ± 0.37 μM and an estimated number of binding sites of 24 ± 10 pmol/mg protein (mean ± SEM, n = 12). Specific binding increases as pH is made alkaline and increases as a function of the calcium concentration in the medium. The rank order for potency of inhibition of ^3H-IP_3 binding is 1,4-IP_2<1,3,4-IP_3<1,3,4,5-IP_4=1,4,5-IP_3<2,4,5-IP_3. Heparin inhibited the binding of IP_3 in a dose-dependent manner with an EC_{50} of 6.7 ± 1.6 μg/ml. The biochemically determined K_d and inhibition of ^3H-IP_3 binding by related inositol phosphates are consistent with the ability of micromolar concentrations of 1,4,5-IP_3 (but not 1,3,4-IP_3) to activate the IP_3-gated calcium currents in whole cell patch clamp and the IP_3-gated calcium channel detected in cilia plasma membrane vesicles fused onto phospholipid bilayers. These results suggest that the specific binding of IP_3 to cilia membranes may represent binding to IP_3-gated calcium channels in catfish olfactory cilia.
Supported by NIH grants DC-00566, DC-00327, DC-00356, BRSG SO7RR05825 and a grant from the Veterans Administration to Dr. J.G. Brand.

W-Pos408

DI-8-ANEPPS FLUORESCENCE IMAGING OF MEMBRANE POTENTIAL CHANGES IN INDIVIDUAL EGF-STIMULATED A431 CELLS: NOVEL KINETICS AND CALCIUM DEPENDENCE
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We have used the fluorescent charge-shift dye di-8-ANEPPS as an indicator of plasma membrane potential (V_m) in the cultured human epidermoid carcinoma cell line A431. We made use of the spectral response properties of the dye to generate ratiometric images of the membrane electric field strength in these cells. Subconfluent cells were incubated with 2.5μM di-8-ANEPPS for 10 minutes in the presence of 0.05% *w/w* Pluronic F-127, a nonionic surfactant. The cells were subsequently washed and examined in a fluorescence microscope. Using a charge coupled device camera, 436 and 546nm excitation time-lapse image pairs were taken and processed as 436nm/546nm ratio images. Upon challenge with 100ng/ml epidermal growth factor (EGF), most A431 cells hyperpolarize on the order of 40 mV, followed by a return to initial values. This process occurs over times of approx. 100 seconds. These changes are indicated by a decrease in fluorescence ratio followed by a return to the initial levels. Removal of extracellular calcium results in a decrease in the magnitude of hyperpolarization. In many of the cells studied, the initial hyperpolarization is followed by a transient depolarization of approx. 20mV. This transient depolarization is not abolished by the removal of extracellular calcium. V_m changes were calibrated using buffers of various [K+] and 3μM valinomycin. The implications of these unusual responses to EGF will be discussed.
Supported by NSF grant DMB-8803826.

W-Pos409

CROSSLINKING INDUCES CYTOSKELETAL ASSOCIATION OF IgE-RECEPTOR COMPLEXES IN TRANSFECTED CELLS
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Crosslinking of IgE-receptor complexes on mast cells by multivalent ligands activates a signal transduction pathway that leads to degranulation. Crosslinked IgE-receptor complexes become associated with the Triton X-100 insoluble cytoskeleton. We used site-directed mutagenesis to truncate each of the five cytoplasmic domains of the tetrameric rat IgE receptor. Cytoskeletal association of the mutant receptors expressed in transfected mastcytoma P815 cells was assessed by staining cells with fluorescein conjugated anti-DNP IgE and examining the capacity of detergent to diminish particle associated fluorescence using flow cytometry. Native transfected receptors and all but one of the mutants became associated with the cytoskeleton when crosslinked by multivalent antigen DNP-BSA. Prior to crosslinking, greater than 99% of IgE-receptor complexes could be solubilized by Triton X-100. After crosslinking, 60% of these molecules associated with the cytoskeleton as evidenced by their detergent insolubility. Truncation of the C-terminal domain of the β chain, created a defect in cytoskeletal association, as only 20% of the mutant receptors became detergent insoluble, suggesting that this domain may be important in this event. IgE receptors expressed in transiently transfected COS cells also demonstrate similar detergent insolubility induced by DNP-BSA.

W-Pos410

DISTRIBUTION OF FC_ε-RECEPTORS ON THE SURFACE OF SINGLE LIVING RAT MAST CELLS (RBL-2H3 LINE): AN ENERGY TRANSFER STUDY
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We have examined the aggregation state of FcεRI on the surface of single living mast cells (RBL-2H3) by resonance energy transfer measurements. By binding to these cells fluorescein and tetramethyl rhodamin labeled FcεRI-specific ligands (IgE or anti-FcεRI-Fab, H10-Fab) we produced two distinct populations of receptors on each cell and measured the energy transfer efficiency between them. Photobleaching kinetics of the donor fluorophor yielded the efficiency of energy transfer between the ligands carrying distinct fluorophors on single cells in a microscope. The data were further analysed using a theoretical model of energy transfer efficiencies as a function of 1) the size of the FcεRI bound ligands, i.e. IgE or H10-Fab, 2) the surface density of FcεRI on the cell, 3) the number of acceptor fluorophors per ligand molecule and 4) the percentage of receptors incorporated into dimers. The model evaluates the energy transfer efficiency between the ligands on the cell surface numerically and considers their distribution by their pair distribution function. Our results clearly suggest the FcεRI are primarily monovalent, randomly distributed plasma membrane proteins. The method used in this study provides a new approach for determination of the aggregation state of cell surface components.

W-Pos411

STABLE EXPRESSION OF FUNCTIONAL FETAL AND ADULT MUSCLE ACETYLCHOLINE RECEPTORS IN FIBROBLASTS.

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Quail fibroblast cells (QT-6) were transfected with the four subunit cDNAs encoding the fetal (α, β, γ, δ) or adult (α, β, ε, δ) type mouse muscle acetylcholine receptor. (P. Blount and J. P., Merlie, Neuron 3:349, 1989.) One stable clone of each type (Q-F18, fetal and Q-A33, adult) was selected for further study on the basis of high expression of surface receptors (5,000 to 10,000 per cell). Sodium butyrate treatment enhanced surface receptor expression up to 8 fold. SDS-PAGE of immunoprecipitated surface receptors resolved all four subunits of Q-F18 and at least three subunits (α, β, δ) of Q-A33. Q-F18 displayed channel properties similar to those of BC3H-1, a mouse myoblast-like cell line, with a slope conductance of approximately 40 pS and a burst duration of 5-10 msec. The larger conductance (about 60 pS) and briefer burst duration (1-5 msec) of Q-A33 channels are characteristic of channels in dissociated adult muscle fibers. The metabolic half-lives of Q-F18 and Q-A33 receptors were approximately eight and nine hours respectively, comparable to the approximate eight hour half-life of BC3H-1 receptors. (J. Patrick, J. McMillan, H. Wolfson and J. O'Brien, J. Biol. Chem. 252:2143, 1977.) The Q-F18 and Q-A33 cell lines provide a model system for biochemical and physiological comparison of fetal and adult type receptor/channel properties.

W-Pos412

LOW MOLECULAR WEIGHT mRNA ENCODES A PROTEIN(S) THAT SUPPRESSES THE ACETYLCHOLINE M_1 RESPONSE IN *XENOPUS* OOCYTES.

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Serotonin and acetylcholine increase the chloride conductance of *Xenopus* oocytes injected with transcipts of the rat M_1 and mouse 5-HT_{1c} receptors. Receptors activate the phosphoinositidase C (PIC) pathway through a G protein. IP_3 hydrolyzed from PIP_2 releases Ca^{++} from intracellular stores, activating Cl^- channels. Facilitation of both receptor responses occurs when 15 s exposures to agonist are repeated at 2 min intervals. We have shown that a low molecular weight fraction of rat brain mRNA (0.3-1.5 kb) encodes a protein(s) that desensitizes the second 5-HT_{1c} response (Walter et al. 1990. Biophys. J. 57:125a). We now show that unlike the first 5-HT_{1c} response, the first M_1 response is suppressed > 90% by a protein(s) encoded by 0.3-1.5 kb mRNA. It is unlikely that this suppression is the result of poor translation by these oocytes for three reasons: 1) the first 5-HT_{1c} response was robust 2) desensitization of the second 5-HT_{1c} response occurred 3) M_1 responses were evoked when the concentration of ACh was increased from 5 X 10^{-8} M to 10^{-6} M. Injection of tRNA from *E. coli* did not result in suppression, thus indicating that a nonspecific effect of low molecular weight RNA was not responsible for suppression. Because the size range of messages encoding the desensitizing and suppressing protein(s) is the same and the two phenomena are similar, it is possible that the same protein is responsible for both effects.

Assuming the IP_3 produced by the activation of either receptors is capable of activating the same calcium stores, suppression of the M_1 response must occur prior to the IP_3 receptor. Therefore, PIC, G protein and receptor are all potential components for modulation by the suppressing protein(s). Noting suppression induced by protein(s) encoded by (0.3-1.5 kb) mRNA resembles that caused by injection of βγ subunits of G protein (Moriarty et al. 1988. Proc. Natl. Acad. Sci. 85:8865), we tested the recently cloned γ_3 (1.0 kb) G protein subunit (Gautam et al., in press). Although our data indicate that $G\gamma_3$ does not suppress the M_1 response, we can not rule out the possibility that other Gγ subunits are responsible. Support: American Heart Association, NIH (NS-11756, GM-10991) and The Markey Trust.

W-Pos413

EXPRESSION AND PROPERTIES OF ϒ-LESS NICOTINIC ACETYLCHOLINE RECEPTOR IN XENOPUS OOCYTES.

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We have expressed nicotinic acetylcholine receptor (nAChR) from BC3H-1 cells by in vitro-synthetized RNA injection in Xenopus oocytes. Acetylcholine (ACh) induced current in oocytes injected with α, ß, ϒ and δ subunit mRNA. However, reliable expression could be obtained when the ϒ, but not the α, ß or δ, mRNA was omitted. This ϒ-less receptor shared many of the properties of the normal nAChR: identical Hill slope, elementary conductance, open time, voltage dependence of the open time, and reversal potential for Na^+, K^+, Cs^+, Li^+, and Tris ions. This suggest that the overall pentameric structure is preserved when the τ subunit is missing, probably due to the replacement of τ by another subunit. However, the open-channel blocker, QX-222, inhibited the ACh-induced current less effectively on the ϒ-less channel than on the normal nAChR. This effect was mainly due to a change in the rate constant of dissociation, the forward-rate constant being similar for both receptors. Thus, the replacing subunit should have a similar patern of peripheral charged amino-acid to preserve the conductance (Imoto et al., 1988), but distinct intrachannel residues to account for the difference in QX-222 binding (Leonard et al. 1988, Charnet et Al., 199ø). The use of the cloned Xenopus ϒ subunit as well as the use of the mutated subunits we have already analyzed on the normal nAChR may enable us to determine the exact stoichiometry of this receptor. This channel is also expressed when α, ß, δ and ε subunits mRNA are injected in oocytes, in conjunction with the normal adult-type nAChR, suggesting that it can play a significant role at the adult endplate. Supported by NS-11756, MDA and FRM.

W-Pos414

REAL-TIME DETECTION OF AGONIST-INDUCED ACETYLCHOLINE RECEPTOR (AChR) ACTIVATION IN TE671 CELLS WITH A SILICON-BASED BIOSENSOR.

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A silicon-based biosensor (microphysiometer) has been previously described (Parce *et al.*, Science 246, 243 (1989)) in which the metabolic activity of living cells can be monitored by measurement of their rates of excretion of acidic metabolites in a micro flow-chamber. It was found that the rate at which the cells acidified their milieu was changed when agents known to affect their physiology were added to their environment. In the present study we have used the microphysiometer to measure changes in acid excretion rates of cells of the medulloblastoma cell line TE671, which expresses a muscle nicotinic AChR, when they have been exposed to agonists and antagonists. The cells respond to agonist (carbachol) in a dose-dependent manner, and this response is blocked by curare. At higher levels of agonist the response is partly transient in nature, indicating a form of desensitization. We also found that the response was enhanced when the cells had been previously exposed to a high nicotine concentration (10 mM) for 2 days, in agreement with biochemical and tracer studies indicating up-regulation of the receptor under such conditions. Interestingly, the kinetics of the response show at least two phases of metabolic activation, one fast and one considerably slower, indicating that activation of the nAChR in this cell line may initiate intracellular changes that last considerably longer than the opening of the receptor, and which might therefore be undetectable using normal electrophysiological methods. Supported in part by DARPA and CRDEC, ARO contract DAAL03-86-C-0009.

W-Pos415

ARE POLYSIALOGANGLIOSIDES RECEPTORS FOR TETANUS TOXIN? A STUDY WITH LIPID MONOLAYERS.

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Lipid monolayers of different compositions were used to study the interaction of tetanus toxin with membrane lipids and to evaluate the role of polysialogangliosides as membrane receptors. The interaction of the toxin with the lipid monolayers was monitored both by measuring the increase in surface pressure with a Wilhelmy plate and by measuring the surface radioactivity with a gas-flow detector.

At neutral pH, the toxin binds to dioleoylphosphatidylcholine monolayers and inserts into the phospholipid layer. This effect is potentiated by acidic phospholipids without an apparent preference for a single class of phospholipids. Polysialogangliosides further increase the fixation and penetration of tetanus toxin in lipid monolayers, but no specific requirement for a particular ganglioside was identified. The ganglioside effect is abolished in the presence of other nervous tissue lipids such as sulphatides and cerebrosides.

The penetration of tetanus toxin in the lipid monolayer is pH dependent. It increases with lowering pH, it is facilitated by acidic lipids and it is mediated both by hydrophobic and electrostatic interactions.

The present results do not support the proposed role of polysialogangliosides as receptors for the toxin on the neuronal cell surface.

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W-Pos416

BINDING AND MOBILITY OF ANTI-DINITROPHENYL MONOCLONAL ANTIBODIES ON FLUID-LIKE, LANGMUIR-BLODGETT PHOSPHOLIPID MONOLAYERS CONTAINING DINITROPHENYL-CONJUGATED PHOSPHOLIPIDS.

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The association of a fluorescently labelled mouse IgG1 anti-dinitrophenyl (DNP) monoclonal antibody (ANO2) with Langmuir-Blodgett monolayers composed of three different binary mixtures of phosphatidylcholine (PC) and DNP-conjugated phosphatidylethanolamine (DNP-PE) has been characterized. Quantitative fluorescence microscopy measurements demonstrated that measurable amounts of ANO2 bound to the monolayers only at high molar fractions of DNP-PE (≥ 5 mol%). Fluorescence pattern photobleaching recovery measurements showed that the apparent translational diffusion coefficients and mobile fractions of a fluorescent lipid were high for all monolayer compositions; and that the ANO2 translational mobility was measurable but slow and depended on the two-dimensional antibody density. These results demonstrate that the ANO2-binding characteristics of Langmuir-Blodgett monolayers consisting of binary mixtures of PC and DNP-PE are substantially different from those of similar haptenated monolayers but that the ANO2, when bound, displays diffusive behavior with characteristics similar to that on other monolayers.

This work was supported by NIH grant GM-31745 and NSF grant DBC-8552986.

W-Pos417

MODULATION OF ADENYLYL CYCLASE ACTIVITY IN DETERGENT-PERMEABILIZED CHICK CARDIAC MYOCYTES BY A_1 ADENOSINE RECEPTOR AGONISTS AND ANTAGONISTS. Hui Ma and R. D. Green (Intro. by P. de Lanerolle) Dept. of Pharmacology, Univ. of Illinois at Chicago, Chicago, IL.

There is now abundant evidence that A_1 adenosine receptors (AdoRs) couple to G-proteins in the absence of an agonist. Work from our laboratory has suggested that AdoR antagonists preferentially bind to free AdoRs and cause the dissociation of "precoupled" AdoR:G-protein complexes (Mol. Pharmacol.38:72, 1990). The possibility that "precoupled" AdoR:G-protein complexes exert an influence on an effector system has now been investigated using detergent-permeabilized cardiac myocytes. Adenylyl cyclase activity (AC) was measured using [^{32}P]ATP or [^{32}P]deoxyATP as the substrate under conditions in which all cyclic nucleotide metabolism was blocked. Adenosine deaminase increased AC when ATP but not dATP was used as the substrate; all experiments were performed in the presence of adenosine deaminase at 2 U/ml, a concentration 5-10X greater than that necessary to maximally increase AC. Under these conditions isoproterenol (ISO) increased AC 4-6 fold; R-PIA, an A_1 AdoR agonist, decreased ISO-stimulated AC by 30-60%. AdoR antagonists (CPX and BW-A844U) increased basal AC; this effect was blocked by pretreatment with IAP which itself increased basal activity. Treatment with cholera toxin (CT) which has been postulated to ADP-ribosylate receptor-coupled, IAP-sensitive G-proteins (BBRC 165:554, 1989), reduced the effect of R-PIA on ISO-stimulated AC. The effect of CT was antagonized by the simultaneous presence of CPX. These results support the hypotheses the A_1 AdoRs spontaneously couple to a G-protein and that this AdoR:G-protein complex exerts a tonic inhibitory effect on AC. (Supported by NSF Grant BNS-8719594).

W-Pos418

A MODEL OF SPARE RECEPTORS AND THEIR EFFECTER

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In the present study, I dealt with the relationship between spare receptors and effecters. An idea of the relationship is as follows: 1) Several receptors connect with an effecter. 2) If one of receptors connecting with one effecter is occupied by an agonist, the effecter works fully. 3) Even if more than one receptor connecting with the same effecter are occupied by agonists, the efficiency of the effecter does not change and is same as at the time when only one of receptors connecting with the effecter is occupied by an agonist. Under the condition, I propose a model of the relationship between the efficiency of the effecter and the occupancy of receptors by agonists. If the number of receptors connecting with one effecter is "m", the relationship between the functional effecters (E) and the concentration of agonists ([A]) is as follows:

$$E = (R_t/m)(1-(1+([A]/K_d))^{-m}),$$

where R_t is the total number of receptors and K_d is the dissociation constant of the agonist to the receptor. If m is one, the equation is absolutely same as Michaelis-Menten equation. If m is larger than one, the apparent saturation in the efficiency of effecters becomes larger; i.e., the concentration of agonists inducing the half maximum effect becomes low as m becomes larger. Further, the dissociation of the fractional efficiency of effecters from the fractional binding of agonists to receptors becomes larger as m becomes larger.

W-Pos419

MOBILITY OF GLYCOPHORIN-A IN RED CELL MEMBRANES: STIFLED BY ANTIBODY BINDING. D. Knowles; M. Narla†; and E. Evans. Pathology and Physics, University of British Columbia, Vancouver, B.C.; †Cell and Molecular Biology, Lawrence Berkeley Lab., Berkeley, California.

With observations of fluorescence recovery after photobleaching, lateral mobilities of glycophorin-A molecules in red cell membranes were measured in relation to extracellular binding of monoclonal antibodies (10F7 & 9A3) to the protein. In one set of tests, unlabelled antibody was bound to native glycophorin-A in red cells labelled with eosin-5-thiosemicarbazide. Initial levels of antibody binding had little effect on the diffusivity but progressively reduced the mobile fraction. At saturation (based on measured binding isotherms), both diffusivity and mobile fraction had markedly decreased. For the other tests, fluorescenated antibody was bound to unlabelled glycophorin-A. Here, even at very low levels of binding, major reductions occurred in both diffusivity and mobile fraction. With increased binding, diffusivity remained almost constant but the mobile fraction was reduced to nearly zero. Since cross-linking of the extracellular surface by the antibody was unlikely at low levels of antibody binding, the implication is that antibody binding promotes increased interaction of the cytoplasmic domain of glycophorin-A with the cytoskeletal network of the membrane. This is consistent with mechanical deformation experiments where binding with either Fab fragments or intact antibodies produces marked rigidification of the cell surface.

W-Pos420

IDENTIFICATION OF A HIGH MOLECULAR WEIGHT GLYCOPROTEIN THAT CO-PURIFIES WITH THE 1,4, DIHYDROPYRIDINE RECEPTOR FROM CARDIAC MUSCLE.
Vita Peri, Brian J. Murphy and Balwant S. Tuana, Dept. of Pharmacology, Univ. Ottawa, Ottawa, Ontario, Canada K1H 8M5.

The dihydropyridine (DHP) receptor in cardiac membranes was photoaffinity labeled with [^{3}H]azidopine and solubilized with digitonin. The purification of the DHP receptor was followed on a DEAE ion exchange column followed by sedimentation through a linear sucrose density gradient. The DHP receptor was further purified by lectin affinity column chromatography. The polypeptide composition of the resulting receptor fraction was analysed in sodium dodecyl sulfate gel electrophoresis. A major polypeptide of Mr 380 kDa was detected in the receptor fraction after silver staining of the SDS gels. Cut and slice analysis of the gel indicated that [^{3}H]azidopine was incorporated into a polypeptide of Mr~185kDa but not the major 380 kDa polypeptide present in the fraction. Staining of nitrocellulose blots of the receptor fraction with wheat germ lectin and concanavalin A indicated that the 380 kDa polypeptide was glycosylated. Polyclonal antibodies were prepared against the 380 kDa glycoprotein and these are being used in immunohistochemical localization and functional studies on the Ca^{2+} channel. The 185 kDa polypeptide that bound [^{3}H]azidopine was a minor component in the receptor fraction and represents the α_1 subunit of the calcium channel. (Supported by a grant from the Heart and Stroke Foundation of Ontario).

W-Pos421

FUNCTIONAL EXPRESSION OF CARDIAC ATP RECEPTOR IN mRNA INJECTED OOCYTES FROM *XENOPUS LAEVIS*.
B. Giannattasio, K. B. Powers and Antonio Scarpa. (Intro. by G. Bright). Dept. of Physiology and Biophysics, Case Western Reserve University, Cleveland, OH 44106.

We expressed the ATP receptor of rat myocardial cells in *Xenopus* frog oocytes by injecting them with a preparation of poly-A enriched RNA extracted from a homogenate of rat heart ventricles.

Xenopus oocytes were injected with 50-100 ng poly-A enriched RNA and 100-150 ng aequorin per cell, either combined or in separate injections. Expression of ATP receptor was determined in single cells by measurement of aequorin luminescence in response to ATP induced Ca^{2+} influx. Alternatively we used an electrophysiological assay, by means of a two electrode voltage-clamp, in cells injected with only poly-A RNA. Substitution of ATP with GTP did not induce a response, as predicted from functional studies of cardiac receptor specificity. Ionomycin was applied following ATP stimulation as a positive control to exclude non-viable or damaged cells.

Positive response was obtained in an average of 40-55% of ionomycin responsive oocytes over days 2-7 after injection; in this case ATP response was correlated with increased activity of baseline luminescence. Cells injected with only aequorin were not responsive to ATP. In preliminary experiments nifedipine reduced both spontaneous oscillation of intracellular Ca^{2+} and ATP induced transients.

We conclude that oocytes are able to express functionally the rat heart ATP receptor and to increase intracellular Ca^{2+} in response to extracellular ATP probably by indirect activation of voltage-dependent Ca^{2+} channels.

This is the first successful step toward size fractionation of the poly-A enriched RNA and cloning of the receptor.

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W-Pos422

DISTRIBUTION AND MOBILITY OF MOUSE SPERM SURFACE β-1,4-GALACTOSYLTRANSFERASE USING MONOVALENT FLUORESCENT PROBES. Richard A. Cardullo, Christine A. McKinnon, and David E. Wolf, Worcester Foundation for Experimental Biology, Shrewsbury, MA 01545.

In mammals, succesful fertilization requires that the sperm plasma membrane overlying the head adhere to the *zona pellucida*, an acellular matrix that surrounds the egg. This adhesion event ultimately triggers the G protein regulated exocytotic event known as the acrosome reaction. A sperm surface β-1,4-galactosyltransferase (GalTase) has been suggested to act as the receptor for the *zona pellucida*. Using a bivalent antibody, sperm surface GalTase has previously been reported to be localized to the anterior portion of the head overlying the acrosome. In this study, we have used two fluorescent, monovalent probes to study the distribution and mobility of sperm surface GalTase. The synthesis and characterization of one of these probes, 2,4,6-trinitrophenyl uridine diphosphogalactose (TUG), was recently described by us (Cardullo *et al.*, 1990, Anal. Biochem., 188:305-309) and modulates its fluorescence emission properties upon binding to either soluble or cell surface GalTase under non-enzymatic conditions. In the absence of GalTase, TUG exhibits a single emission maximum at 530 nm upon excitation at 360 nm. In the presence of either soluble or membrane bound GalTase, TUG exhibits excitation maxima at 430 nm and 530 nm. Using this probe, as well as rhodamine labeled α-lactalbumin (Rh-α-lac), we found that sperm surface GalTase was localized over the entire head and only was seen overlying the acrosome upon cross-linking with a bivalent antibody against GalTase. Induction of the acrosome reaction with the calcium ionophore A23187 shwoed that all antigen from the anterior head was removed with no detectable loss in fluorescence over the posterior head. Mobility of these antigens was measured using Fluorescence Recovery After Photobleaching (FRAP). Using either TUG or Rh-α-lac, sperm surface GalTase was characterized by a diffusion coefficient of approximately 10^{-9} cm^2/sec and 60% - 70% recovery. These studies suggest that sperm surface GalTase is a mobile sperm surface protein which redistributes upon cross-linking. This finding may be significant in that recent reports have shown that cross-linking the sperm receptor for the zona pellucida is necessary for the initiation of signal transduction events leading to the acrosome reaction.

W-Pos423

DIACYLGLYCEROL SURROGATES INDUCE MEMBRANE CURRENTS IN BALANUS PHOTORECEPTORS. H. Mack Brown, J. Burnham and J. Smolley. Dept. Physiol., Univ Utah School of Med., Salt Lake City, Utah.

We reported that dimethylsulfoxide (DMSO) initiates a small inward current in Balanus photoreceptors (Biophys. J. 57:370, 1990). DMSO is known to produce a large increase in isozymes of protein kinase C (PKC). Consequently, we investigated effects of phorbol esters, potent activators of PKC, on membrane properties of the photoreceptor. Micromolar concentrations were used in the injection pipettes. The order of efficacy for producing a membrane depolarization in darkness was phorbol, 12-myristate, 13-acetate (PMA) > 1-oleoyl-2-acetylglycerol (OAG) > phorbol. Voltage clamp experiments showed that PMA injection induced an inward current proportional to the injection pulse duration. Injections interspersed between light flashes at different membrane potentials showed that the two inward currents produced had similar I-V properties. These observations coupled with the known increase in Ca_i with light suggest that the Ca/phospholipid dependent enzyme, PKC, may activate light-sensitive channels in the photoreceptor.

W-Pos424

ADAPTATIONAL CHANGES IN BALANUS PHOTORECEPTOR ASSOCIATED WITH ALTERATIONS IN Na/Ca EXCHANGE. J. Smolley and H. Mack Brown (Sponsor: J.W. Woodbury). Dept. Physiology, Univ. Utah School of Medicine, Salt Lake City, Utah, USA

In the lateral photoreceptor of Balanus, some 15% of the light induced current is carried by Ca ions. This poses a large challenge to intracellular calcium (Ca_i) homeostasis which is met in part by a Na_o dependent Na/Ca exchange. By altering the gradients of these two ions, Ca_i can be reversibly manipulated from resting levels. Those maneuvers that should slow or block Na/Ca exchange lead to a readily reversible diminution in both the peak and plateau phases of the photoresponse similar to that seen in light adaptation. The rate and extent to which this occurs varies systematically with Ca_o. On the other hand, manipulations designed to lower Ca_i retard the development of adaptational processes. Similar to the hypothesis that Ca underpins background adaptation in vertebrate photoreceptors, our findings in Balanus suggest that the adaptational state of the photoreceptor is highly dependent on changes in Ca_i and that recovery from light adaptation is governed to a large extent by Na/Ca exchange.

W-Pos425

WHOLE-CELL AND ON-CELL PATCH RECORDINGS OF THE LIGHT-SENSITIVE CONDUCTANCE IN HYPERPOLARIZING INVERTEBRATE PHOTORECEPTORS. Maria del Pilar Gomez and Enrico Nasi (Intro. by G. Waloga). Department of Physiology, Boston University School of Medicine and Marine Biological Laboratory, Woods Hole

While the majority of invertebrate eyes are composed of rhabdomeric photoreceptors that depolarize with light, in some mollusks a second retina with hyperpolarizing ciliary visual cells is also found. We have investigated the membrane current in non-rhabdomeric cells enzymatically dissociated from the eye of the flame scallop (Lima scabra) and the bay scallop (Pecten irradians). Solitary ciliary cells from Lima have a spherical soma, 15-20 μm in diameter, with a prominent bundle of fine processes up to 30 μm long. Whole-cell tight seal recording shows that light stimulation gives rise to an outward current graded with light intensity, several hunderd pA in amplitude, accompanied by an increase in membrane conductance. The photocurrent increases in magnitude with depolarization, and becomes inward if the cell is hyperpolarized to -100 mV, consistent with the notion that it is mediated by K-selective light-activated channels. Intracellular perfusion with Cs blocks outward but not inward ion movement, causing the photocurrent to become inward over a wide range of voltages. On-cell patch recordings of unitary currents were obtained from non rhabdomeric Pecten cells. Some patches displayed depolarization-activated channels in the dark, which were suppressed by photostimulation, suggesting that light caused the cell to hyperpolarize. A strong rebound of activity usually followed light termination. In other patches channels could be activated by light but not by voltage steps alone. These were outwardly directed at resting potential, and rectified markedly, with a two-fold conductance increase per 20 mV depolarization. The number of openings increased dramatically as the patch was depolarized, with little effect on the open times, indicating that the outward rectification is due at least in part to the voltage dependence of the opening rate of the light-sensitive channels.
Supported by NIH grant RO1 EY07559.

W-Pos426

CYCLIC GMP OPENS LIGHT-DEPENDENT CHANNELS IN EXCISED PATCHES OF *LIMULUS* VENTRAL PHOTORECEPTORS
J. Bacigalupo*, E. C. Johnson†, C. Vergara* & J.E. Lisman§. *Dept. Biologia. Univ. of Chile, Santiago, Chile. †Dept of Physiology, Marshall Univ, Hungtington, WV. §Biology Dept. Brandeis Univ., Waltham, MA.

Some evidence suggests that cGMP opens the light dependent channels in invertebrate photoreceptors; other evidence suggests these channels may be opened by IP_3 mediated Ca^{2+} release. To resolve this issue, we have applied these putative second messengers to excised patches of *Limulus* photoreceptor membrane and found that inside-out patches containing light-dependent channels are opened by cGMP but not Ca^{2+}. Excision was performed directly into internal solution (300 mM KCl, 2 mM $MgCl_2$, 5 mM BAPTA, $CaCl_2$ to reach a pCa of 7.0, 5 mM Hepes, pH 7.0). Application of 100 μM 8-Br cGMP reversibly activated inward channel currents. This 8-Br-cGMP induced activity occurs in the absence of nucleotide triphosphates, indicating a direct action of cGMP on the channel. Analysis with data at a limited number of 8-Br-cGMP concentrations indicate that the channel activity is half maximally activated at about 20 μM cGMP and has a Hill coeficient greater than 1. Similar results were observed in 16 patches with either 8-Br-cGMP or cGMP, while 21 patches that were silent upon excision did not respond to either. These unresponsive patches were presumably due to vesicle formation. The single channel conductance, mean open time and reversal potential of the light-induced and cGMP induced activity are quite similar as is the strong voltage dependence of the probability of being in the open state. We have exposed inside-out patches containing light-dependent channels to internal solutions with free Ca^{2+} concentrations within a range of 10 nM to 100 μM and found that within this range Ca^{2+} never induced channel activity (tested in 5 patches that responded to cGMP, and 23 that did not). This is strong evidence that the light-dependent conductance seen in cell-attached patches is opened by cGMP and not by Ca^{2+}.

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W-Pos427

LIGHT-DEPENDENT CHANNEL ACTIVITY OF *Limulus* VENTRAL PHOTORECEPTORS IS STRONGLY AFFECTED BY VOLTAGE. E.C. Johnson[+], J. Bacigalupo[*], C. Vergara[*] & J.E. Lisman[$]. [+]Dept. of Physiology, Marshall Univ School of Medicine, Hungtington, WV. [*]Dept. Biology, Faculty of Sciences, Univ. of Chile, Santiago, Chile. [$]Biology Dept. Brandeis Univ., Waltham, MA.

Membrane potential transiently reaches positive values during bright illumination, largely due to the inward current through the light-dependent channels (LDC). We have investigated whether the properties of the LDCs change in the positive voltage range by recording LDC activity in depolarized patches under bright light. Previously, we have shown that LDC activation is preceded by a latency of variable duration that can often be longer than that of the macroscopic light-dependent current. Here we show that in depolarized patches the latency becomes less variable and considerably shorter. We also found that LDC activity decays to dark level at a much slower rate after light is turned off if the patch is kept depolarized. We have previously shown that the probability of the channel being open increases dramatically at depolarizing potentials. In addition, often patches that are unresponsive to light in the negative voltage range can develop LDC activity if the patch potential is held at a positive value, suggesting that the light sensitivity of the patch is enhanced by voltage. Altogether, these evidence is consistent with the notion that the binding of the excitatory messenger to the LDC is voltage dependent.

We also found that the LDC chiefly has 2 conductance states, of 15 and 40 pS at negative potentials, whereas at positive potentials 2 additional states, of about 8 and 58 pS are often observed. As previously observed in the negative potential range, at positive potentials these different conductance levels are also grouped in modes. Supported by NIH EY01496, NSF BNS-8812455, NSF INT-8610625 and FONDEYCT 90/1116

W-Pos428

EXCITATION OF *LIMULUS* PHOTORECEPTORS BY cGMP.
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cGMP has been shown to induce a depolarization when injected into the ventral photoreceptor cells of *Limulus*. However, the light-sensitive rhabdomeral-lobe (R-lobe) is not uniformly responsive to cGMP injection; that is, there are "hot spots" of cGMP sensitivity[1]. To test the idea that a potent phosphodiesterase which rapidly hydrolyses cGMP contributes to the existence of these "hot spots", we injected hydrolysis resistant cyclic nucleotide analogues into ventral photoreceptors. For injections into the R-lobe, we found no response to cGMP (10 mM, 4 cells), while there was a reproducible depolarization to 8-br-cGMP (10 mM, 6 cells) and 8-fluro-cGMP (10 mM, 2 cells). To test the specificity of responses to these cGMP analogues, we injected 8-br-cAMP (10 mM) into the R-lobe of 4 cells. Three gave reproducible depolarizations, the other cell did not respond. Depolarization of the photoreceptor by cyclic nucleotide analogues was not seen for injections into the light-insensitive arhabdomeral lobe (10 mM, 10 cells). Furthermore, the depolarization produced by 8-br-cGMP was not caused by a rise in intracellular Ca^{++}, as it was not blocked by the Ca^{++} buffer BAPTA (2 cells).

Our findings are consistent with the explanation that a potent phosphodiesterase is responsible for the existence of "hot spots". However, the depolarization by 8-br-cAMP was unexpected, as cAMP did not depolarize these cells[1]. It could be that the responses to both 8-br-cGMP and 8-br-cAMP are merely epiphenomena, resulting from substitution on the 8th position of the molecule.

1. Johnson et al., *Nature* 324, 468-470, 1986.

W-Pos429

INHIBITION OF THE IP_3-INDUCED Ca RELEASE IN LIMULUS VENTRAL PHOTORECEPTORS REQUIRES THE CONTINUOUS PRESENCE OF ELEVATED RESTING Ca_i.

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Injection of inositol 1,4,5 trisphosphate ($InsP_3$) into Limulus ventral photoreceptors releases Ca^{2+} from intracellular stores and depolarizes the photoreceptors. Using aequorin to monitor changes in Ca_i, Payne *et al* (Neuron 4:547, 1990) have shown that the IP_3-induced elevation of Ca_i can be inhibited by prior injection of Ca^{2+} or IP_3 delivered a few seconds earlier (feedback inhibition). As aequorin is fairly insensitive to small elevations of Ca_i, they were unable to determine whether the decline of the residual elevation of Ca_i following the injection of Ca^{2+} or IP_3 correlated with the recovery of sensitivity to IP_3. We report here that the recovery of sensitivity to IP_3, following a strong flash of light (which elevates Ca_i) or prior injection of IP_3, seems to be related to the decline of resting Ca_i. We used double-barrelled Ca selective microelectrodes to pressure-inject IP_3 and measure resting Ca_i at the same site (Levy, Biophys. J. 55:436a, 1989); the membrane potential was recorded with a separate micropipette. With the double-barrelled Ca microelectrode in the most light-sensitive lobe (the so-called R lobe) a strong flash depolarized the cell and induced a large increase in Ca_i. By giving injections of IP_3 (100 μM in the injection barrel) during the decay of the resting Ca_i, we found that the IP_3-induced Ca_i increase was totally inhibited immediately following the flash of light and started to recover as the resting Ca_i approached the dark level. The same result was obtained when Ca_i was first elevated by prior injection of IP_3. Again recovery was complete after resting Ca_i reached the dark level. We also found that the recovery of the IP_3-induced membrane depolarization corrolated with the decay of resting Ca_i. Thus the feedback inhibition by Ca^{2+}, which is thought to protect the photoreceptors' stores, may be in part mediated by the level of resting Ca_i. Supported by NSF BNS-8820001 and NIH EY03793.

W-Pos430

MODULATION OF THE VOLTAGE-DEPENDENT MAINTAINED POTASSIUM CONDUCTANCE IN *LIMULUS* VENTRAL PHOTORECEPTORS. Peter M. O'Day and Cynthia L. Phillips. Institute of Neuroscience, University of Oregon, Eugene, OR 97403.

We report that the voltage-dependent maintained (delayed rectifier) K^+-currents (i_K) in *Limulus* ventral photoreceptors[1] were reduced by agents that increase intracellular levels of cyclic-AMP and by a phosphatase inhibitor. These TEA-sensitive currents are reduced by bright prolonged illumination and by calcium.

Using 2-microelectrode voltage-clamp, we measured the effects on i_K of agents designed to induce phosphorylation of photoreceptor proteins. In *Limulus* there is Ca^{2+}-dependent and cAMP-dependent protein kinase activity[2]; octopamine increases intracellular [cAMP], as does forskolin + IBMX[3]. We found that extracellular introduction of 200μM octopamine reduced i_K ~32%. 100μM forskolin + 1mM IBMX had a similar effect on i_K. These results are similar to observations made using whole-cell recordings[4] in *Limulus* ventral photoreceptors.

We examined the effect of okadaic acid, OKA, an inhibitor of protein phosphatase 2A. OKA suppresses dephosphorylation of at least one protein in *Limulus* ventral photoreceptors[5]. We found that extracellular introduction of 200nM OKA irreversibly diminished i_K ~25%.

Our observations are consistent with the idea that i_K modulation involves a key photoreceptor phosphoprotein if resting levels of kinase and phosphatase activity are non-zero.

1. Pepose & Lisman. 1978. *J. Gen. Physiol.* 71:101-120.
2. Zhang, Kass, Pelletier, & Renninger. 1990. *Invest. Ophth. Vis. Sci.* 31:389.
3. Edwards, Wishart, Wiebe, Battelle. 1989. *Vis. Neurosci.* 3:95-105.
4. Brown, Kaupp, & Malbon. 1984. *J. Physiol.* 353:523-539.
5. Edwards. 1990. *Soc. Neuroscience Abstr.* 16:405.
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W-Pos431

Depolarization of *Limulus* ventral photoreceptors by acetylcholine after microinjection of type M1 muscarinic receptor mRNA

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Limulus ventral nerves were dissected free, desheathed, treated with 1% Pronase for 1 min and subsequently kept in an organ culture medium[1] at room temperature. Between experiments ventral nerves were stored in the dark. RNA was transcribed *in vitro* from a cDNA encoding the human m1 muscarinic receptor (courtesy of T. Bonner, N.I.H.) and was injected into ventral photoreceptor cells on day 1. Two days after mRNA injection some of the injected cells were found to depolarize in response to acetylcholine (100-400 μM) whereas uninjected cells did not respond to acetylcholine. Under voltage clamp mRNA injected cells exhibited an acetylcholine-induced inward current. These experiments are consistent with the m1 muscarinic receptor being expressed in the photoreceptor. Since the light-induced depolarization of ventral photoreceptors results from activation of an inward current it may be that the expressed receptor is substituting for rhodopsin and is capable of activating the transduction pathway normally activated by light.

[1] L. Kass and G.H. Reninger 1988. Visual Neuroscience **1**: 3-11

W-Pos432

The Fusogenic Properties of Rod Outer Segment Disk Membranes. Kathleen Boesze-Battaglia, Arlene D. Albert and Philip L. Yeagle, Department of Biochemistry, University at Buffalo School of Medicine (SUNY), Buffalo, NY 14214.

Retinal rod outer segments (ROS) contain flattened disks which hold the photoreceptor, rhodopsin. These disks undergo continual renewal through formation of new disks at the base of the ROS and the shedding of old disks at the apical tip of the ROS. Fusion processes likely play a key role in the formation of new disks and the shedding and subsequent phagocytosis of old disks by the overlying pigment epithelium. The ROS disk membrane is comprised of nearly 50 mole % phosphatidylethanolamine (PE), an unusually high PE content for biological membranes. Since this lipid due to its low headgroup hydration, is highly fusogenic, the ability of ROS disk membranes to fuse with large unilamellar vesicles (LUV) was examined. The initial rates of fusion of disks with a number of LUV was measured by following the relief of self quenching of R_{18} labelled disk membranes. Fusion was initiated by dropping the pH of the mixture to 7.4. ROS disks were found to fuse readily with these LUV. The initial rates of fusion of disk membranes with palmitoyl-oleolyl PE (POPE), transphosphatidylated transPE and disk lipid LUV was measured as a function of temperature. The initial rates of fusion were found to increase with increasing temperature. Fusion of the two membranes was confirmed by sucrose density gradient centrifugation and electron microscopy. The effect of calcium, EGTA and papain proteolysis of the disk membranes on disk membrane-LUV fusion was measured. ^{31}P NMR was used to characterize the morphology of the LUV as a function of temperature. The relationship between isotropic ^{31}P NMR resonances and the initial rates of membrane fusion was examined. This work was supported by grants from the National Eye Institute (EY03328 & EY06241).

W-Pos433

Functional Implications of Cholesterol Distribution in Bovine Rod Outer Segment Membranes. Kathleen Boesze-Battaglia and Arlene D. Albert, Department of Biochemistry, University at Buffalo (SUNY), Buffalo, N.Y. 14214

Retinal rod cells are responsible for vision under conditions of dim light. The rod outer segment (ROS) consists of an array of flattened membrane sacs, ROS disks, surrounded by the plasma membrane. The photoreceptor protein, rhodopsin is embedded in the membranes of both the plasma membrane and the disk membranes. Upon activation by light, the rhodopsin initiates the activation of transducin which in turn activates a phosphodiesterase (PDE). This culminates in the reduction of cGMP concentration. Under conditions of low cGMP the cation channels in the plasma membrane close and the membrane becomes hyperpolarized. The ability of rhodopsin in the isolated plasma membrane and in disk membranes to activate the enzymatic cascade was compared. Rhodopsin in the plasma membrane could not initiate cGMP hydrolysis to the maximal level observed for the same disk rhodopsin concentration under any light levels used. We have shown that disk membranes are not homogeneous with respect to cholesterol content (Boesze-Battaglia, K, et al. (1989) *J. Biol. Chem.* 264, 8151) and that this cholesterol heterogeneity is related to the age and thus the position of the disks in the rod outer segment. (Boesze-Battaglia, K. et al (1990) *J. Biol. Chem.* in press). The cholesterol to phospholipid mole ratio of the disks ranged from 0.05 to 0.35 and that of the plasma membrane was 0.40. Therefore the role of cholesterol in modulating this enzymatic activity was investigated. Plasma membrane samples were treated with cholesterol oxidase which converts cholesterol to cholestenone. When these membranes were used, the PDE activity was restored. The role of cholesterol was further investigated in recombinant membranes. Rhodopsin was reconstituted into membranes composed of phosphatidylcholine and cholesterol. The ability of rhodopsin to initiate the activation of PDE decreased with increasing levels of cholesterol. These results suggest that the ability of rhodopsin to activate the enzymatic cascade is inhibited in the plasma membrane by the presence of cholesterol. This may be an important factor in controlling rhodopsin receptor activity. (Supported by NEI Grant EY 03328 (ADA) and FFS-NSPB (KBB)).

W-Pos434

DIFFUSION IN SALAMANDER ROD OUTER SEGMENTS OF FLUORESCENTLY LABELLED MOLECULES INTRODUCED FROM A PATCH PIPETTE

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From analysis of electron micrographs, the lamellar disks of salamander rod outer segments (ROS) occupy > 98% of the outer segment cross-sectional area and necessarily hinder longitudinal cytoplasmic diffusion. Hindered diffusion of cGMP likely plays a role in limiting the spread of activation in ROS [Lamb et al. (1981), *J. Physiol.* 319:463-96].

We have developed a method, using fluorescence microscopy and digital image analysis, for measuring the longitudinal diffusion in ROS of fluorescently labeled molecules introduced from a whole-cell patch pipette. The diffusion process is monitored by exposing the cell briefly to epi-fluorescence illumination at regular intervals after obtaining whole-cell access. Both video and electrical data are recorded for later analysis. We have developed software for image analysis which includes time averaging, rotation and translation to a common coordinate frame, background subtraction and spatial averaging of fluorescence intensity.

For our initial studies, we chose 5(6)-carboxyfluorescein (CF), a molecule with high fluorescence quantum efficiency and with molecular weight and charge similar to cGMP. Within the time resolution of our system (30 ms), we are unable to detect any radial variation of fluorescence intensity which cannot be accounted for by differences in path length arising from the circular cross-section. Thus CF diffusion in the ROS is effectively one-dimensional. An apparent longitudinal diffusion coefficient, D_L=0.1-1.0x10^{-7} cm^2sec^{-1}, is obtained by fitting solutions of the diffusion equation (with appropriate initial and boundary conditions) to the fluorescence intensity data. This range is in fair agreement with a measurement, using the FRAP technique, of diffusion of CF in mud puppy ROS (D_L=1.7x10^{-7} cm^2sec^{-1}, [E.S. Phillips (1985) PhD thesis, Johns Hopkins]) and with an estimate (based on analysis of the time course and spatial distribution of current induced by introduction of cGMP) for cGMP diffusion into salamander rods (D_L=3-10x10^{-7} cm^2sec^{-1}, [Cameron and Pugh (1990), *J. Physiol.* 430:419-39]).

We are currently employing this method to measure the diffusion of FITC-labeled transducin (kindly provided by T. Wensel) in ROS to test the hypothesis that cytoplasmic diffusion of this protein could serve to spread excitation between disks.

Supported by NIH grant EY-02660.

W-Pos435

Light-Induced Membrane Protein Phosphorylation in the Bovine Rod Outer Segment as a Function of Disk Age: A Magic Angle Spinning ^{31}P NMR Study. Philip L. Yeagle*, Arlene D. Albert*, James S. Frye† and Kathleen Boesze-Battaglia*, (Intro. by R. Spangler) *Department of Biochemistry, University at Buffalo School of Medicine (SUNY), Buffalo, NY 14214; †Department of Chemistry, Colorado State University, Fort Collins, CO 80523.

Magic angle spinning ^{31}P nuclear magnetic resonance (MAS ^{31}P NMR) spectra of bovine rod outer segments, unphosphorylated and phosphorylated, were obtained. In the phosphorylated samples the spectra showed new resonances not assignable to phospholipids. These signals were present only when stimulation of receptor phosphorylation occurred. These resonances were not due to exogenous, soluble phosphorus-containing compounds. Limited proteolysis to remove the carboxyl terminal region of the photoreceptor that contains the phosphorylation sites removed these resonances. The chemical shifts were in the usual range for serine phosphate and threonine phosphate. The pKa obtained from a pH titration of the ^{31}P chemical shift was typical of serine phosphate. Therefore, these ^{31}P NMR resonances were assigned to the phosphorylation sites on membrane proteins in the rod outer segment disk membranes. Static ^{31}P NMR measurements revealed that at least some of these sites gave rise to relatively narrow resonances, indicative of considerable motional freedom of the carboxyl terminal segment of the photoreceptor when phosphorylated. Using this approach, the extent of phosphorylation along the rod outer segment was examined. Phosphorylation was found to be inhomogeneous with age of the disk. This work was supported by grants from the National Eye Institute (EY03328 & EY06241).

W-Pos436

THE EFFECT OF RHODOPSIN PHOSPHORYLATION ON THE FORMATION AND DECAY OF METARHODOPSIN II, THE G_t-ACTIVATING PHOTOINTERMEDIATE

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The light-activated form of the integral membrane receptor rhodopsin, metarhodopsin II (meta II), serves as the substrate for rhodopsin kinase. Previous studies demonstrated that increasing levels of phosphorylation, up to 9 phosphates per rhodopsin (P/R), resulted in a graded desensitization of rhodopsin relative to its G_t activating function[1]. These results showed that the level of rhodopsin phosphorylation regulates visual signalling. Similar phosphorylation induced desensitization has been observed in signal modulation in other G-protein interactive receptors. In order to elucidate the mechanism whereby phosphorylation modulates the efficacy of rhodopsin's receptor function, we have studied the decay of meta II and the meta I-meta II equilibrium in POPC vesicles containing rhodopsin with either 0 P/R or $\geq$ 4 P/R. Spectral measurements were carried out at 25 °C, pH 7.0. Equilibrium spectra were acquired within 3 s after a bleaching flash on a diode array spectrometer. These spectra were deconvolved to determine the amounts of meta I and meta II at various times after bleaching. The K_{eq} for the meta I $\leftrightarrow$ meta II equilibrium was determined to be $\approx$1 for both the 0 P/R and $\geq$4 P/R species. The meta II decay times were also found to be the same for the two rhodopsin samples. Hence, phosphorylation of sites on the cytoplasmic surface of rhodopsin does not affect the equilibrium associated with the conformation change which leads to the formation of meta II, nor the stability of meta II, as indicated by its decay to meta III. Thus, the mechanism of phosphorylation induced down regulation of rhodopsin's receptor function is not associated with changes in the photointermediate decay sequence. These results suggest that the effect of phosphorylation is at the level of the G-protein-receptor interaction, a question which will also be addressed in these studies. (Supported by NIH grant EY00548)

[1]J. L. Miller, D. A. Fox, and B. J. Litman. (1986) Biochemistry 25, 4983.

W-Pos437

SINGLE CHANNEL STUDY OF THE cGMP-DEPENDENT CONDUCTANCE OF RETINAL RODS FROM INCORPORATION OF NATIVE VESICLES INTO PLANAR LIPID BILAYERS. Michèle Ildefonse and Nelly Bennett (Intro. by S. Crouzy), Lab. Biophys. Molec. & Cell., C.E.N.-G 85X, 38041 Grenoble cedex, France.

Unitary currents through cGMP-dependent channels of retinal rods are observed following incorporation into planar lipid bilayers of native vesicles from purified rod outer segment membranes, washed free of soluble and peripheral proteins. The influence of the concentration of cGMP, inhibitors (*cis*-diltiazem and tetracaine) and divalent cations (Ca^{++}, Mg^{++}) on the conductance and open probability of the channel is described, as well as the voltage dependence of these effects. The cGMP-dependence reveals the existence of four discrete conductance levels; the smaller levels are predominent at low cGMP concentrations while the proportion of maximal level increases with increasing [cGMP]. Hill plots and theoretical curves which fit the experimental data suggest that four cGMP molecules bind cooperatively to the channel, and that the conductance of the channel increases stepwise with each cGMP molecule bound. Diltiazem and tetracaine increase the proportion of the closed state, total closure being observed around 50 - 100 uM; before total inhibition, the proportion of larger conductance levels decreases to the benefit of the smallest level, whose conductance is clearly resolved as 1/4 of the maximal level. Cytoplasmic calcium and magnesium have no effect on the inward current; on the other hand, external calcium and magnesium markedly reduce the probability of the channel being open and, at physiological divalent concentrations, the conductance of the channel for the inward current is too small to be measured. Finally, we provide conclusive evidence that activated G-protein does not directly inactivate the cGMP-dependent channels of bovine retinal rods.

W-Pos438

LONG-TERM CHANGES IN THE cGMP-ACTIVATED CONDUCTANCE IN EXCISED PATCHES FROM ROD OUTER SEGMENTS. Sharona E. Kantrowitz-Gordon and Anita L. Zimmerman Section of Physiology, Brown University, Providence, RI 02912.

Previous studies have suggested that excised rod outer segment patches have adherent intracellular structural elements that restrict diffusion in the vicinity of the channels (Zimmerman *et al*, Biophys. J. 54:351 (1988)), and that they have the necessary enzymes for phototransduction (Ertel *et al*, Biophys. J. 55:458a (1989)). It is conceivable that such enzymes and structural elements (perhaps including attached disks) may leave the patch with continued perfusion during excised patch experiments. This change in patch structure or composition may in turn affect the local concentration of cGMP and/or channel function. Using salamander and frog rods, we have looked for such time-variant effects in the cGMP-activated conductance of inside-out excised outer segment patches continuously perfused for up to two hours. We have examined: 1) the concentration dependence of activation by cGMP; 2) the concentration dependence of activation by the hydrolysis-resistant analog, 8-Br-cGMP; and 3) the effect of Ca^{2+} on activation by cGMP. On a time scale of minutes to tens of minutes, the dose-response curve for activation of the channels by cGMP was often found to shift to the left, approaching a limiting value. For example, in one patch, the $K_{0.5}$ decreased from about 28 μM to 7 μM within 37 minutes. In interleaved trials on the same patch, a comparable shift was seen in the concentration dependence for activation by 8-Br-cGMP. This suggests that the shift seen in the cGMP data did not result from the loss of patch-associated phosphodiesterase (PDE) that had initially reduced the amount of cGMP reaching the channels. With symmetrical 130 mM Na^+ and no external divalent cations, the presence of 200 to 400 μM internal Ca^{2+} blocked the channels as expected from many earlier studies and shifted the cGMP dose-response curve slightly to the right (Zimmerman *et al*, Neurosci. Research, Suppl. 12:S165 (1990)). This latter effect was studied further here: it was not seen in all patches, and in one patch the effect was seen initially but disappeared by the end of the 2-hour experiment, coincident with a slow increase in all currents. This last finding suggests an involvement of (initially) patch-associated PDE, weakly stimulated by Ca^{2+}, although this remains to be tested. We are now investigating whether the channel itself undergoes any time-variant changes following excision, or if instead all our observations merely reflect changes in patch structure or chemical composition. (Support: NIH grant EY07774 & NSF grant RCD-9054722)

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METAL CATIONS INFLUENCE PHOTOTRANSDUCTION IN EXCISED PATCHES OF VERTEBRATE ROD OUTER SEGMENTS

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Excised patches of *Gekko gekko* rods retain a functional phototransduction cascade (Ertel E.A., *PNAS* 87, 4226 1990). This preparation was used to investigate the modulation of phototransduction by metal cations. In intact rods and detached outer segments, calcium has been shown to regulate the activity of guanylate cyclase but the role of other ions has not been investigated. We find that:

1) External calcium flowing into the cytoplasmic milieu of the patch inhibits the *de novo* synthesis of cGMP from MgGTP by guanylate cyclase. The addition of the calcium chelator BAPTA to the cytoplasmic solution removes the inhibition.

2) The dark MgGTP-induced current and the kinetics of light responses are modulated by the monovalent cations present in the cytoplasmic solution. Solutions containing potassium as the main cation produce recordings similar to those obtained in intact rods. Sodium, and to an even larger degree lithium, significantly reduce the size of the current and impair response recovery. This suggests that one or more enzymes are sensitive to monovalent cations and require potassium for normal function. This phenomenon might be a safety mechanism to reduce dark current, if the cytoplasmic sodium concentration reaches excessively high levels.

3) If patches are prepared with micromolar external divalent cations and perfused with millimolar MgGTP, the movement of magnesium across the patch membrane influences the cytoplasmic cGMP level in a manner similar to that proposed for calcium. Under these conditions, phenomena typical of light adaptation are observed. Responses to steps of light exhibit peak-to-plateau relaxation and flashes on a background are desensitized and have accelerated kinetics. These effects disappear when the magnesium gradient across the membrane is reduced, suggesting that this ion is responsible. They cannot be assigned to calcium since calcium fluxes are negligible under these conditions

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Ca REGULATION OF GUANYLATE CYCLASE IS RESPONSIBLE FOR LOW FREQUENCY LIGHT-SENSITIVE NOISE IN RETINAL RODS.

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There are continuous low frequency undulations in the amplitude of dark current recorded from intact rods (Baylor et al., J. Physiol., 309, 591-621, 1980) and detached lizard rod outer segments (DOS) dialyzed in whole-cell voltage clamp with standard internal solution containing 5 mM ATP and 1 mM GTP (control solution). The noise is suppressed by light and has been attributed to fluctuations in internal cGMP.

In DOS dialyzed with control solution the power spectrum of the low frequency noise was fitted by a squared Lorentzian; corner frequency Fc = 0·46 ± 0·04 Hz, zero frequency asymptote $S(0) = 2{\cdot}0 \pm 0{\cdot}3 \times 10^{-25}$ A^2/Hz (mean ± S. E. M., n experiments = 5). This noise was not present in DOS dialyzed with 50 μM cGMP and 0 triphosphates (n = 4), indicating that noise generation requires guanylate cyclase activity and does not arise from changes in basal PDE activity.

The addition of a guanylate cyclase inhibitor (10 mM pNHp) to dialysis solution containing 50 μM cGMP, 500 μM ATP, 100 μM GTP and 10 mM BAPTA caused a substantial reduction in low frequency noise (n = 4). Since the presence of pNHp did not block the light response, this result suggests that the low frequency noise is not generated by spontaneous activation of the transduction cascade.

Low frequency noise became slower and larger in DOS dialyzed with control solution containing 1-10 mM BAPTA. The resulting power spectum was fitted by a squared Lorentzian; Fc = 0·14 ± 0·01 Hz and $S(0) = 6{\cdot}8 \pm 1{\cdot}1 \times 10^{-24}$ A^2/Hz (n = 5), indicating that the spectral properties of the noise are influenced by the Ca buffer capacity of the outer segment which affects the time course of changes in internal Ca.

These results suggest that the low frequency noise is generated by the negative feedback loop that couples the rate of cGMP synthesis by guanylate cyclase to Ca influx through cGMP-gated channels.

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VOLTAGE DEPENDENCE OF LIGHT-SENSITIVE AND Na:Ca EXCHANGE CURRENTS IN DETACHED LIZARD RETINAL ROD OUTER SEGMENTS.

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The effect of voltage on whole-cell dark current (I_d) recorded from detached outer segments (DOS) dialyzed with standard internal solution containing 5 mM ATP and 1 mM GTP was studied by stepping from -29 mV (V_{hold}) to voltages that ranged from -109 to +68 mV.

Large voltage steps triggered a time dependent process that caused the instantaneous (I_i) and steady state (I_{ss}) currents to be different. In the vicinity of the reversal voltage there was little difference between I_i and I_{ss} but with steps to more positive or negative potentials the initial change in current was followed by a slower increase that reached a steady state level in 5 to 40 sec. Depolarization evoked a much larger delayed increase in current than did hyperpolarization; e. g. I_{ss} evoked by step to 41 mV could exceed I_i by as much as 3 fold.

Since I_d is the sum of light-sensitive, Na:Ca (I_{exch}), and leakage (I_{leak}) currents, the voltage and time dependence of the later two components was investigated. I_{leak} in saturating light was ohmic and time independent. Saturated I_{exch} was recorded from DOS dialyzed with 10-40 mM Ca. It increased with hyperpolarization and decreased with depolarization from V_{hold} in a time independent manner that showed a nearly linear dependence on voltage from -109 to +11 mV; 50% change in 40 mV. The voltage dependence of I_{exch} was less than linear at potentials > +11mV and was not reversed at +68 mV.

The effect of voltage on the light-sensitive component of whole-cell I_d was determined by subtracting I_{exch} and I_{leak} from I_d. Both the instantaneous and steady-state currents showed slight inward rectification, reversed at ~ 9 mV and rectified markedly in the outward direction. The slow voltage dependent increase in current which causes the I_{ss} to be larger than the initial current did not occur in 0 external Ca nor in nucleoside triphosphate depleted DOS bathed in normal Ringer. Neither of these recording conditions affected the form of the instantaneous IV curve. These results suggest that the time dependent increase in current is triggered by a voltage-induced fall in internal Ca and increased cGMP synthesis by guanylate cyclase.

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Regulation of internal free Ca^{2+} concentration in bovine retinal rod outer segments (ROS) by Na^+ gradient, K^+ gradient and membrane potential.

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Internal free Ca^{2+} concentration in isolated bovine ROS was measured with the fluorescent Ca^{2+}-indicating dye fluo-3. Loading of fluo-3 appeared to occur exclusively in the ROS cytoplasm and not in the intradiskal space. The initial internal free Ca^{2+} concentration in bovine ROS could range between < 10 nm and up to 50 uM depending on the Na^+ and Ca^{2+} concentrations in the media used to isolate ROS. Changes in internal free Ca^{2+} concentration by manipulations of the external ion concentrations were examined and the following results were obtained
1. Lowering of internal free Ca^{2+} absolutely required external Na^+. 2. A rise in internal free Ca^{2+} required both internal Na^+ and external Ca^{2+}. 3. A progressive increase in external K^+ caused a progressive increase in internal Ca^{2+}. 4. External Cs^+ caused an increase in internal free Ca^{2+}, but only in the presence of the electrogenic Cs^+ carrier valinomycin, most likely due to depolarization of the plasma membrane. These results are consistent with regulation of internal free Ca^{2+} by an electrogenic Na-Ca exchanger that couples both Na^+ and K^+ gradient to Ca^{2+} extrusion. The Na-Ca exchanger worked more efficeintly in the presence of K^+, but a large and rapid rise in internal free Ca^{2+} mediated by Na-Ca exchange was also observed in the absence of external K^+. The efficiency of the exchange protein was such that it could mediate changes in internal free Ca^{2+} over the full usable range of fluo-3 (10 nM to 2 uM) within a few seconds even when the exchange protein was exposed to free external Ca^{2+} concentrations well below 1 uM.
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A Ca^{2+}-SENSITIVE LOW K_m INORGANIC PYROPHOSPHATASE IN RETINAL ROD OUTER SEGMENTS

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Retinal rod outer segments contain high levels of the enzyme inorganic pyrophosphatase (PPase). This enzyme is essential for hydrolysis of the inorganic pyrophosphate produced at high rates as a byproduct of cyclic GMP turnover. We have purified PPase from bovine rod outer segments to greater than 90% electrophoretic homogeneity, and to a specific activity of >600 μmol pyrophosphate hydrolyzed min^{-1}-mg^{-1}. The protein migrates as a single polypeptide of ~36 kd on SDS polyacrylamide gels. Results from centrifugation on a sucrose gradient indicate a native size of ~70 kd, suggesting that, like other eukaryotic PPases, it is a homodimer. This enzyme differs from previously studied PPases in having a lower K_m (.9-1.5 μM), and in displaying a greater sensitivity to Ca^{2+} in the sub-micromolar range. Imidodiphosphate is a competetive inhibitor of PPase, with K_i = 1.2 μM. The enzyme is also inhibited by fluoride ion with $C_{1/2}$ of ~ 20 μM. Because guanylate cyclase is inhibited by inorganic pyrophosphate, regulation of PPase by Ca^{2+} may play a role in Ca^{2+}-mediated recovery of rod cells from stimulation by light. Supported by USPHS grants RR05425 and EY07981.

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THE PERMEABILITY TO ORGANIC CATIONS OF THE cGMP-ACTIVATED CHANNEL OF RETINAL RODS.

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The permeability of cyclic GMP-activated channels to organic cations was determined by recording macroscopic currents in excised inside-out patches from retinal rods of the tiger salamander. The patch pipette contained 110 mM NaCl and low divalents. Current-voltage relations were measured as the NaCl of the bathing medium was replaced by salts of organic cations and permeability ratios relative to Na ions were calculated from the reversal potentials. The following ions were found to be permeable: ammonium, guanidinium, formamidinium, aminoguanidinium, hydroxylammonium. Permeabilities calculated from reversal potentials in some cases disagreed with the ratio of the amplitudes of outward currents. Such disagreements are expected if these ions influence the gating of the channel or if the pore contains binding sites with different affinities for different ions. Moreover it is known that the cGMP-activated channel is not permeable to a variety of other organic cations as tetraethylammonium, tetramethylammonium, arginine, lysine, choline, glucosamine (Menini, J. Physiol. 424:167-185, 1990).

A comparison with similar experiments on other ion channels indicates that the cGMP-activated channel has selectivity properties for organic cations similar to those of the Na channel.

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Na TO Li SUBSTITUTION DOES NOT AFFECT THE GATING OF THE cGMP-ACTIVATED CHANNEL OF RETINAL RODS.

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Li and Na currents through the cGMP-activated channels in excised, inside-out membrane patches from salamander rod outer segments were studied in the absence of divalent cations. The permeability ratio for these two ions determined from reversal potential measurements is close to 1 (Nunn 1987 J. Physiol. 394, 17P). However the ratio of the outward currents measured at +60 mV with 110 mM LiCl or 110 mM NaCl cytoplasmic solutions is about 0.3 (Menini 1990 J. Physiol. 424, 167-185). The amplitude of macroscopic currents is determined both by the mean number of open channels and by the amplitude of the single channel current. To investigate if Li and Na ions have a different influence on the gating of the channel we have estimated single channel conductances from noise analysis. We studied stationary fluctuations of the membrane current as a function of cGMP concentrations at +60 mV. The relation between variance and mean current is well described by a parabola both in Li and in Na solutions. The ratio of single channel currents obtained from these relations is about 0.3. The power spectra of the fluctuations in Li and Na solutions are identical apart for a scaling factor.

We conclude that the difference between Li and Na macroscopic currents is entirely due to the different permeation of these ions through the open pore and not to modifications of the gating of the cGMP-activated channel.

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MONOVALENT CATION SELECTIVITY OF THE cGMP-DEPENDENT CURRENT OF THE CONE OUTER SEGMENT MEMBRANE.

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Using tight-seal electrodes in detached membrane patches we measured the ionic selectivity of the cGMP-activated current in the outer segment membrane of cone photoreceptors. Single and twin cones were isolated from the retina of the striped bass *(Morone saxatilis)* and the macroscopic current was recorded. In symmetrical ionic solutions containing 157 mM NaCl, 0.2 mM CaCl2 and 10 mM HEPES, cGMP activated membrane currents that exhibited outward current rectification and whose I-V characteristics were the same as those of the dark-current in intact cells. The dependance of current amplitude on cGMP concentration was well described by a Hill equation with approximate values of Km= 55 uM and n= 2.2. In bionic conditions the permeability ratio sequence of monovalent cations, determined by changes in the reversal potential of the current activated with 40 uM cGMP in the presence of 0.2 mM Ca was K:Rb:Li:Na:Cs = 1.31:1.24:1.11:1:0.99. The conductance ratio sequence derived from the macroscopic steady-state currents for the same ions measured at +80 mV was: Na:K:Li:Rb:Cs = 1:0.83:0.77:0.77:0.48. In the absence of Ca (or any multivalent ion) the I-V curve of the cGMP-dependent current was linear, but the dependence of its amplitude on cGMP concentration was unaffected. The ionic selectivity of the channel changed as a function of Ca concentration. The permeability sequence of monovalent cations in the absence of multivalent cations (with 1 mM each EGTA and EDTA) and activated with either 40 or 200 uM cGMP was: K:Li:Na:Rb:Cs = 1.13:1.09:1:0.99:0.77. The conductance sequence for the same ions measured at +80 mV was: Na:Rb:K:Cs:Li = 1:0.97:0.94:0.68:0.54. These permeability and conductance sequences are different than those of the cGMP-dependent current in rod photoreceptors. Supported by N.I.H. grant EY05617 to J.I.K.