W-Pos260 OPTICAL SPECTROSCOPIC STUDIES OF HEMOGLOBINS AND MYOGLOBIN AT HIGH PRESSURE.
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Hemoglobin and myoglobin in various forms of ligation (deoxy, CO, O_2) have been studied at high pressure (0-34 kbar) using resonance Raman and optical absorption spectroscopies. As noted by earlier workers, deoxyhemoglobin and deoxymyoglobin first undergo high-to-low spin state changes at relatively low pressures. As pressure is further increased, both proteins precipitate from solution. CO-hemoglobin also precipitates at about 3.4 kbar but is observed to go back into solution upon release of the pressure. The spin-state changes are characterized by frequency shifts of the spin-state marker bands in the Raman as well as demonstrable changes in the optical absorption spectra. Precipitation of these hemeproteins is accompanied by a red shift and line-broadening of the Soret band. As pressure is increased the Raman features above 1200 cm^{-1} increase in frequency and the relative intensities of several of the Raman modes change. It is proposed that the spin-state change in deoxyhemoglobin and deoxymyoglobin is accompanied by coordination of distal histidine residue and that the surprising difference in reversibility of precipitation of deoxy- and CO-hemoglobin is also related to this effect. Resonance Raman spectra show that photolysis of CO is suppressed at elevated pressures. The insights into the hemepocket geometries and heme electronic state of the various hemoglobin species at high pressures provided by the structural sensitivity of resonance Raman scattering will be discussed.
This work supported by the NIH (GM33330).

W-Pos261 STRUCTURAL AND SPIN-STATE DYNAMICS OF HEMOGLOBIN VALENCE HYBRIDS.
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The kinetics of structural transitions and CO binding to human hemoglobin valency hybrids with mixed-spin ligands on the ferric subunits have been studied using laser photolysis and either optical absorbance or Time REsolved Magnetic Susceptibility (TREMS) to follow the kinetics. Contrary to results for cyanomet hybrids, with other ferric ligands the removal of CO from the ferrous subunits does cause a switch to the low-affinity T structure, and this switch to the T structure is quite rapid (>5,000/sec). The rate of the R->T conformational change can be determined from both characteristic changes in the heme spectra and by analysis of the CO rebinding kinetics at high CO concentrations (where there is strong competition between rapid CO binding to the R state and the R->T conversion). The kinetics have been studied for hybrids with a variety of ferric ligands, with spin-state equilibria ranging from mostly low-spin (azide) to mostly high-spin (fluoride). The Perutz stereochemical mechanism predicts a strong coupling between the quaternary transitions and the spin equilibria of the ferric subunits. In these kinetic experiments this coupling should manifest itself in two ways: (1) as the ferric ligand is varied, the extent of switching to T should be much less for predominantly low-spin ligands; and (2) as the switch to T occurs, the spin equilibrium of the ferric subunits should shift strongly toward high-spin. These predictions are not supported by the data. Neither the allosteric constant nor the rate of the R->T switch vary systematically with the ferric spin distribution. Furthermore, the direct TREMS magnetic data fail to show significant spin-state shifts upon R->T conversion.
(Supported by N.I.H. HL-24644)

W-Pos262 AN APPROACH TO THE STRUCTURAL AND DYNAMIC INVESTIGATION OF LOW-SPIN FERRIC HEMEPROTEINS BY PROTON NMR SPECTROSCOPY.
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In paramagnetic hemeproteins-such as metcyano sperm whale myoglobin-the short range electron-nucleus interactions cause hyperfine shifts for the protons in the heme cavity and improve the resolution of the corresponding signals from the intense diamagnetic envelope. Increased resolution is accompanied by accelerated relaxation rates which create severe difficulties in reaching the peak assignments necessary for structural and dynamic studies. We have applied the following strategies to elucidate the spectrum. 1) Nuclear Overhauser Effects (NOEs) in H_2O solution: NOEs from the known ring NHs of the proximal and distal His allow us to distinguish peaks from cavity protons "above" and "below" the heme plane. 2) NOEs in D_2O solution: The NMR experiment and the interpretation of its results are simplified by eliminating the exchangeable protons. Data complementary to those in H_2O are collected. 3) NOEs on reconstituted proteins: a)with selectively deuterated hemins, connectivities to non-resolved heme protons are confirmed as NOEs to a group disappear upon deuteration of that group. Likewise, secondary NOEs mediated by such group are suppressed. b)with modified hemins, substitution of vinyl or propionate sidechains by methyl groups provides additional markers. NOEs to and from such methyl peaks are readily interpretable. Furthermore if a symmetrically substituted hemin is used, the heme orientational disorder complication is alleviated. Once assignments are ascertained, specific sidechain geometry and mobility information is extracted from truncated NOE data.

W-Pos263 CROSS-LINKED Fe-Co HYBRID Hbs AS MODELS OF MONO-, DI- & TRI-LIGATED SPECIES. C.D'Ambrosio, T.Inubushi, M.Ikeda-Saito & T.Yonetani, U.of Penn., Phila., PA 19104.

Mono-, di-, and tri-cobalt substituted hybrid Hbs have been successfully prepared as models of Hb species with three, two and one ligand respectively, since only Fe-subunits are ligated to CO at atmospheric pressure. 1H NMR studies of the paramagnetically induced, downfield hyperfine shift of the proximal histidine imidazole proton and the resonances of inter- and intra-subunit hydrogen-bonded protons reveal different structural natures for species ligated in different subunits. EPR studies examining the electronic state of the Co metal ion in the hybrid hemoglobins as a function of the number of ligands also exhibit different spectral characteristics depending on which subunits are ligated. This structural and physicochemical information supports the existence of more than two quaternary structures in the course of oxygen binding to Hb. Integrated with equilibrium oxygen binding data, this information allows us to postulate that each oxygenation step of Hb choses a favorable intermediate among the possible partially saturated species. Supported by the NIH grant HL14508-17.

W-Pos264 PROBING THE INTER-SUBUNIT INTERFACES IN HUMAN NORMAL ADULT HEMOGLOBIN BY PROTON NUCLEAR OVERHAUSER EFFECTS. Irina M. Russu, Nancy T. Ho, and Chien Ho. Department of Biological Sciences, Carnegie-Mellon University, Pittsburgh, PA 15213, U.S.A.

Previous work from this laboratory has demonstrated that several exchangeable proton resonances in the ^1H nuclear magnetic resonance (NMR) spectra of human normal adult hemoglobin (Hb A) originate from the inter-subunit hydrogen bonds at the $\alpha_1\beta_1$ and $\alpha_1\beta_2$ interfaces of the protein molecule. Using appropriate mutant and chemically modified Hbs, some of these resonances have been assigned by this laboratory to specific amino acid residues in the Hb A molecule. The inter-subunit hydrogen bonds at the $\alpha_1\beta_2$ interfaces are currently thought to play a key role in the cooperative oxygenation of Hb A.

We have now extended these early studies by an investigation of the truncated-driven nuclear Overhauser effect (NOE) for each of the exchangeable proton resonances of Hb A, in both deoxy and ligated forms. The results allowed us to make detailed assignments of all the exchangeable proton resonances to specific inter-subunit hydrogen bonds in the Hb molecule. Using these NOEs, we are able to characterize the local conformations and environments for several other amino acid residues situated at or near the $\alpha_1\beta_1$ and $\alpha_1\beta_2$ interfaces of Hb A. The NOEs on the exchangeable proton resonances of Hb A have also been compared to the corresponding effects in normal and mutant Hb tetramers for which the cooperativity in oxygen binding is either reduced or abolished. The relationship between these NMR results and the molecular mechanisms for cooperativity and allosteric effects in Hb A will be discussed. [Supported by a research grant from the NIH (HL-24525)].

W-Pos265 A PROTON NUCLEAR MAGNETIC RESONANCE INVESTIGATION OF THE MOLECULAR MECHANISMS INVOLVED IN THE ANION BOHR EFFECT OF HUMAN NORMAL ADULT HEMOGLOBIN. Gregory Kellogg, Irina M. Russu, and Chien Ho. Department of Biological Sciences, Carnegie-Mellon University, Pittsburgh, PA 15213, U.S.A.

Extensive experimental evidence over the last decade has demonstrated that a large fraction of the Bohr effect of human normal hemoglobin (Hb A) originates from the heterotropic interactions between Hb A and inorganic anions. In the present work, we have used high-resolution proton nuclear magnetic resonance (NMR) spectroscopy to detect and characterize the electrostatic interactions between chloride or inorganic phosphate ions and Hb A, in both deoxy and ligated states. The effects of chloride and inorganic phosphate ions upon the individual pH-titration curves of twenty-two surface histidyl residues of deoxy Hb A and HbCO A have been measured by ^1H NMR at 21° and 29°C. The results indicate that some of the ionizable sites of the Hb A molecule involved in the chloride-linked Bohr effect are different from those involved in the inorganic phosphate- or organic phosphate-linked Bohr effect. Hence, the detailed molecular mechanism responsible for the anion Bohr effect of Hb A is not unique and depends on the nature of the anion. The two anions investigated have also been found to affect the individual ionization properties of certain histidyl residues by altering the intra-molecular electrostatic interactions within the protein charge array. The relationship between these results and the molecular mechanisms for allosteric effects in Hb will be discussed. [Supported by a research grant from the NIH (HL-24525)].

W-Pos266 CHANGES IN THE STABILITY OF HEMOGLOBIN A_2 AND ABNORMAL HEMOGLOBINS DUE TO SEQUENCE DIFFERENCES AND CROSS-LINKING. Kenneth W. Olsen, Thao Yang, Mark Nathan, Stamatia Zimianitis and Frank L. White, Department of Chemistry, Loyola University of Chicago, 6525 North Sheridan Road, Chicago, IL 60626.

The thermal denaturation of hemoglobin A_2 was studied and compared with that of hemoglobin A. The hemoglobin A_2 was first oxidized to methemoglobin by KFe(CN)6. The thermal denaturation was done in 0.01M MOPS, 0.9M guanidine, 0.001M NaCN, at pH 7.0. The changes in absorbance between 200-650nm were monitored by a diode array spectrophotometer, while heating the sample at a rate of 0.3° C/m between 25°C to 70°C. As expected, hemoglobin A_2 was more stable than hemoglobin A. The transition temperature for hemoglobin A_2 was 46°C and that of hemoglobin A was 42°C. This difference corresponds to a change in ΔG of 0.8 kcal/mole. The increased stability for hemoglobin A_2 is probably due to an additional hydrogen bond between Arg δ-116 and Pro α-114 and an additional nonpolar contact between Met δ-126 and Val δ-11 of the same subunit. The ability of cross-linking reagents, such as bis (3,5-dibromosaclicyl) fumarate or dimethyl suberimidate, to further stabilize hemoglobin A_2 or abnormal hemoglobins will be presented. When it was cross-linked by these reagents, hemoglobin A showed a second structural transition at 57°C, in addition to one at 42°C. The length of the cross-link or the ligand state of the hemoglobin also affected the resulting stability. (Supported by a Biomedical Research Support Grant, NIH.)

W-Pos267 INFLUENCE OF SITE SPECIFIC INCORPORATION OF NEGATIVE CHARGE ON ELECTROSTATIC STABILIZATION OF HUMAN HEMOGLOBIN. Bo Hedlund, Dight Labs., Univ. of Minnesota, Minneapolis, MN 55455; Bertrand Garcia-Moreno and Frank Gurd, Chemistry Dept., Indiana Univ., Bloomington, IN 47405; George Turner, Benjamin Turner and Gary Ackers, Biology Dept., The Johns Hopkins Univ., Baltimore, MD 21218

Reductive carboxymethylation leading to covalent introduction of negative charge at specific sites of human Hb is achieved by chemical reduction of Schiff bases formed between the glyoxylate anion and primary amino groups (Saunders & Hedlund Biochemistry 23:1457, 1984). This approach can be considered an affinity labelling method for the localization of small anion binding sites. In the presence of millimolar concentration of glyoxylate (pH 7.0, 0.1 M P_i), there are three sites of modification. These are the α- and β-N-terminal amino groups, and a single lysine residue, lys α-127. The pattern of modification is insensitive to the state of ligation of the protein. IHP blocks the modification of the β-1 val amino group in both oxy- and deoxyHb. The dissociation of deoxyHb, measured under standard conditions, is unaffected by the introduction of negative charge at the N-termini. OxyHb, on the other hand, is destabilized when the α-, β-, or both N-termini are modified (Δ^4G_2=0.8, 0.2 and 1.4 kcal/mole resp.). The electrostatic consequences of carboxymethylation of the three sites were calculated with the static accessibility modified Tanford-Kirkwood algorithm, using models of the carboxymethylated structures. The approach handles anion binding explicitly, through scrutiny of the electrostatic potentials at the protein-solvent interface.

W-Pos268 INVESTIGATION OF MULTIPLE HEME CONFIGURATIONS IN NORMAL AND DISTAL SUBSTITUTED MYOGLOBINS D.E. Bartnicki, P.L. Pingerelli, C. Lentz and H. Mizukami. Division of Regulatory Biology and Biophysics, Department of Biological Sciences, Wayne State University, Detroit, Michigan 48202.

Most distal substitutions in hemoproteins prove to be deleterious; however, a notable exception is the distal glutamine of Asian elephant myoglobin. Using elephant and whale myoglobins, the comparative difference spectra obtained by computer subtraction in the visible region reveal a similar proton-linked spectral change having a pK = 5.7 for the carbon monoxy derivatives. However, the pH dependent spectral change seen with oxy whale and elephant myoglobins is different, and suggests that distal histidine contributes to the proton-linked spectral change in normal oxy myoglobin, while a second histidine, probably FG2, is exclusively responsible for the spectral change in the carbon monoxy forms. Two optical species were also observed in thermal equilibrium for both elephant and whale myoglobins. The temperature-induced difference spectra were reversible and ligand dependent, with elephant and whale myoglobins showing the same spectral changes for deoxy, carbon monoxy (pH 6.0) and oxy forms. Only met (pH 6.5) and carbon monoxy (pH 8.0) derivatives gave dissimilar temperature difference spectra. In the case of elephant myoglobin, it is proposed that CO and H_2O (ferric heme) can interact with the distal amide to form two interconverting configurations, while the 2-state equilibrium observed in the other ligand forms and in the deoxy form result from a mechanism which affects the position of the heme iron.

W-Pos269 OPTICAL PUMPING OF THE GEMINATE STATE OF CARBOXYMYOGLOBIN. B. Chance, *Powers, L., Zhou, C., Naqui, and *Chance, M. Dept. Biochem.Biophys, Univ. of Penna, Phila., PA 19104 and *AT&T Bell Labs, Murray Hill, NJ 07974

Previous work gives an EXAFS structure of the photoproduct at 4^{o} where the ligand remains near the iron ($\lesssim$0.05 A) (1,2) with significantly increased bending of the CO bond (3) and an increase of the iron pyrrole nitrogen (Np) distance. Kinetic analysis of the optical signal of the geminate state at 765 nm shows a distinct slow phase of recombination at ($k = 5 \times 10^{-5}$ sec^{-1}) at 40 K which decreases on one hour's pumping to 2.5×10^{-5} sec^{-1}. The pump light is manually regulated to give 50% FeCO and 50% of the geminate states as determined by periodic optical spectrophotometry. Pumping apparently accumulates a structure in which the ligand is in a chemically or sterically constrained state(s). Preliminary X-ray absorption spectroscopy of this compound gives FeNp and FeNe parameters that are unaltered within the error from the 4^{o} geminate state and the present data are best fitted with no ligand present in the first shell. It appears so far that the EXAFS method provides FeNp and FeNe distances on two different geminate states of myoglobin. (1) Chance, B., Fischetti, R., Powers, L. (1983) Biochem. 22: 3820-3829; (2) Chance, B, Korszun, R., Kumar, C. & Zhou, C. (1984) Biophys. J. 45:368; (3) Powers, L., Sessler, J.L., Woolery, G.L., Chance, B. (1984) Biochem.23:5519-5523. Supported in part by NIH Grants HL 18709, GM 31992, RR 01633, SSRL Proj. 825B (supported by DOE,OBES;NSF,DMR; & NIH,BRP,DRR).

W-Pos270 NEAR INFRARED OPTICAL ABSORPTION SPECTRA OF PHOTODISSOCIATED HEMOGLOBIN AND MYOGLOBIN. M. Sassaroli and D. L. Rousseau, AT&T Bell Laboratories, Murray Hill, NJ 07974.

The wavelength of the near infrared change transfer transition at $\sim$760 nm has been obtained with 10 nsec pulses in photodissociated hemoglobin and myoglobin and compared to that of the corresponding deoxy preparations. We find that these transitions are located 762 and 759 nm for deoxy myoglobin and deoxy hemoglobin, respectively. In the photoproducts generated by photolysis of the carbon monoxide bound proteins we find that in myoglobin the wavelength is unchanged from its position in the deoxy preparation. However, in the hemoglobin photoproduct the transition shifts from 759 to 765 nm. In studies of metastable species generated at low temperatures ($\sim$10K) large shifts in this transition have been reported for both hemoglobin and myoglobin. In transient studies of these proteins, carried out at room temperature, however, all spectroscopic indicators show that the myoglobin photoproduct is fully relaxed by 10 nsec but the hemoglobin photoproduct relaxes with a much longer time constant. The data reported here are therefore consistent with the other spectroscopic indicators of the time dependence of the heme relaxation in the two proteins. Comparison of this data with that of other techniques allows consideration of the structural basis for the change in wavelength of the charge transfer transition and a better understanding of the differences between metastable species obtained at room temperature and those generated at cryogenic temperatures.

W-Pos271 AMINO TERMINAL SEMISYNTHESIS OF HEMOGLOBIN, Stanley A. Hefta, Stephen B. Lyle, Mark R. Busch, David E. Harris, and Frank R. N. Gurd, Department of Chemistry, Indiana University, Bloomington, IN 47405; Jeremy Johnson, George J. Turner, and Gary K. Ackers, Department of Biology, The Johns Hopkins University, Baltimore, MD 21218.

The amino termini of the α-chains of human hemoglobin are known to play an important role in the alkaline Bohr effect and to be involved in the binding of chloride ion and carbon dioxide. In order to characterize further this region of the molecule we have prepared des-$Val^{\alpha 1}$-hemoglobin by methods described earlier (Lyle et al., Biophys. J. 47, 85a, 1984) and hemoglobin rebuilt with substitution at the amino terminal position of the α chains by ^{13}C enriched glycine. Structural characterization of the truncated protein included amino terminal sequencing, amino acid analysis, electrophoresis, isoelectric focusing, tetramer-dimer dissociation studies and UV/visible spectroscopy. Functional characterization included oxygen equilibrium studies. The ^{13}C-$Gly^{\alpha 1}$-hemoglobin was examined similarly and with ^{13}C NMR to determine the amino terminal pK value in the presence and absence of various effectors. The enrichment with ^{13}C-glycine begins a series of experiments in which other ^{13}C enriched aliphatic amino acids are to be incorporated at the amino terminal position. (Supported by Public Health Service Research Grants HL-14680 and HL-05556.)

W-Pos272 ELECTRON TRANSFER MECHANISMS IN HEMOGLOBIN. Celia Bonaventura, Joseph Bonaventura, John Harrington, and Robert Cashon; Duke University Marine Laboratory, Beaufort, NC 28516.

It has recently been proposed that metalloproteins can, under some circumstances, act as an electrical conduit, avoiding the necessity for direct contact between oxidizing or reducing agents and the metal center. Hemoglobin can be used as a model protein for further studies of electron transfer reactions, in light of its ability to be reduced by NADPH and to catalyze NADPH-driven hydroxylation reactions. We are making use of variant human hemoglobins and hemoglobins with specialized functions found in a number of marine species to probe the role of specific structural elements in the electron transfer process. We have found that the reduction of ferric HbA by reduced nicotinamide nucleotides can occur under strictly anaerobic conditions and in the absence of mediators or enzymatic reductases. The rate of the reaction is decreased at high pH and at high ionic strength, and is inhibited by the co-addition of 2,3-diphosphoglycerate or inositol hexaphosphate. Reduction does not occur with Hb Providence Asp (beta 82 Lys→Asp) or sperm whale myoglobin. These results indicate that the beta chain anion binding site is involved and that the electron transfer does not involve activated oxygen species. Differences between various NADPH-reducible hemoglobins suggest an internal electron transfer mechanism that is modulated by protein conformation.

W-Pos273 Lifetimes and Correlation Times Associated with Tryptophan Fluorescence in Human Apohemoglobin. C. Fronticelli and E. Bucci. Biochemistry, University of Maryland School of Medicine, Baltimore, Maryland 21201.

The experiments were conducted at 7°C in 0.05 M phosphate buffer at pH 6.5 at protein concentrations near 2 mg/ml. Human apohemoglobin contains 2 tryptophans in the beta and 1 tryptophan in the alpha subunits. Their emission shows the presence of at least 3 lifetime components of 1.4 nsec, 3.5 nsec and 7.2 nsec respectively. A similar heterogeneity is present in samples unfolded in 6 M GuHCl, suggesting that the lifetime heterogeneity is intrinsic to the chromophores, rather than due to the three different emitters. The correlation times associated with these emissions were also heterogeneous and included a very short component of 1 nsec and a longer one of 12 nsec, (data corrected for water at 25°C). In the presence of 1 mM inositolhexaphosphate the lifetimes remained practically the same, however three correlation times were detectable of 1 nsec, 10 nsec and 25 nsec respectively. The longer correlation time of apohemoglobin in the presence of IHP is consistent with the polymerization of apohemoglobin produced by polyphosphates, as shown by Chu and Bucci (J. Biol. Chem. (1979) 254: 371). It was not possible to detect in the system correlation times of about 5 nsec as present in liganded hemoglobin (Sassaroli, M., Bucci, E., Steiner, R.F., J. Biol. Chem. (1982) 257: 10136).

W-Pos274 KINETICS OF THE GROWTH OF DOMAINS OF SICKLE HEMOGLOBIN POLYMERS S. Basak, F. A. Ferrone, A. J. Martino and H. X. Zhou, Department of Physics, Drexel University, Philadelphia, Pa. 19104.

Upon deoxygenation, sickle hemoglobin molecules assemble into multistranded fibers which themselves form well ordered spherulitic arrays called domains. These domains are thought to arise from the heterogeneous nucleation pathway for polymer growth. In static observations employing birefringence and linear dichroism domains have previously been found to be isotropic and homogeneous, with a high degree of polymer alignment. We have used a technique employing laser photolysis of COHbS on microscopic samples to initiate polymerization and subsequent domain formation. The evolution of the spatial character of the domain is monitored by light scattering (45° backscatter at 514 nm) or birefringence (light transmission through crossed polarizers at 488 nm). Using an externally triggered filter changer the two signals are alternately collected by a SIT Vidicon as a function of time. Spatial growth is analyzed by observing a 62.5μm square area of the sample and storing this image as 2500 discrete elements. Preliminary results indicate that domain formation consists of two phases. In the early phase both symmetric and asymmetric growth of domains has been observed. During this phase even symmetric domains are not radially isotropic. Although the domain expands (as measured by its radius of gyration) during this period, the increase in scattering is much more than can be accounted for by the increase in domain size. In the second phase the domain becomes structurally symmetric and spatially homogeneous and the changes in the scattered intensity, including pronounced overshoots, occur uniformly across the monitored region.

W-Pos275 TOWARD A KINETIC/EQUILIBRIUM MODEL FOR THE FORMATION OF SICKLE CELL HEMOGLOBIN NUCLEI.
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We have formulated an equilibrium and kinetic model to describe the time course for the formation of sickle cell hemoglobin (HbS) nuclei. Using this model, we have also predicted the length distribution of the polymers formed.

The solubility of deoxygenated sickle cell hemoglobin has been studied extensively. Upon establishing conditions where precipitation occurs, there exists a delay time before the presence of polymers can be detected. This delay time is indicative of an energetically unfavorable nucleation mechanism. During the delay time, progression toward an equilibrium distribution of unstable prenuclear complexes occurs until a significant concentration of the metastable nuclei are formed. The process is followed by polymer growth. We have observed that during slow deoxygenation of HbS, the total amount of birefringence arising from the HbS polymers is dependent upon the deoxygenation rate. More birefreingence is observed with slower deoxygenation rates, implying that slower deoxygenation forms more macroscopic domains. The free energy of alignment is greater for longer polymers; therefore, we postulate that deoxygenation rate alters the final polymer length distribution.

Our model describes the time course of nuclei formation which determines the total number of nuclei formed and consequently the polymer length distribution. The length distribution of the polymers could have profound effects in terms of establishing macroscopic domains that could distort the deoxygenated sickle cell. (Supported by NIH HL16008)

W-Pos276 PROTON HYPERFINE NUCLEAR MAGNETIC RESONANCE ASSIGNMENTS OF THE GLYCERA DIBRANCHIATA MONOMERIC MET-AQUO HEMOGLOBINS AND THEIR PH AND TEMPERATURE PROPERTIES.

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Glycera dibranchiata is a marine annelide which contains both a polymeric and a monomeric hemoglobin. NMR studies along with column chromatography and isoelectric focusing have determined the extent of heterogeneity of the monomeric fraction. It should be noted that the importance of the Glycera monomeric hemoglobins resides in the fact that both crystal structure and amino acid analysis of one of the components has demonstrated that it lacks the distal histidine at position E-7. Assignments of proton resonances which appear in the region 10-100 ppm and 0-30 ppm in the NMR spectra of the met (aquo) monomeric hemoglobins will be presented. These resonances have subsequently been studied over the pH range 4.0-10.0 and over the temperature range 5°-40°C.

W-Pos277 CYANIDE LIGAND BINDING AND PROTON NMR STUDIES OF GLYCERA DIBRANCHIATA MET-CYANO MONOMER HEMOGLOBINS.

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The monomer hemoglobin fraction from the marine annelid Glycera Dibranchiata has been shown to possess a three-dimensional structure similar to vertebrate hemoglobins with the exception that the distal histidine (primary sequence E-7) is replaced by leucine. Several investigators have discussed the influence such a substitution may have on ligand binding and the spectroscopy of static ligated states.

The results which will be presented here are for our studies of CN^- (as KCN) binding to the oxidized (met) forms of the proteins and subsequent NMR studies of the met-cyano forms. For Myoglobin, our values for K_{Diss} and Hill coefficient were in accordance with the literature. For the Glycera hemoglobin, however, the results were not comparable to the literature values. For both proteins it was found that the rate at which CN^- is bond is not instantaneous.

Furthermore, we have found that at different pH values a change in the apparant K_{Diss} was observed for the Glycera as well as for the myoglobin. Competing equilibria and a possible protonation of the ligand could account for this observation.

W-Pos278 THE NON-COVALENT COMPLEX BETWEEN CYTOCHROME C PEROXIDASE AND HORSE CYTOCHROME C.

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The mechanism of cytochrome c peroxidase (CcP) catalysis of the hydrogen peroxide oxidation of ferrous cytochrome c is thought to involve a molecular docking complex between the two proteins. We have initiated proton NMR studies of this complex. In contrast to other spectroscopic techniques NMR is remarkably sensitive to the complex being formed. Large changes in proton resonance linewidths and shifts are observed. These changes allow us to define the stoichiometry of the complex, reveal that the proteins are in fast exchange on the NMR time scale and allow us to estimate the off rates for this exchange.

W-Pos279 THE COVALENTLY CROSSLINKED COMPLEX BETWEEN CYTOCHROME C PEROXIDASE AND HORSE CYTOCHROME C.

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Cytochrome c peroxidase (CcP) catalyzes the oxidation of ferrous cytochrome c by hydrogen peroxide. Previous studies performed in other laboratories using a variety of techniques, have shown that cytochrome c binds to the peroxidase. In most of the studies, a 1:1 complex has been indicated although evidence for the existence of a second cytochrome c binding site on CcP has been obtained. In this study, CcP has been chemically crosslinked to cytochrome c through the use of a water soluble carbodiimide (EDC) by a modification of the method of Waldemeyer and coworkers. The crosslinking reaction has been performed using both ferrous and ferric cytochrome c and several differences were noted in the resulting crosslinked products.

The 1:1 CcP-cytochrome c complex has been purified in high enough yields to permit NMR studies to be performed. Our proton NMR results indicate large changes in the linewidths and chemical shifts of the heme methyl resonances of cytochrome c in addition to changes in the high-spin heme methyl resonances of CcP.

W-Pos280 KINETIC STUDIES OF REDUCTION OF THE CYTOCHROME C : CYTOCHROME C PEROXIDASE COMPLEX BY FREE FLAVIN SEMIQUINONES. James T. Hazzard and Gordon Tollin, Dept. of Biochemistry, University of Arizona, Tucson, AZ, 85721.

The kinetics of one-electron reduction by free flavin semiquinones, generated by laser flash photolysis, of the components of the preformed cytochrome c(Cyt c):cytochrome c peroxidase(CcP) complex have been studied. The reduction rate constant for complexed Cyt c decreases 73% to 90% for neutral flavin semiquinones relative to the free Cyt c, indicating that steric access of free flavins to the complexed Cyt c heme is significantly restricted. The rate constant for reduction of complexed Cyt c by the negatively charged FMNH· decreases with increasing ionic strength, implying an attractive electrostatic interaction of FMNH· with the interfacial region of the protein complex. This demonstrates that the electrostatic environment near the heme c in the complex is only minimally affected by CcP. The accessibility of the CcP heme to reductants is less affected by complexation, with a 45% decrease being found in the reduction rate constant for complexed CcP (III) relative to free CcP(III). Reduction of the two-electron oxidzed form of CcP(Fe(IV)O,$R^{+}\cdot$), by 5-deazariboflavin did not occur on fast time scales ($<$ 100ms). In the presence of Cyt c(III), however, one-electron intramolecular transfer from Cyt c was observed (k= 750 s^{-1}), suggesting a specific requirement for Cyt c in order to transfer electrons into the peroxide oxidized CcP. The intramolecular electron transfer from Cyt c to CcP(Fe(IV)O,R) gave a rate constant of 450 s^{-1}, suggesting that the oxidized species, $R^{+}\cdot$, has a small effect on the intramolecular electron transfer process. These results are analyzed in terms of the hypothetical Cyt c:CcP structural model proposed previously by Poulos and Kraut (1980).

W-Pos281 RAMAN AND OPTICAL STUDIES OF PULSED, PULSED PEROXIDE AND RESTING CYTOCHROME OXIDASE. M. Sassaroli, Y. Ching and D. L. Rousseau, AT&T Bell Laboratories, Murray Hill, NJ 07974.

The resonance Raman and optical absorption spectra of reduced cytochrome oxidase after exposure to oxygen was followed as a function of time. The enzyme was observed to pass through the "pulsed peroxide", the pulsed and then finally back to the resting states. The spectra of the initial resting state, prior to reduction, and the final resting state, after the functional turnover, are identical. In all of our Yonetani- or Caughey-type preparations we find that the resting enzyme shows the presence of some reduced cytochrome a. This reduced contribution is absent in both the pulsed and the pulsed peroxide intermediates. Resonance Raman spectra were obtained in both the low and high frequency regions to assess coordination, spin, and redox states at all stages of the functional cycle.

W-Pos282 CHARACTERIZATION OF RAT AND OPOSSUM MICROSOMAL CYTOCHROMES P-450 AND P-420 BY LOW TEMPERATURE VISIBLE AND FT-IR SPECTRA. **F.G. Fiamingo, R.A. Altschuld, K.A. Powell, and J.O. Alben. Dept. of Physiol. Chem. Ohio State Univ. Columbus, OH 43210**

Cytochrome P-450 is identified by its Soret absorption at 450nm when reduced and complexed with CO. This form reportedly can convert to a non-enzymatic form absorbing at 420 nm. We compared low temperature (10-80 K) visible and infrared spectra of dithionite reduced, CO exposed microsomes from rat and opossum livers. Both show Soret peaks near 422 and 450 nm that are at least partially quenched by photolysis with visible light (at 10 K). Infrared vibrational absorptions of the CO bound to cytochromes P-450 and P-420 are observed at 1948 and 1968 cm-1, respectively in microsomes from rats pre-treated with phenobarbital. The photodissociated form at 1968 cm-1 relaxed much faster than did the 1948 cm-1 form and was not observed in the Dark vs Light absorbance difference spectra above 40 K, whereas the 1948 cm-1 band could be observed at 80 K. Opossum liver microsomes are different in that while both P-450-CO and P-420-CO are present and photodissociable in the visible spectra, only a single, symmetric CO vibrational absorption is observed in the infrared at 1949 cm-1. This band is significantly narrower than those observed for the rat. These data suggest there are two types of P-450-CO. It is not known if these are due to species or metabolic differences. P-450-CO appears to be spectrally similar in both species, but with the narrower bandwidth from the opossum suggesting that it contains fewer isozymes.

W-Pos283 PROTON NMR SPECTRA OF CYTOCHROME c PEROXIDASE AND FLUORO-CYTOCHROME c PEROXIDASE: pH DEPENDENCE. James E. Erman, Lidia B. Vitello, and James D. Satterlee. Department of Chemistry, Northern Illinois University, DeKalb, IL, 60115 and Department of Chemistry, University of New Mexico, Albuquerque, NM 87131.

In solution, cytochrome c peroxidase exists in two distinct conformations related by an apparent pK_a near 5.5. This apparent ionization constant is markedly dependent upon ionic strength. The enzyme conformation stable at high pH is the active form of the enzyme, reacting rapidly with hydrogen peroxide, while the low pH form is inactive. The proton NMR spectrum of cytochrome c peroxidase is dependent upon the pH, with the upfield shifted resonances broadening significantly at low pH. Three histidine C-2 protons titrate and single ionizations between pH 4.5 and 10 with apparent pK_a values of 6.7, 6.7, and 7.9. Based on ring current calculations, the resonance with pK of 7.9 is tentatively assigned to His-181. Addition of fluoride to cytochrome c peroxidase causes substantial alterations in the NMR spectrum of the enzyme. However, the pH titration behavior of the three observable histidine residues in the enzyme-fluoride complex is essentially identical to that of the native enzyme, both in terms of the chemical shift and apparent pK_a values.

W-Pos284 THEORETICAL EVALUATION OF THE NON-COPLANARITY OF THE VINYL GROUPS IN DIVINYL PORPHYRINS Leonore A. Findsen and Robert R. Birge Department of Chemistry, Carnegie-Mellon University, Pittsburgh, PA 15213

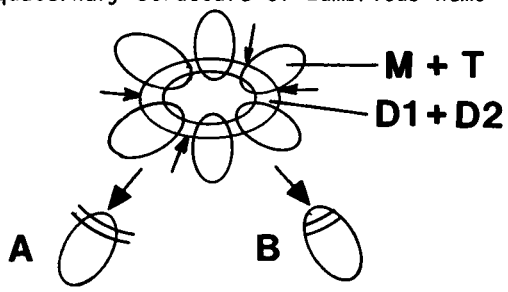
Porphyrins having vinyl substituents are common in nature (e.g., heme a, protoporphyrin). The extent to which vinyl groups conjugate into the porphyrin macrocycle (perturbing porphyrin electronic properties) and the steric interaction experienced by porphyrins inside a protein are strongly dependent upon vinyl-macrocycle dihedral angles. Recent NMR studies by Bothner-By, et al. (Org. Mag. Res., in press) examined the coplanarity of the free base and zinc complex of vinylphylloerythrin methyl ester. They determined a vinyl-macrocycle dihedral angle of 51^{o} for both of the above molecules. INDO semi-empirical molecular orbital theory was used to generate the potential energy surface of dihedral rotation of the vinyl group around the vinyl-porphyrin bond in several vinyl porphyrins, and predicts dihedral angles of approximately 50^{o} for the vinyl groups at room temperature. Although vinyl-macrocycle conjugation favors coplanarity of the vinyl group with the macrocycle, our calculations indicate that the close proximity of the vinyl hydrogens and the porphyrin meso hydrogens (<1Å) forces the vinyl group out of the plane of the macrocycle.

W-Pos285 ISOLATION OF THE SUBUNITS AND POLYPEPTIDE CHAINS OF THE EXTRACELLULAR HEMOGLOBIN OF LUMBRICUS TERRESTRIS, T. Gotoh, M. Mainwaring, F. Shishikura, D. Walz and S. Vinogradov, Departments of Physiology and Biochemistry, Wayne State University School of Medicine, Detroit MI 48201. The hemoglobin (M_r 3,900 kDa) consists of four subunits: M (16 kDa), D1 (31 kDa), D2 (37 kDa) and T (50 kDa). Gel filtration of the on Sephadex or Sephacryl columns at pH 9 or above results in the elution of two heme-containing fragments, one consisting of subunit M and the other of subunits D1+D2+T. Gel filtration of the latter on Sephadex G-100 at pH 4 resulted in the elution of the oxidized forms of subunits T and D2; subunit D1 appeared to adsorb irreversibly to the matrix. Gel filtration of the oxyhemoglobin on Sephadex G-100 at pH 4 resulted in the elution of the undissociated methemoglobin and of the oxidized subunits T, D1 and M; subunit D2 was irreversibly adsorbed to the matrix. Subunits T and D2, but not subunit D1, possessed absorbance at 415nm, suggesting that D2 but not D1 contained heme. Subunit T was reduced with DTT and subjected to either chromatofocusing on a PBE 94 column (Pharmacia) or to ion-exchange chromatography on a Mono Q column (Pharmacia) . By both methods subunit T was dissociated into three polypeptide chains which were identified by SDS PAGE to be chains II, III and IV possessing an M_r of 16 to 19 kDa. Of the three chains isolated by chromatofocusing, chains II and IV but not III had absorbance at 415nm, suggesting that chains II and IV but not III contained heme. Subunits M, D1 and D2 correspond to chains I, V and VI, respectively, observed in the SDS PAGE pattern of the reduced hemoglobin. The presence of heme in four out of the six chains explains why the 0.22% iron content of Lumbricus hemoglobin is about 2/3 of the iron content of all other vertebrate and invertebrate hemoglobins and myoglobins. Supported by USPHS Grants AM-30382 (to DAW) and HL-25952 (to SNV).

W-Pos286 PRIMARY STRUCTURE OF THE MONOMERIC SUBUNIT OF LUMBRICUS TERRESTRIS HEMOGLOBIN: HOMOLOGY WITH OTHER EXTRACELLULAR HEMOGLOBIN SUBUNITS. Fumio Shishikura, Toshio Gotoh, Daniel A. Walz, June Snow and Serge N. Vinogradov, Departments of Physiology and Biochemistry. Wayne State University, Detroit MI 48201. Annelid extracellular hemoglobins are giant (3,000 kDa) molecules having hexagonal bilayer structures. The hemoglobin of Lumbricus (3,900 kDa) consists of four subunits: a monomeric subunit M (16 kDa), two dimeric subunits D1 (31 kDa) and D2 (37 kDa) and a disulfide-bonded trimer T (50 kDa). The amino acid sequence of one of the three chains of subunit T has been determined by R. Garlick and A. Riggs (J. Biol. Chem. 257, 9005, 1982). We have established the amino acid sequence of Lumbricus subunit M globin chain by alignment of overlapping peptides derived from tryptic, S. aureus protease and cyanogen bromide fragmentation. The sequences of individual, highly purified peptides were determined with a Beckman spinning cup sequenator. We have compared the sequence of the Lumbricus subunit M to the amino acid sequences of the Lumbricus trimer chain, of the Tylorrhynchus monomer chain (S. Suzuki et al., Biochim. Biophys. Acta 708, 253, 1982) and of the three chains of the Tylorrhynchus trimer subunit (S. Suzuki et al., J. Biol. Chem. 260, 3145, 1985; ibid., 260, 11481, 1985; in preparation). There are at least 16 positions of amino acid identity among the six globin chains compared, including the proximal and distal histidine residues found in vertebrate hemoglobins and myoglobins. When the six globin chains were aligned for maximum homology it was found that most of the homology was located in the amino terminal halves of the chains and that the carboxyl terminal sequences were highly variable. There was about 37% sequence homology between the two Lumbricus globin sequences, similar to the extent of homology between the four Tylorrhynchus sequences. Supported by U. S. Public Health Service Grant AM 30382

W-Pos287 THE DISSOCIATION OF THE EXTRACELLULAR HEMOGLOBIN OF LUMBRICUS TERRESTRIS AT ACID pH AND ITS REASSOCIATION AT NEUTRAL pH. M. Mainwaring, S. Lugo, O. Kapp*, A. Crewe* and S. Vinogradov, Biochemistry Department, Wayne State University School of Medicine, Detroit, MI 48201 and *Enrico Fermi Institute, University of Chicago, Chicago, IL 60637. The oxy and CO forms of the Hb (M_r 3900 kDa) dissociated below pH 5.5. The extent of dissociation was unaffected by the presence of Ca(II) but was decreased by an increase in ionic strength. The oxy and CO hemoglobins exposed to pH 4.0 to 4.8 were centrifuged to give dissociated supernatant (S1) fractions, which were reassociated at pH 7.0 in sodium phosphate, Tris chloride, sodium cacodylate and imidazole chloride buffers and then centrifuged to give the reassociated (P2) and unreassociated (S2) fractions. Reassociation was possible only if S1 was dialyzed against water prior to the return to neutral pH; otherwise, precipitation occurred starting at pH about 5.3 . The extent of reassociation into whole molecules was 40% to 80% and was not affected by increase in ionic strength or the presence of Ca(II). Gel filtration of fractions P2 and S2 on Sephacryl S-300 in 0.1M Tris.Cl buffer pH 7.0, gave one peak (IaR) and four peaks Ia, Ib, II and III, respectively. IaR eluted at a slightly smaller volume than the native hemoglobin but had an identical SDS PAGE pattern. The M_r of peaks Ib, II and III were 200 kDa, 65 kDa and 18 kDa, respectively. The SDS PAGE of Ib, II and III showed that they consisted of all four subunits, subunits T+D1+D2 (chains II-VI) and subunit M (chain I), respectively. However, peak Ib was not a one twelfth of the whole molecule since it had a much reduced content of subunits D1 and D2 . Although the reassociated hemoglobin had a STEM appearance similar to that of the native oxyhemoglobin its vertex-to-vertex diameter was 25nm instead of 30nm and its height was 16nm instead of 20nm.. Supported by USPHS Grant HL-25952 (SNV) and by a grant from the Department of Energy (AVC).

W-Pos288 BRACELET PROTEIN: A NEW MODEL OF THE QUATERNARY STRUCTURE OF LUMBRICUS TERRESTRIS HEMOGLOBIN. S. Vinogradov, S. Lugo, M. Mainwaring and O. Kapp*, Biochemistry Department, Wayne State University School of Medicine, Detroit, Mi 48201 and *Enrico Fermi Institute, University of Chicago Chicago, IL 60637. The dissociation of the hemoglobin at alkaline and acid pH and at neutral pH in the presence of denaturants, chaotropic salts and complex anions always results in the formation of three fragments. The two smallest fragments are II (65 kDa) consisting of subunits D1+D2+T and III (ca. 18 kDa) consisting of subunit M; together, they reassociate to the hexagonal bilayer structure of the whole molecule. The third fragment, Ib (200 kDa, is always deficient in subunits D1 and D2 and is not in equilibrium with the whole molecule; thus, it is not a one twelfth of the whole molecule. To explain these facts, we propose a model of the quaternary structure of Lumbricus hemoglobin consisting of a "bracelet" of D1 and D2 subunits to which are attached twelve complexes of M and T subunits. In this model, shown as the top view of half a molecule, the subunit composition of a Ib fragment obtained by dissociation of the hexagonal bilayer structure, will be dictated by the location of the points at which occurs the scission of the "bracelet" of D1 and D2 subunits (indicated by arrows). In case A, but not in case B, the Ib fragment can reassociate to the whole molecule because it contains a sufficient number of D1 and D2 subunits. Supported by USPHS Grant HL-25952.



W-Pos289 THE DISSOCIATION OF THE EXTRACELLULAR HEMOGLOBIN OF LUMBRICUS TERRESTRIS AT NEUTRAL pH S. Lugo, O. Kapp*, M. Mainwaring and S. Vinogradov, Biochemistry Department, Wayne State University School of Medicine, Detroit, MI 48201 and *Enrico Fermi Institute, University of Chicago, Chicago, IL 60637. The dissociation of the hemoglobin (M_r 3900 kDa) at pH 7 in the presence of 1-4M urea, 1-2M guanidine hydrochloride, 0.5-5mM sodium dodecyl sulfate, 10-50mM sodium phosphotungstate, 20-100mM sodium phosphomolybdate, 0.7-1.5M sodium perchlorate, 0.7-1.5M potassium thiocyanate and 1-2M magnesium chloride was followed by centrifugation, gel filtration on Sephacryl S-200 columns and FPLC on Superose 6 columns (Pharmacia) in 0.1M sodium phosphate buffer pH 7, 1mM EDTA and by SDS PAGE and scanning transmission electron microscopy (STEM). Gel filtration of the supernatants obtained by centrifugation, in cases where dissociation was not complete, provided four peaks, Ia, Ib, II and III. Peak Ia eluted at a volume close to that of the native hemoglobin and consisted of all four subunits M, D1, D2 and T. The M_r of Ib, II and III were ca. 220 kDa, 65 kDa and 18 kDa, respectively. Peak Ib in all cases was found to be deficient in subunits D1 and D2 and hence was not a one twelfth of the native hemoglobin; in addition, it was not in equilibrium with the whole molecule. Peaks II and III consisted of subunits D1+D2+T and M, respectively, and when mixed together, reassociated to the hexagonal bilayer structure of the native hemoglobin. STEM images of peak Ia showed the presence of incomplete hexagonal structures missing a "pseudoprotomer", an apparent one twelfth. Repeated dissociations and gel filtration of peaks Ia and Ib demonstrated an increase in the presence of incomplete hexagonal bilayer molecules in the former and in the latter a decrease in the content of subunits D1 and D2, leading to the formation of fragments containing only the M and T subunits. Supported by U.S. Public Health Service Grant HL 25952 (to SV).

W-Pos290 OXYGENATION OF INDIVIDUAL SUBUNITS, TRIMERS AND PARTIALLY REASSEMBLED HEMOGLOBIN OF LUMBRICUS TERRESTRIS. Kenzo Fushitani and Austen F. Riggs, Department of Zoology, University of Texas, Austin, Texas 78712.

Hemoglobin from Lumbricus terrestris comprises four major, heme-containing subunits designated a, b, c, and d in equimolar proportions. Subunits a, b, and c form a disulfide-linked trimer. Oxygen-binding by whole molecules which contain about 200 subunits is highly cooperative with a pH-dependent Hill coefficient as high as 7.9. Oxygen equilibria of the individual subunits are neither cooperative nor pH dependent. Measurements made in 0.11 M bis-tris-propane, 0.11-0.13 M NaCl at 15 °C show that O_2-binding by the trimer has very low cooperativity but has a Bohr effect about 2/3 as large as that of whole molecules. Measurments in 0.11 M bis-tris-propane, 25 mM $CaCl_2$, 0.01-0.03 M NaCl show that Ca^{2+} ions almost double the Bohr effect bringing it closer to that of whole molecules which also show an enhanced Bohr effect under the same conditions. Since calcium ions have no effect on oxygen binding by the subunits, an oxygenation-linked calcium effect is evidently created by trimer formation. Addition of subunit d to the trimer results in substantial cooperativity which is absent from both the trimer and subunit. The oxygen equilibrium curve of the trimer-subunit d complex is very similar to that of whole molecules but the Hill coefficient is lower. The results indicate that conformational changes accompany formation of both trimer and the trimer-subunit d complex because each association is accompanied by new properties not present in the constituents themselves. (This work has been supported by grants from NSF PCM 8202760, DNB-8502857 and Welch Foundation grant F 213.)

W-Pos291 MONOCLONAL ANTIBODIES TO THE EXTRACELLULAR HEMOGLOBIN OF LUMBRICUS TERRESTRIS. J. Lightbody, E. Ziaja, S. Lugo, M. Mainwaring and S. Vinogradov, Biochemistry Department, Wayne State University, Detroit, MI 48201. Murine monoclonal antibodies to Lumbricus hemoglobin were prepared by a modification of the method of Kohler and Milstein (Nature 256, 495, 1975). Approximately 230 clones were obtained that were reactive towards the hemoglobin using an ELISA assay. These clones were tested for activity towards the four subunits of the hemoglobin, M, D1, D2 and T isolated by gel filtration at acid pH and by gel filtration in SDS at neutral pH. About 150 clones reacted with the native hemoglobin and with all four subunits and about 50 clones reacted with the hemoglobin and with various combinations of subunits. The remaining 30 clones were found to react with the native hemoglobin but not with any of the subunits. Our interpretation of the latter result is that the antibodies secreted by these clones are reactive towards conformation-dependent epitopes characteristic of a complete hexagonal bilayer structure. In addition, we examined the reactivity of the 30 clones towards the reassociated whole molecules IaR obtained subsequent to dissociation at alkaline and at acid pH and dissociation at neutral pH in the presence of chaotropic salts, denaturants and complex anions using an ELISA assay. Although the IaR obtained subsequent to dissociation at alkaline pH and subsequent to removal of dissociating agents at neutral pH reacted with all 30 clones, the IaR obtained subsequent to dissociation at acid pH reacted with only 10 of the 30 clones. It is known that although the IaR obtained subsequent to dissociation at acid pH have a hexagonal bilayer structure their vertex-to-vertex diameter and height are about 20% smaller than the corresponding dimensions of the native hemoglobin (see abstract by Mainwaring et al.). Supported by U. S. Public Health Service Grant HL 25952.

W-Pos292 MONOCLONAL ANTIBODIES TO THE EXTRACELLULAR HEMOGLOBIN OF LUMBRICUS TERRESTRIS. J. Lightbody, E. Ziaja, S. Lugo, M. Mainwaring and S. Vinogradov, Biochemistry Department, Wayne State University, Detroit, MI 48201. Murine monoclonal antibodies to Lumbricus hemoglobin were prepared by a modification of the method of Kohler and Milstein (Nature 256, 495, 1975). Approximately 230 clones were obtained that were reactive towards the hemoglobin using an ELISA assay. These clones were tested for activity towards the four subunits of the hemoglobin, M, D1, D2 and T isolated by gel filtration at acid pH and by gel filtration in SDS at neutral pH. About 150 clones reacted with the native hemoglobin and with all four subunits and about 50 clones reacted with the hemoglobin and with various combinations of subunits. The remaining 30 clones were found to react with the native hemoglobin but not with any of the subunits. Our interpretation of the latter result is that the antibodies secreted by these clones are reactive towards conformation-dependent epitopes characteristic of a complete hexagonal bilayer structure. In addition, we examined the reactivity of the 30 clones towards the reassociated whole molecules IaR obtained subsequent to dissociation at alkaline and at acid pH and dissociation at neutral pH in the presence of chaotropic salts, denaturants and complex anions using an ELISA assay. Although the IaR obtained subsequent to dissociation at alkaline pH and subsequent to removal of dissociating agents at neutral pH reacted with all 30 clones, the IaR obtained subsequent to dissociation at acid pH reacted with only 10 of the 30 clones. It is known that although the IaR obtained subsequent to dissociation at acid pH have a hexagonal bilayer structure their vertex-to-vertex diameter and height are about 20% smaller than the corresponding dimensions of the native hemoglobin (see abstract by Mainwaring et al.). Supported by U. S. Public Health Service Grant HL 25952.

W-Pos293 STIMULATION OF SODIUM-CALCIUM EXCHANGE ACTIVITY IN CARDIAC SARCOLEMMAL VESICLES BY INTRAVESICULAR CALCIUM. Reeves, J.P. and Poronnik, P., Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ 07110 USA.

When cardiac sarcolemmal vesicles were incubated at 37°C in 160 mM NaCl containing 0.5 mM $CaCl_2$ and subsequently assayed for Na-Ca exchange activity, they exhibited a 3-fold increase in the initial rate of $^{45}Ca^{2+}$ uptake (at 20 μM $^{45}Ca^{2+}$) compared to vesicles incubated without added $CaCl_2$. Removal of endogenous Ca^{2+} by incubation of the vesicles with 0.1 mM EGTA resulted in a 35% inhibition in exchange activity. The pretreatment with $CaCl_2$ produced an acceleration of Na-Ca exchange activity rather than an increase in Ca^{2+} uptake due to Ca-Ca exchange; this was shown by the stimulation of Ca^{2+} uptake in the Ca-treated vesicles by K-valinomycin (a reflection of the electrogenic nature of Na-Ca exchange) as well as by the inability to detect Ca-Ca exchange under these conditions using vesicles preloaded with $^{45}Ca^{2+}$. Pretreatment of the vesicles with $CaCl_2$ lowered the apparent K_m of the exchange system for Ca^{2+}. The effects of the Ca-treatment were reversed by subsequently incubating the vesicles with EGTA. These effects of intravesicular Ca^{2+} undermine the application of Michaelis-Menten kinetics to Na-Ca exchange activity in sarcolemmal vesicles. Since exchange activity necessarily alters intravesicular Ca^{2+} levels, the kinetics of the exchange system will be altered as a consequence of its own activity and the conventional concept of a defined K_m and V_{max} is not meaningful under such circumstances. This phenomenon may contribute to the large variability that has been reported among different vesicle preparations in their apparent K_m values for Ca^{2+} (1.5 to >100 μM).

W-Pos294 SODIUM-INDEPENDENT Ca^{2+} UPTAKE IN CARDIAC SARCOLEMMAL (SL) VESICLES: Ca^{2+} CHANNEL OR Na^+-Ca^{2+} EXCHANGE? Kinya Otsu and Jeffrey Froehlich GRC, NIA, NIH, Baltimore, Maryland 21224.

Rapid mixing was used to investigate the time course of Na_i^+-independent Ca^{2+} uptake in bovine cardiac SL membrane vesicles at 21°C. The vesicles were washed with EGTA prior to assay in order to minimize the probability of Ca^{2+}-Ca^{2+} exchange. When valinomycin-treated SL vesicles loaded with 160 mM KCl and 20 mM MOPS-Tris (pH 7.4) were diluted 1:20 into 160 mM choline chloride containing 50 μM $^{45}CaCl_2$ (to generate an inside negative potential) and subsequently mixed with 1 mM EGTA, the resulting time course of Ca^{2+} uptake was triphasic: (1) a fast exponential (FE) phase (110 s^{-1}), (2) a slow exponential (SE) phase (11 s^{-1}), and (3) a linear (L) phase (.113 nmol mg^{-1} s^{-1}). The amount of Ca^{2+} taken up in the first two phases was .09 nmol mg^{-1} with 20% and 80% in the FE and SE phases, respectively. A similar pattern was observed when 160 mM choline-loaded vesicles were diluted into either 160 mM KCl (inside positive potential) or 160 mM choline chloride (zero potential) except that the SE phase was slower (4-5 s^{-1}) in both cases. Additional effects noted in the absence of depolarization were a reduction in the amount of Ca^{2+} taken up in the FE and SE phases (.03 nmol mg^{-1}) and a decrease in the velocity of the L phase (.054 nmol mg^{-1} s^{-1}). Addition of NaCl to the dilution medium inhibited Ca^{2+} uptake to a maximum extent of 60% with a K_I of 10 mM and Hill coefficient of 1.8. In contrast, verapamil and nitrendipene had no effect on Ca^{2+} uptake. The marked similarity between the inhibitory effects of extravesicular Na^+ on Na_i^+-independent Ca^{2+} uptake and Na^+-Ca^{2+} exchange and the lack of effect of Ca^{2+} channel blockers on the Na_i^+-independent pathway indicate that a major portion of the activity measured in choline- and K^+-loaded vesicles is due to the Na^+-Ca^{2+} exchanger.

W-Pos295 INTERACTION OF BEPRIDIL AND AMILORIDE WITH THE NA-CA ANTIPORTER IN CARDIAC SARCOLEMMAL VESICLES. M.L. Garcia, V.F. King and G.J. Kaczorowski, Merck Institute, Rahway NJ 07065.

Bepridil (BPD), N-3,4-dichlorobenzylamiloride (DCB) and N-1-naphthylmethylamiloride (NMA) are all potent inhibitors of Na-Ca exchange in cardiac sarcolemmal membrane vesicles with K_i's of 30, 22 and 20 μM, respectively. Their mechanism of inhibition appears similar since all are noncompetitive *vs.* Ca but competitive *vs.* Na, which suggests an interaction at the carrier's substrate binding sites. However, inhibition by BPD is partial (ie. Na_i-dependent Ca uptake is blocked maximally 50%). To determine the relationship between these inhibitors, exchange was monitored with mixtures of blockers present. At low fixed DCB or NMA concentrations and varying BPD present, Dixon plots display parallel lines suggesting a competitive interaction between inhibitors. As either amiloride is increased, however, the data no longer yield parallel lines. Dixon plots of amiloride inhibition with either DCB or NMA fixed and the other analog varied yield nonlinear data curving upward, indicating a complicated interaction of inhibitor with the carrier. Inhibition by BPD displays a Hill coefficient n=1, but similar analysis with DCB or NMA yields biphasic Hill plots with n=1 and n=1.5-2.2 at low and high inhibitor concentrations, respectively. Analysis of inhibition with both DCB and NMA present yields n values of ca. 2.3. These results suggest that DCB and NMA interact with more than one type Na binding site on the carrier while BPD interacts at only one common Na site. At low amiloride concentrations, interaction occurs preferentially at the site where either the third transported Na or BPD binds, while at higher concentrations, DCB and NMA bind in addition to another site which may be common for Na and Ca. This is consistent with kinetic data on amiloride inhibition which reveal mixed kinetics *vs.* Ca with a major noncompetitive component. The partial inhibition of exchange activity by BPD could be related to its interaction at only one Na binding site.

W-Pos296 IDENTIFICATION OF Na-Ca EXCHANGE IN SARCOLEMMAL FRACTIONS FROM TRACHEAL SMOOTH MUSCLE: COMPARISON WITH CARDIAC SARCOLEMMAL Na-Ca EXCHANGE. Robert Slaughter, Ann Welton, and Douglas Morgan, Hoffmann-La Roche Inc., Nutley, NJ 07110.

A Na^+ dependent Ca^{2+} exchange activity differing from that in heart has been identified in sarcolemmal (plasma membrane) vesicles prepared from bovine tracheal smooth muscle. These vesicles were separated from other membrane types through differential and sucrose density step gradient centrifugation. The exchange process co-purified with 5'-nucleotidase, a plasma membrane marker enzyme, and was significantly enriched (>100-fold) compared to mitochondria (cytochrome c oxidase) but only slightly enriched (4-fold) compared to sarcoplasmic reticulum (NADPH cytochrome c reductase). The Na^+ dependence of Ca^{2+} transport was demonstrated through both uptake and efflux procedures. The uptake profile with respect to Ca^{2+} was monotonic with a linear v_o vs $v_o S^{-1}$ plot. The resultant K_m from the airway sarcolemmal vesicles (20 μM) was similar in magnitude to the K_m of cardiac sarcolemmal vesicles (30 μM). Tracheal vesicles demonstrated a V_{max} of 0.3 to 0.5 $\mu mol.mg^{-1}.sec^{-1}$ which corresponds to 15 to 50% of activity found in cardiac vesicles, a known source of high Na-Ca exchange activity. However, two processes found to stimulate cardiac Na-Ca exchange, pretreatment with either dithiothreitol and Fe^{2+} or with chymotrypsin, were ineffective on the tracheal smooth muscle. Thus, the Na-Ca exchanger identified in tracheal smooth muscle appears to be different from that observed in cardiac muscle implying that regulation of this activity may also be different.

W-Pos297 CALCIUM INFLUX AND SODIUM EFFLUX MEDIATED BY THE Na/Ca EXCHANGER IN GIANT BARNACLE MUSCLE CELLS ARE PROMOTED BY INTRACELLULAR Ca^{2+}. H. Rasgado-Flores, E. Santiago, and M.P. Blaustein. Dept. of Physiol., Univ. of Maryland Sch. Med., Baltimore Md, 21201.

The external Ca (Ca_o)-dependent ^{22}Na efflux and internal Na (Na_i)-dependent ^{45}Ca influx were studied in internally-perfused giant barnacle muscle cells. The external superfusion solution (ESS) contained (mM): 456 Li, 10 K, 25 Mg, 11 Ca, 538 Cl, 6 Tris (pH 7.8) and 0.1 ouabain. The internal perfusion fluid (IPF) contained (mM): 40 Na, 200 K, 10 Mg, 202 glutamate, 58 Cl, 285 sucrose, 3 ATP (and an ATP regenerating system), 60 HEPES (pH 7.3), 8 EGTA, and various amounts of Ca to obtain the desired free Ca^{2+} concentratrions. To study the Na_i-dependent Ca influx, $[Na^+]_i$ was reduced to 10 mM (Na glutamate replaced by sucrose); to study the Ca_o-dependent Na efflux, Ca_o was replaced by Mg. To reduce the background Ca influx, 0.5-1 mM $LaCl_3$ was usually added to the ESS. With $[Ca^{2+}]_i$ = 10 nM, removal of Ca_o had no effect of Na efflux, and reduction of $[Na^+]_i$ to 10 mM had no effect on Ca influx. However, when $[Ca^{2+}]_i$ was increased, both the Na_i-dependent Ca influx and the Ca_o-dependent Na efflux were activated; half-maximal activation for both fluxes occured at a $[Ca^{2+}]_i$ of about 700 nM. Both fluxes were also inhibited by external La^{3+}, with half-maximal inhibition at about 0.5 mM. The Ca_o-dependent Na efflux was also observed when the ESS contained Tris instead of Li, and when the ATP was omitted and apyrase (3 U/ml) was added to the IPF. These data clearly indicate that the Ca_o-dependent Na efflux and the Na_i-dependent Ca influx are coupled; the fluxes appear to be mediated by the Na/Ca exchanger working in the Ca influx/Na efflux ("reverse") mode. This exchange is not fueled by ATP, although it is possible that ATP alters the kinetics of the exchange. Supported by AHA, MDA and NIH.

W-Pos298 MEAN BLOOD PRESSURE IS ELEVATED IN RATS AFTER TREATMENT WITH STREPTOZOTOCIN. J.H. Pizzonia, D.G. Brunder, J.J. Oleynek, and R.D. Moore. SUNY, Plattsburgh, N.Y. and Dept. of Physiology and Biophysics College of Medicine, University of Vermont, Burlington, Vt.

In the present study the effect of a 60 mg/kg dose of streptozotocin (injected intraperitoneal) on blood pressure was examined in Sprague-Dawley rats. Blood pressure measurements were made using the tail cuff method every 4 to 7 days over a period of 28 days. The mean blood pressure $\pm$SE for 14 sham injected controls was 112.4$\pm$1.7 while the mean blood pressure for 8 streptozotocin treated animals was 123.4$\pm$4.5. This 9% increase is significant ($P < 0.05$, two tailed). Intraperitoneal injection of streptozotocin lowers plasma insulin producing a diabetic state, and elevates intracellular sodium by about 30% (Moore and coworkers J. Physiol. 1983 338:277-294). An increase in intracellular sodium represents a decrease Δ_{Na} which would be expected to decrease Na/Ca exchange (see review by Moore:Biochim. Biophys. Acta 1983 737:1-49). Elevated intracellular sodium of arteriolar smooth muscle cells has been implicated in essential hypertension. There is now considerable evidence that Na:Ca exchange exists in arteriolar smooth muscle cells. Thus either an increase in intracellular sodium should decrease Na:Ca exchange resulting in an increase in intracellular Ca^{2+} or activation of voltage sensitive pathways by a decrease in membrane potential should allow an increase in intracellular free Ca^{2+}. The latter would tend to increase contractile tone of these muscle cells thus elevating blood pressure (see Moore:The Role of Intracellular pH in Insulin Action and Diabetes Mellitus; In: Na^+-H^+ Exchange, Intracellular pH, and Cell Function. P.S. Aronson and W.F. Boron eds. A.P., N. Y. (In Press). [This work was supported by NIH grant # AM21059 and N. E. Sect. N. Y. Heart Assn. 329-C172-A.]

W-Pos299 THE ROLE OF CYSTEINE-154 IN THE *ESCHERICHIA COLI LAC* PERMEASE AS DETERMINED BY OLIGONUCLEOTIDE-DIRECTED, SITE-SPECIFIC MUTAGENESIS. D.R. Menick, H.K. Sarkar, J.A. Lee, M.S. Poonian*, and H.R. Kaback. Roche Institute of Molecular Biology, Roche Research Center, and *Department of Molecular Genetics, Hoffmann-La Roche Inc., Nutley, NJ 07110.

In previous studies (W.R. Trumble, P.V. Viitanen, H.K. Sarkar, M.S. Poonian, and H.R. Kaback [1984] *Biochem. Biophys. Res. Commun.* **119**, 860-867; P.V. Viitanen, D.R. Menick, H.K. Sarkar, W.R. Trumble, and H.R. Kaback [1985] *Biochemistry*, in press), the *lac* permease of *Escherichia coli* was modified by site-directed mutagenesis such that cys_{148} was replaced with either *gly* or *ser*. It was demonstrated that cys_{148} is not obligatory for lactose:proton symport, but is essential for substrate protection against sulfhydryl inactivation. In this work, another cysteine residue, cys_{154}, was replaced by either a *gly* or *ser* residue. Permease with *gly* in place of cys_{154} exhibits essentially no transport activity, and substitution of cys_{154} with *ser* also causes marked, though less complete loss of activity. Immunoblot analysis with Mab 4A10R and [^{125}I]-labeled protein A demonstrates that the permease molecules encoded by the mutated *lac Y* genes are incorporated into the membrane at levels comparable to that observed in wild-type membranes. The findings suggest that cys_{154} plays an important role in lactose:H^+ symport.

W-Pos300 OLIGONUCLEOTIDE-DIRECTED, SITE-SPECIFIC MUTAGENESIS OF HISTIDINE RESIDUES IN THE *LAC* PERMEASE OF *ESCHERICHIA COLI*. H.K. Sarkar, I. Püttner, E. Padan, P. Viitanen, M.S. Poonian*, and H.R. Kaback. Roche Institute of Molecular Biology, Roche Research Center, and *Department of Molecular Genetics, Hoffmann-La Roche Inc., Nutley, NJ 07110.

The *lacY* gene of *Escherichia coli* which encodes the *lac* permease has been modified by oligonucleotide-directed, site-specific mutagenesis such that each of the four *his* residues in the molecule is replaced with an *arg* residue. Replacement of his_{35} and his_{39} with *arg* has no apparent effect on permease activity. In contrast, replacement of either his_{205} or his_{322} with *arg* causes a dramatic loss of transport activity. Interestingly, although substitution of his_{205} or his_{322} with *arg* results in loss of ability to catalyze active lactose transport, permease molecules with *arg* at residue 322 appear to facilitate downhill lactose movements at high concentrations of the disaccharide in cells, membrane vesicles and in proteoliposomes reconstituted with purified permease molecules. However, downhill movement of lactose does not lead to alkalization of the medium (i.e., lactose and H^+ translocation are uncoupled in this mutant). Finally, a number of spontaneous revertants of the his_{205} mutant have been isolated, and partial sequencing of *lacY* in each of the plasmids reveals back mutations from *arg* to *his* in codon 205. The results provide support for the contention that *his* residues in the *lac* permease play an important role in the coupling between lactose and proton translocation.

W-Pos301 **STUDIES OF CALCIUM BINDING AND ACTIVATION IN Ca^{2+}ATPase PURIFIED FROM THE ERYTHROCYTE MEMBRANE**

Danuta Kosk-Kosicka, Sonia Scaillet, and Giuseppe Inesi; University of Maryland, School of Medicine, Baltimore, MD 21201

Ca^{2+}-ATPase was purified from erythrocyte ghosts on calmodulin column in the presence of the nonionic detergent $C_{12}E_8$ and supplemented with phosphatidylcholine. The activity of the purified enzyme was comparable to its activity in the ghost membrane at 37°C with respect to the velocity dependence on Ca^{2+} and Mg^{2+} concentration, calmodulin concentration, calmodulin stimulation on Ca^{2+} and Mg^{2+} concentrations, and pH. Further, it was shown that in appropriate conditions, the purified ATPase at high protein concentration in the absence of calmodulin exhibits Ca^{2+} concentration dependence identical to that in the presence of calmodulin at low ATPase concentration. Calcium binding to the purified ATPase in the less diluted state was then assessed by measurements of the ATPase intrinsic tryptophan fluorescence. Ca^{2+} binding isotherms obtained by measuring fluorescence intensity were identical to curves representing the Ca^{2+} concentration dependence of ATPase activity, and demonstrated that cooperativity is in fact a feature of the binding mechanism, rather than an apparent effect of enzyme kinetics. Loss of cooperativity and a reduction of the ATPase affinity for Ca^{2+} observed for the highly diluted purified ATPase was found to be temperature dependent. It is demonstrated that specific Ca^{2+} binding by erythrocyte membrane Ca^{2+}-ATPase is influenced by hydrophobic interactions of binding domains, and that this interaction is regulated by calmodulin. (Supported by USPHS (HL 27867) and MDA).

W-Pos302 ^{3}H-BUMETANIDE BINDING TO DUCK RED CELLS AND INHIBITION OF Na+K+Cl COTRANSPORT. Mark Haas and Bliss Forbush III, Departments of Pathology and Physiology, Yale University School of Medicine, New Haven, CT 06510.

Bumetanide (BU) is a potent inhibitor of Na+K+Cl cotransport in many cell types, including duck red cells (RBC). Previous studies have demonstrated marked similarities between ^{3}H-BU binding to membranes from dog kidney (Forbush and Palfrey, J.B.C. 258: 11787, 1983) and BU inhibition of duck RBC cotransport (Haas and McManus, Am. J. Physiol. 245: C235, 1983). Thus we studied equilibrium binding of ^{3}H-BU to intact duck RBC under conditions known to affect Na+K+Cl cotransport. We found ~1000 ^{3}H-BU binding sites per RBC, implying a turnover number of ~5000/sec for the cotransporter. Saturable binding of ^{3}H-BU to duck RBC is stimulated 17-fold by norepinephrine or 8-fold by cell shrinkage, conditions under which cotransport is stimulated to a similar extent. Under optimal ionic conditions, binding of ^{3}H-BU was half-maximal at 0.15 uM, the same level required for half-maximal BU inhibition of Na+K+Cl cotransport under similar conditions. Binding of ^{3}H-BU to duck RBC is stimulated in a saturable manner by increasing extracellular Na and K, as was the case with inhibition of Na+K+Cl cotransport by BU (Haas and McManus, Biophys. J. 37: 214a, 1982). The level of $[K]_o$ required to half-maximally stimulate ^{3}H-BU binding is dependent on $[Na]_o$, and vice versa. Chloride has a biphasic effect on ^{3}H-BU binding; increasing $[Cl]_o$ (by replacement of methanesulfonate at constant $[Cl]_c/[Cl]_o$) from zero to 16 mM markedly enhances binding, whereas increasing $[Cl]_o$ to 160 mM inhibits binding. These findings convincingly demonstrate that the ^{3}H-BU binding sites are inhibitory sites on the cotransporter, and suggest the stable inhibited conformation of the cotransporter is (Na+K+Cl+BU). (Supp. by AM-17433 and GM-07562).

W-Pos303 OUABAIN-SENSITIVE Na/K EXCHANGE, Na/Na EXCHANGE and UNCOUPLED Na EFFLUX ARE INSENSITIVE TO CHANGES IN MEMBRANE POTENTIAL, Em, IN INTACT HUMAN RED BLOOD CELLS. M.A. Milanick and J.F. Hoffman, Dept. of Physiology, Yale Univ. Sch. of Med., New Haven, CT 06510

By treatment with DIDS, Em in red cells can be varied while maintaining pH constant (pHi=pHo= 7.4). When Em was varied by external anion substitution [$(sulfate)_i$ cells: Em= -50 mV in $(nitrate)_o$ and + 100 mV in $(gluconate)_o$ or $(p\text{-amino hippuric acid})_o$; $(Cl)_i$ cells: Em= -10mV in $(Cl)_o$ and + 100 mV in $(gluconate)_o$] the ouabain-sensitive efflux of Na ($^{o}M^{ouab}_{Na}$) and influx of K ($^{1}M^{ouab}_{K}$) did not vary. In a Na free medium, the $^{o}M^{ouab}_{Na}$ was independent of K_o from 2 to 180 mM (E_K varied from -100 to + 10 mV) with or without valinomycin. Varying Em by varying external anions had little or no effect on the rate of uncoupled Na efflux ($Na_o=K_o=0$) and Na/Na exchange ($K_o=0$). These results imply that the rate limiting steps for these modes [perhaps K deocclusion (Na/K), E-P breakdown (uncoupled Na), and ADP binding(Na/Na)] do not involve net charge movement across a domain of the membrane where the electric field varies. Though the $^{1}M^{ouab}_{K}$ into cells loaded by the nystatin technique with Li (150 mM) was about 1/3 of the rate into cells that contained 150 mM Na, Em had no effect on the rate of the $^{1}M^{ouab}_{K}$. The range of conditions studied was evidently not sufficient to detect effects of Em on the different transport processes. In all these experiments in intact cells, the energy of hydrolysis of ATP exceeds the work the pump must do. (Indeed the ion movments are downhill for the uncoupled Na efflux, Na/Na exchange and Li/K exchange.) Thus we are currently studying the effect of Em under other conditions including varying ATP/ADP ratios, orthophosphate concentrations and the Na and K gradients and on these and other modes including K/K and pump reversal.(Supported by NIH-HL09906 and AM 17433)

W-Pos304 CALMODULIN STIMULATES RESTING Ca^{2+} TRANSPORT RATE IN GHOSTS CONTAINING AN ATP REGENERATING SYSTEM AND QUIN-2. M. James-Kracke and J. Chai. (Intr. by G. Kracke) SUNY Upstate Medical Center, Syracuse, N.Y., 13210.

Calmodulin has been demonstrated to stimulate Ca^{2+} transport extensively at high $[Ca^{2+}]_i$ but the extent of its contribution has been in question at the resting level. Although the differences are small, a lower steady state level of $[Ca^{2+}]_i$ (approx. 20 nM) is attained when calmodulin (200 ng/ml) is incorporated into ghosts along with 3 mM ATP, 5 mM phosphocreatine and 40 ug/ml of phospocreatine kinase and 250 uM Quin-2 (or 25 uM Fura-2). Transport is demonstrable when ATP is incorporated alone, (steady state level of approx. 35 nM) but the rate of transport is greatly stimulated by maintaining higher ATP concentrations with an ATP regenerating system (steady state level of 25 nM). The effect of calmodulin is only demonstrable when mM ATP levels are maintained with the regenerating system. The same steady state level of $[Ca^{2+}]_i$ is approached whether the initial $[Ca^{2+}]_i$ is high (ghosts loaded with Ca^{2+}) or low (no Ca^{2+} incorporated into ghosts) indicating that the balance between passive influx and active efflux sets the steady state level of $[Ca^{2+}]_i$. This observation demonstrates that the fluorescent chelator is not artificially setting the steady state level. Plots of $d[Ca^{2+}]_i/dt$ versus the $[Ca^{2+}]_i$ curve upward rapidly and appear to be the lower portion of a sigmoid but because of the saturation of Quin-2 above 1 uM, only the lower portion of the curve (physiological end) can be analyzed. Linear transformation of the curve requires the inverse of the rate to be plotted versus $1/[Ca^{2+}]_i^2$ supporting a cooperative transport of 2 calciums per pump cycle,. Supported by the Muscular Dystrophy Association and the N.Y. Affiliate of the American Heart Association/Mid-Hudson Chapter.

W-Pos305 THE EFFECT OF HEART, KIDNEY, AND BRAIN EXTRACTS ON CA-DEPENDENT INHIBITION OF THE Na,K-ATPase D. R. Yingst, T. M. Jones, and D. M. Polasek, Dept. of Physiology, Wayne State University, Detroit, MI 48201

Ca-dependent inhibition of the Na,K-ATPase of human red blood cells was markedly increased by extracts of bovine brain and kidney and human red cells purified by means of Ca-dependent hydrophobic-interaction chromatography (Walsh, M. P. et. al., 1984. Biochem. J. 224:117-127), indicating they may contain calnaktin. Calnaktin is a proposed cytoplasmic protein from red cells that reversibly increases Ca-dependent inhibition of the Na,K-ATPase (Yingst, D. R. et al., 1985. Biophys. J. 47:342a). Cells from bovine heart, kidney, and brain and human red cells were hemolyzed and homogenized and the subsequent supernatant applied to DEAE sephadex. Active fractions were eluted with NaCl. $CaCl_2$ was added to the fractions from kidney, brain, and red cell which were then applied to phenyl sepharose columns equilibrated with Ca. The sepharose was washed with 1 M NaCl and Ca to elute proteins that were nonspecifically bound, and then eluted with a low ionic strength solution containing EGTA to chelate the Ca. After washing all the fractions free of salt and EGTA, they were tested for their ability to increase Ca-dependent inhibition of the Na,K-ATPase of human red blood cells at 2 uM free Ca. The proteins eluted in the presence of EGTA increased Ca-dependent inhibition of the Na,K-ATPase, whereas those that either did not bind to the sepharose at all or were eluted with 1 M NaCl had no effect. Thus, calnaktin interacts with phenyl spharose as predicted for other Ca binding proteins and calnaktin may also be present in bovine heart, kidney, and brain. (Supported by NIH (GM 3223-02) and a RCDA (AM 01253-02) from NIH to D.R.Y.)

W-Pos306 WHERE DO THE DEPLETED PLASMA AMINO ACIDS GO IN PKU? H. N. Christensen, Department of Biological Chemistry, The University of Michigan, Ann Arbor, MI 48109

Although the hyperphenylalaninemia of PKU is believed to interfere with transport of certain amino acids into the developing brain, an accompanying lowering of the plasma levels of several amino acids is unexplained. The following observations I now bring into focus: 1. Unless transport is perturbed, spontaneous variations of the levels of a given amino acid tend to occur together in plasma, liver, and muscle, as shown for glycine in the guinea pig. 2. An excess of a single amino acid of one group (ala,ser,thr,pro) lowered the muscle and liver glycine levels, relative to plasma; a different group (trp,phe,leu,ileu,tyr,his) tended to elevate muscle glycine (J.Biol.Chem.172:515, 1948). 3. This partition into two groups corresponds to steeply uphill, Na^+-dependent inward amino acid transport, inhibited by the first group, and downhill, outward transport by System L, by the second group, a directional cooperation between these systems shown in other instances. 3. Intraperitoneal administration to the rat of an amino acid analog largely specific to the uphill inflow sharply lowers the hepatic levels of most of the neutral amino acids, whereas a bicyclic amino acid largely specific to System L elevates the hepatic levels of pro, ala, met, leu, val, ileu, and orn. These opposite actions show that the effects of Item 2 are not restricted to glycine. 5. System L operates inward across the blood-brain barrier for most of its substrate amino acids. These effects allow excess phenylalanine, and also leucine as shown by Nyhan et al., to lower plasma levels of various amino acids, this action arising I propose from their sequestration in the liver, muscle, & other tissues. Accelerated amino acid catabolism probably results in these tissues. This trapping thus adds a second factor tending to deprive the brain of amino acids in PKU, although in a less predictable pattern, and perhaps also in the leucinemia of maple syrup urine disease. Ackn.HD01233,NIH.

W-Pos307 MAGNESIUM REGULATION IN SQUID GIANT AXONS STUDIED OPTICALLY WITH ERIOCHROME BLUE. R. E. Chandler, H. Gonzalez-Serratos, H. Rasgado, and R. A. Sjodin. Department of Biophysics, University of Maryland School of Medicine, Baltimore, MD 21201.

Squid giant axons were microinjected with eriochrome blue dye (EB) for monitoring the internal ionized magnesium concentration. The sensitivity of the value of $[Mg^{2+}]_i$ so monitored to the external value of $[Na]_o$ was determined for several axons. Responses to removal of $[Na]_o$ and replacement with $[Li]_o$ were variable, sometimes showing a shift in EB absorbance in the direction of an increase in $[Mg^{2+}]_i$ and sometimes a shift in the opposite direction. Measurement of internal pH with phenol red under the same conditions always showed an acidification upon changing to Li. The EB signal is sensitive to pH and decreases in the EB signal (apparent drop in $[Mg^{2+}]_i$) could be explained as due to acidification overriding the increase in $[Mg^{2+}]_i$. In axons showing an increase in $[Mg^{2+}]_i$ upon replacing Na with Li, the result cannot be due to pH change as the observed change in absorbance is in the opposite direction. In axons showing an increase in $[Mg^{2+}]_i$ upon replacing $[Na]_o$ with $[Li]_o$, the increase was sensitive to both $[Mg^{2+}]_o$ and $[Ca^{2+}]_o$. Either divalent cation could increase the EB signal in the absence of the other. As the EB signal is insensitive to changes in $[Ca^{2+}]_i$ we conclude that increased Ca influx can lead to increases in $[Mg^{2+}]_i$, possibly by an internal Ca/Mg exchange. Replacing $[Na]_o$ with other substitutes such as tris also gave rise to increases in $[Mg^{2+}]_i$. Restoration of Na to the medium resulted in Mg extrusion. Limitation in the rise of $[Mg^{2+}]_i$ is consistent with internal buffering of Mg, changes in $[Mg^{2+}]_i$ with $[Na]_o$ are consistent with Mg/Na exchange, and results $\pm$ $[Ca^{2+}]_o$ are suggestive of internal Mg/Ca exchange.

W-Pos308 DEPENDENCE OF THE SQUID-AXON INTRACELLULAR-pH REGULATING MECHANISM ON EXTRACELLULAR pH. Walter F. Boron and Roger C. Knakal. Department of Physiology, Yale University School of Medicine, New Haven, CT.

Intracellular pH (pH_i) of the squid axon is regulated by a transport mechanism that mediates the influx of one Na^+ and two HCO_3^- (or an equivalent species), and the efflux of one Cl^-. The results of previous studies, in which the transport rate was examined as a function of $[Na^+]_o$, $[HCO_3^-]_o$ and $[DNDS]_o$, suggested that the species transported into the cell might actually be the ion pair $NaCO_3^-$. As in earlier studies, the axons for the present work were internally dialyzed with a pH-6.5 fluid containing 0 mM Na^+ and 400 mM Cl^- until pH_i fell to ~6.6. The acid-extrusion rate (J, equivalent H^+ efflux) was calculated from the rate of pH_i recovery, measured with a microelectrode. We measured J as pH_o was altered in three different ways. First, we varied $[HCO_3^-]_o$ at a constant $[CO_2]_o$ of 0.5%, allowing $[NaCO_3^-]_o$ to float. As pH_o was reduced in steps of 0.3 from 8.6 to 7.1, J sigmoidally fell from 20.7 pmol $cm^{-2}s^{-1}$ (pcs) to 1.6 pcs, with a half-maximal J at $pH_o \cong 7.75$. Second, we varied $[CO_2]_o$ at a constant $[HCO_3^-]_o$ of 12 mM, allowing $[NaCO_3^-]_o$ to float. As pH_o was reduced from 8.6 to 7.1, J linearly fell from 21.5 pcs to 6.6 pcs. Finally, we altered pH_o by varying $[HCO_3^-]_o$ and $[CO_2]_o$ at an approximately constant $[NaCO_3^-]_o$ of 285 μM. As pH_o was reduced from 8.6 to 7.4, J showed no trend, falling from 16.1 pcs to 15.9 pcs. These results could be explained if $NaCO_3^-$ were the true substrate, and the transporter were pH_o insensitive. Indeed, in preliminary experiments, in which we determined the dependence of J on $[HCO_3^-]_o$ at pH_o values from 7.4 to 8.3, both the apparent J_{max} and the apparent K_m with respect to the calculated $[NaCO_3^-]_o$ were insensitive to changes in pH_o. (Supported by NIH grant NS18400.)

W-Pos309 ATP-DEPENDENCE OF ASTROCYTE VOLUME RECOVERY IN HYPO-OSMOTIC MEDIUM
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We have studied ion and volume control properties of rat cerebral astrocytes from primary culture. Astrocytes suspended in hypo-osmotic phosphate buffered saline (PBS) swell initially and then exhibit a regulatory volume decrease (RVD) over the next 30 min. The RVD is blocked by ouabain and inhibitors of electron transport (Olson et al., Biophys J 47:475a, 1985). We now report volume changes and cellular concentrations of ATP following hypo-osmotic exposure with and without inhibitors of specific pathways of energy metabolism.

With 5 mM glucose as substrate, an inhibitor of mitochondrial electron transport, 1 ug/ml antimycin-a (AA), reduces astrocyte RVD. The mitochondrial uncoupler, 50 uM dinitrophenol (DNP), has no effect on RVD. With 5 mM lactate as substrate, the glycolytic inhibitor, 0.1 mM iodoacetic acid (IAA), also has no effect on RVD. Only AA produces a significant change in the ATP level (76 ± 7% of the level in control cells, $p < .02$) after a 30 min incubation in hypo-osmotic PBS. Thus astrocyte RVD is reduced by metabolic inhibitors only if a substantial reduction in cellular ATP occurs, indicating a dependence on the concentration of cellular ATP. The ATP utilized for RVD may be manufactured by mitochondrial electron transport or cytosolic glycolytic pathways. The data also indicate that astrocyte ATP levels can be maintained by electron transport in the absence of glycolysis and by glycolysis alone when mitochondria are uncoupled.

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W-Pos310 THE ROLE OF PROTONS IN THE SODIUM PUMP MECHANISM. Gilles Fellous and Rhoda Blostein, Depts. of Biochemistry and Medicine, McGill University, Montreal, Canada.

The effects of protons on the sodium pump have been studied using inside-out vesicles derived from human red cells. To facilitate the maintenance of pH differences across the membrane, the cells were first treated with DIDS (4,4'-diisothiocyano-2,2'-stilbene disulfonate). Assays of ATP-dependent, strophanthidin-sensitive ^{86}Rb efflux and/or ^{22}Na influx were used to measure (i) normal Na/K exchange (^{22}Na influx into K-containing vesicles or ^{86}Rb efflux into Na-containing medium), (ii) ^{86}Rb efflux into alkali cation-free medium and (iii) ^{22}Na influx into vesicles free of alkali cations. As shown previously (J. Biol. Chem. 260, 829, 1985), normal Na/K exchange is decreased markedly as pH is reduced, e.g. from pH 7.4 to 6.2. In contrast, the other fluxes, particularly (ii), are increased. The sidedness of the pH effects are consistent with the idea that when the proton concentration is raised sufficiently, e.g. from pH 7.4 to pH 6.2, the sodium pump can effect H/K exchange in the absence of cytoplasmic Na and, at least to some extent, Na/H exchange in the absence of extracellular K. Further indirect support of the role of protons as congeners of cytoplasmic Na and extracellular K comes from studies of the Na,K-ATPase activity. Thus, when the pH is decreased in the absence of alkali cations, we observe significant strophanthidin-sensitive ATP hydrolysis as well as phosphoenzyme intermediate (Supported by MRC, Canada).

W-Pos311 ISOLATION AND CHARACTERIZATION OF PROTON PUMPING VESICLES FROM CORN ROOT CELLS. Shu-I Tu, Janine N. Brouillette, and Gerald Nagahashi. U. S. Department of Agriculture, ARS, Eastern Regional Research Center, Philadelphia, PA 19118.

The microsomal fraction of corn root cells isolated by a standard differential centrifugation procedure, was further separated by a linear sucrose density (15-40%) centrifigation. The Mg-ATP dependent proton pumping (uptake) activity of each fraction was assayed by the movement of acridine orange (AO) in a buffered (pH 6.3) KCl solution. Proton pumping vesicles were found in a sharp peak at 20-21% sucrose (1.080 g/cc) in the density gradient. EM data showed the fraction contained a relatively homogeneous population of small vesicles. Kinetic measurements indicated that the K_m of the ATP hydrolysis and the pumping were 0.55 and 0.82 mM, respectively. Both the ATP hydrolysis and the pumping activity were inhibited by the presence of nitrate anion. However, the sensitivity of these two processes to the concentration of nitrate was different. The presence of ionophores generally caused an inhibition of proton pumping but only slightly affected the ATP hydrolysis. Fluorescamine modification of the vesicles caused a significant reduction of both activities. However, the functional link between the ATP hydrolysis and proton pumping has yet to be established.

W-Pos312 RELEASE OF OCCLUDED ^{86}Rb FROM Na,K-ATPase IN THE PRESENCE OF PHOSPHATE OR ARSENATE. Bliss Forbush III, (Intro. by R. W. Mercer), Dept. of Physiology, Yale University School of Medicine, New Haven, CT 06510.

Previous experiments have shown that the rate of ATP-stimulated dissociation of ^{86}Rb from the Na,K-ATPase is rapid enough to be consistent with the "occluded state" as an intermediate involved in the inward transport of Rb^+ or K^+ (Biophys.J. 45:76a,1984). We have found that arsenate (see Kenny and Kaplan, J.Gen.Physiol. 84, 31a) (K_{app} 0.2mM) can substitute for Pi (K_{app} 1.0mM) in promoting rapid release of ^{86}Rb, presumbly by reversing the direction of the pump cycle. At low concentrations of AsO_4 at pH 6.6, the rate of ^{86}Rb release rises for 20-200 ms and then decreases. Analyzed as the result of two sequential reactions, the rate constant of the rising phase increases with [AsO_4] between 0 and 2 mM, while the rate constant of the falling phase is independent of [AsO_4]. The data can be modelled by the reactions E(Rb) + Pi === E-P·Rb --- E-P + Rb, with $k_1 \cong 4x10^4/s/M$, $k_{-1} \cong 5/s$, $k_2 \cong 4/s$. This fit predicts that the dearsenylation of Na,K-ATPase (k_{-1}) is very slow compared to the rate of dephosphorylation, which is generally thought to be >>100 s^{-1}. To examine this directly, we watched the rate of ^{86}Rb deocclusion stimulated by ATP, Pi, or AsO_4, as it decreased upon removal of the appropriate ligand. With ATP, the rate of deocclusion fell as rapidly as could be detected with the rapid filtration apparatus (>150 s^{-1}), indicating that ATP dissociates rapidly from the low affinity binding site. With Pi, the rate was marginally detectable in the apparatus (60-120 s^{-1} at pH 6.6), somewhat slower than expected for the rate of dephosphorylation. With AsO_4, the deocclusion rate fell at a rate of 5-10 s^{-1}, presumably limited by dearsenylation (or AsO_4 dissociation), in support of the above model. (Supported by GM-31782).

W-Pos313 CYTOPLASMIC pH REGULATION IN ACTIVATED NEUTROPHILS. P.E.Nasmith, W.Furuya, W.D.Biggar and S. Grinstein. Research Institute, The Hospital for Sick Children, Toronto, Ontario, Canada M5G 1X8

Cytoplasmic pH (pHi) was measured in resting and activated human neutrophils using the trapped fluorescent indicator bis(carboxyethyl)carboxyfluorescein. When treated with 12-O-tetradecanoyl-phorbol 13, acetate normal neutrophils undergo a biphasic change in pHi:an initial acidification is superseded by a moderate alkalinization. The latter reflects activation of Na^+/H^+ exchange, inasmuch as it requires extracellular Na^+ and is inhibited by micromolar amiloride. The initial acidification, which is fully expressed in Na^+-free media, is accompanied by efflux of H^+ equivalents, suggesting a metabolic origin. Four lines of evidence suggest that the cytoplasmic acidification is associated with superoxide synthesis and the attendant stimulation of the hexose monophosphate shunt: 1) Inhibition of these pathways by N-ethylmaleimide, iodoacetamide or deoxy-glucose prevents the acidification; 2) When neutrophils are activated by a variety of soluble stimuli in Na^+-free medium, the rate and magnitude of oxygen consumption are proportional to rate and magnitude of the intracellular acidification; 3) Activation of the hexose monophosphate shunt in resting cells by addition of phenazine methosulfate or methylene blue produces cytoplasmic acidification; 4) Neutrophils from patients with chronic granulomatous disease, which lack the superoxide generating system, failed to acidify their cytoplasm in response to phorbol esters. The results indicate that large amounts of H^+ are generated metabolically in the cytoplasmic compartment of activated neutrophils. However, pHi is maintained constant, and can even be slightly alkalinized by the operation of a pHi homeostatic system: the Na^+/H^+ antiport.

W-Pos314 STRUCTURAL AND MECHANISTIC IMPLICATIONS OF THE PRIMARY STRUCTURE OF SARCOPLASMIC RETICULUM Ca^{2+}-ATPases. C.J. Brandl[1], N.M. Green[2] and D.H. MacLennan[1]. (Intr. by A. Rothstein). C.H. Best Institute[1], University of Toronto, Canada and MRC, Mill Hill, U.K.[2].

A neonatal rabbit skeletal muscle cDNA library was screened for the Ca^{2+} ATPase of sarcoplasmic reticulum using oligonucleotide probes. Full-length clones for two forms of the enzyme were isolated. The first coded for the fast twitch skeletal muscle Ca^{2+} ATPase except that the terminal Gly was replaced by a polar sequence of eight amino acids. The second shared over 80% nucleotide and amino acid sequence homology with the fast twitch enzyme. Northern blot anlysis has identified this second form as the slow twitch muscle Ca^{2+} ATPase but a similar transcript appears in cardiac muscle mRNA. The slow twitch and cardiac ATPases appear to be products of the same gene, although they may differ as a result of alternative splicing. Hydropathy plots and predictions of secondary and tertiary structure are virtually identical for the two Ca^{2+} ATPases. The enzymes have three cytoplasmic domains, joined to ten transmembrane helices by an amphipathic penta-helical stalk. The stalk is rich in negatively charged residues and provides the most likely structure for the high affinity Ca^{2+} binding sites. Ca^{2+} bound to these sites could be occluded in the stalk by rotation of one or more of the stalk helices. These rotations could be triggered by phosphorylation-induced conformational changes in the globular domains and transmitted to the stalk. The conformational changes could be accompanied by disruption of the high affinity Ca^{2+} binding sites and the creation or reorientation of a transmembrane channel formed from the membrane spanning helices. (Supported by the Ontario Heart Foundation and the MRC of Canada).

W-Pos315 PHOSPHORYLATION OF THE Ca^{2+}-ATPase OF SARCOPLASMIC RETICULUM: RATE LIMITING CONFORMATION CHANGE FOLLOWED BY RAPID PHOSPHORYL TRANSFER. Joanne R. Petithory and William P. Jencks (Intr. by Christopher Miller). Dept. of Biochemistry, Brandeis University, Waltham MA 02254

We report kinetic experiments demonstrating that 2 forms of the $E \cdot Ca_2 \cdot ATP$ complex interconvert via a conformational change prior to phosphoryl transfer by monitoring formation of $[^{32}P]$-labeled phosphoenzyme using the rapid mix-quench technique. Enzyme with exterior Ca^{2+} binding sites occupied ($E \cdot Ca_2$) reacts with Mg·ATP forming covalent phosphoenzyme ($E\text{-}P \cdot Ca_2$) to completion with a rate constant of 220 s^{-1} (pH 7.0, 25°C, 20 μM exterior Ca^{2+}, 250 μM ATP, 5 mM $MgSO_4$, 100 mM KCl, intact SR vesicles passively loaded with 20 mM Ca^{2+}). If phosphorylation were a single step after substrate binding, the rate constant for $E\text{-}P \cdot Ca_2$ formation to equilibrium would equal the sum of the forward and reverse phosphoryl transfer steps ($k_{obs} = k_p + k_{-p}$). We observe 270 s^{-1} under conditions in which the equilibrium amount of $E\text{-}P \cdot Ca_2$ is 50%. This is not consistent with the k_{obs} expected for a single step mechanism of 220 + 220 = 440 s^{-1} ($k_p = k_{-p}$ at $K_{eq} = 1$). This shows phosphorylation does not occur in a single step and is consistent with a slow conformational change of $E \cdot Ca_2 \cdot ATP$ followed by rapid equilibrium phosphate transfer. ATP dissociates from $E \cdot Ca_2 \cdot ATP$ with a rate constant of 120 s^{-1} when this complex is formed from $E \cdot Ca_2$ plus ATP, but with a rate constant of 35 s^{-1} when $E \cdot Ca_2 \cdot ATP$ is formed in the reaction of $E\text{-}P \cdot Ca_2$ plus ADP. The conformation change of $E \cdot Ca_2 \cdot ATP$ changes the catalytic specificity of the enzyme which is required in order to couple ATP hydrolysis to Ca^{2+} transport. The very rapid (>500 s^{-1}) phosphoryl transfer step alters the vectorial specificity of the enzyme, rendering Ca^{2+} inaccessible to the membrane exterior.

W-Pos316 ION CHANNELS WITHIN THE MONOVALENT CATION TRANSPORTING ATPase SYSTEMS ARE MONITORED BY THE ASSOCIATED K^+-pNPPase ACTIVITIES. Tushar K. Ray, Amit Ray, Pratap K. Das and Sandip Bandopadhyay, Department of Surgery, SUNY-Upstate Medical Center, Syracuse, New York 13210.

Recent reports (Ray and Nandi, FEBS Lett. 185:24-28, 1985; Biochem.J.-in press) from our laboratory revealed that the K^+-pNPPase activity associated with both the Na^+,K^+-ATPase and gastric H^+,K^+-ATPase systems does not represent the partial (phosphatase) step of the total ATPase reaction as was widely believed. In this report we present evidence suggesting that the K^+-pNPPase monitors the ion-channel activity of the monovalent cation transporting ATPase systems. Several different ionic species such as spermine, Zn^{+2} and SCN^- inhibit specifically the K^+-pNPPase without having any effect on the H^+,K^+-ATPase activity. However, the H^+,K^+-ATPase mediated transport of H^+ within the gastric microsomal vesicles is nearly abolished under similar conditions demonstrating a strong correlation between the K^+-pNPPase activity and ion-translocation process. The inhibitory effects of spermine and SCN^- on both the K^+-pNPPase and ion transport could be reversed by elevation of K^+ suggesting regulation of both processes by a common K^+ site which is unrelated to H^+,K^+-ATPase activity. Reversal of the Zn^{+2} effect by DTT suggested involvement of some -SH groups critical for the K^+-pNPPase but not for the H^+,K^+-ATPase activity. Contrary to the differential inhibition of the K^+-pNPPase by several ions as above, two drugs namely furosemide and floridzin were found to compete with ATP in inhibiting the H^+,K^+-ATPase but cause significant stimulation of the associated pNPPase. Similar to Zn^{+2} the effect on the latter activity can also be reversed by DTT indicating -SH involvement. The vesicular uptake of H^+ was also abolished by the drugs. The data suggest the K^+-pNPPase to monitor the ion translocation process which although present within the same enzyme complex can be kinetically separated from the ATP hydrolytic activity.

W-Pos317 PRESTEADY STATE KINETICS OF Na^+-H^+ EXCHANGE AT 0°C IN KIDNEY BRUSH BORDER MEMBRANE VESICLES (BBMV). Kinya Otsu, James Kinsella, Jeffrey Froehlich, and Bertram Sacktor. GRC, NIA, NIH, Baltimore, Maryland 21224.

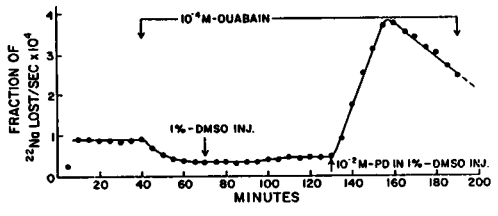
Initial time course studies of $^{22}Na^+$ uptake via the amiloride-sensitive Na^+-H^+ exchange system in BBMV were carried at 0°C in order to resolve the kinetics of the presteady state and to evaluate the sequence and rates of intermediate reactions in the exchange mechanism. Amiloride-sensitive $^{22}Na^+$ uptake between 1 and 15 sec consisted of 3 distinct phases: (1) an initial lag, (2) a rapid uptake or "burst", and (3) a linear steady state phase. A slow linear rate of Na^+ uptake was observed in the presence of amiloride whereas addition of monensin to the stop solution completely eliminated Na^+ uptake. Control experiments with $^{22}Na^+$-loaded vesicles showed that the reduced rate of uptake following the burst was not due to $^{22}Na^+$ backflux. Decreasing the internal pH (pH_i) from 7.5 to 6.0 at constant external pH (pH_o=7.5) and $[Na^+]$ (1 mM) increased the burst size (40 to 120 pmol Na^+/mg) and steady state velocity (2.3 to 65 pmol Na^+/mg/sec) without affecting the duration of the lag (<0.5 sec) or apparent burst rate (0.6 sec^{-1}). Similar results were obtained by raising the $[Na^+]$ from 1 to 5 mM at pH_i=6.0 and pH_o=7.5. In contrast, raising pH_o from 7.5 to 8.5 ($[Na]$=1 mM, pH_i= 6.0) shortened the lag and increased the steady state velocity without changing the burst size. Generation of an inside negative membrane potential with FCCP (pH_i=6.0, pH_o=7.5) increased the amplitude of the burst but had no affect on the steady state velocity. The results are compatible with a model for Na^+-H^+ exchange that includes a rapid Na^+ translocation step (burst phase) preceded (lag phase) and followed by (linear phase) slower transitions possibly involving transformations between Na^+-and H^+-specific carrier states.

W-Pos318 THE INFLUENCE OF GTP AND PDE INHIBITORS ON THE BEHAVIOR IN BARNACLE MUSCLE FIBERS OF THE OUABAIN-INSENSITIVE SODIUM EFFLUX TOWARD HIGH Ke AND INJECTED Ca^{2+}. Jude Nwoga* and E. Edward Bittar, Department of Physiology, University of Wisconsin, Madison, WI 53706.

Previous experiments showed that in single barnacle muscle fibers high Ke always induces a transitory stimulation of the ouabain-insensitive Na efflux which can be rendered diphasic by preinjecting GTP. This work has now been confirmed e.g. injection of 0.1M or 0.25M-GTP (dilution in these fibers being ~ 100-fold), followed by raising Ke from 10 mM to 100 mM always leads to a diphasic stimulatory response, viz, an early phase which is prompt and small, and a delayed phase which is large and sustained e.g. with 0.25M-GTP sustained stimulation averages 488 ± 47%, n=8. This delayed response is partly reversed by post-injecting 0.25M-EGTA (63 ± 8% reversal, n=4), but not by injecting 10^{-4}M-PKI (n=4). Pre-application externally of the PDE inhibitors, 1-propyl-3-methyl-7-(5-hydroxylhexyl)-xanthine-PMX(10^{-4}M), as well as 1-isoamyl-3-isobutylxanthine-IAX-(10^{-6}M) stops the response to 100 mM-Ke from decaying completely, the residual stimulation being in the order of 322 ± 44%, n=10. Injection of 10^{-4}M-PKI reduces this persistent response by 50 ± 3% (n=4), while injection of 0.25M-EGTA is without effect (n=4). In parallel experiments, injection of Ca^{2+} (e.g. 0.25M) in the presence of PMX-IAX results in sustained stimulation averaging 471 ± 23%, n=8 vs 302 ± 52% in 5 controls, $P < .01$. PKI (10^{-4}M), when postinjected, reduces the sustained response by 81 ± 1% (n=4), but not 0.25M-EGTA (n=4). Taken together, these results support at least two views: one is that excess GTP exerts a permissive, persistent effect mainly through Ca^{2+} rather than cAMP. The other is that whereas the Ca^{2+} message is enhanced by cAMP, Ca^{2+} shortens the message by activating PDE ("sequential control"-Rasmussen).

W-Pos319 PHORBOL ESTER-INDUCED STIMULATION OF THE OUABAIN-INSENSITIVE SODIUM EFFLUX IN BARNACLE MUSCLE FIBERS. E.E. Bittar, *K. Ueno** and *C. Li**, Department of Physiology, University of Wisconsin, Madison, Wisconsin 53706.

Studies with the barnacle muscle fiber as a preparation have led to the view that the ouabain-insensitive Na efflux can be divided operationally into four distinct components, three of which are regulated by protein phosphorylation catalyzed by cAMP-PK, cGMP-PK and CM-Ca^{2+}-PK. The possible existence of a fifth component has now been investigated using 4-β-phorbol-12, 13-dibutyrate (PD) as a probe. The results show that injection of 10^{-2}M-PD in 1%-DMSO into unpoisoned or ouabain-poisoned fibers always leads to a stimulatory response. As illustrated in the accompanying Figure, injection of 1%-DMSO once (or twice in controls) is without effect (n=8), whereas injection of 10^{-2}M-PD causes a prompt and sharp rise in the ouabain-insensitive Na efflux which reaches a peak some 30 mins later (555 ± 83% stimulation, n=4), and then decays. A comparable response is obtained following external application of 10^{-5}M-PD, the minimal effective concentration being ~10^{-8}M. However, decay of the response is more slow or the response is often of a sustained nature. Since PL/Ca^{2+}-PK c is found in barnacle fibers (J.F. Kuo, priv. comm.) and since protein kinase c is the internal receptor for phorbol esters, it seems likely that the observed responses to PD are the result of protein phosphorylation catalyzed by kinase c. Whether 1,2-diacylglycerol mimics the effect of PD is not yet known.



W-Pos320 INHIBITION OF THE RABBIT SKELETAL MUSCLE TRANSVERSE TUBULE Mg^{2+}-ATPase BY DIETHYLPYROCARBONATE. T.L. Kirley, Department of Pharmacology and Cell Biophysics, University of Cincinnati, Cincinnati, OH 45267-0575

Transverse tubule membranes from rabbit fast skeletal muscle contain a Ca^{2+} or Mg^{2+}-ATPase (termed Mg^{2+}-ATPase) which is distinct from mammalian cation transporting ATPases such as sarcoplasmic reticulum (SR) Ca^{2+}-ATPase or (Na,K)-ATPase. Fluorescein isothiocyanate (FITC), which at micromolar concentrations inactivates Ca^{2+} and (Na,K)-ATPase by covalently labelling the ATP binding site, does not inactivate the Mg^{2+}-ATPase. Also, unlike the (Na,K)-ATPase, the Mg^{2+}-ATPase is fairly insensitive to inactivation by sulfhydryl, carboxyl, arginyl, tryptophanyl and tyrosyl directed reagents. The only reagent found to be an effective inhibitor of the Mg^{2+}-ATPase at concentrations less than 1.0 mM is diethyl pyrocarbonate (DEPC), a reagent fairly specific for histidine modification. The inactivation by DEPC does not allow first order kinetics, but appears to be biphasic over the entire pH range studied (5.5 to 7.5). The slower phase of inactivation seems to be quantitatively related to the amount of Mg^{2+}-ATPase activity restored upon treatment with hydroxylamine. Since hydroxylamine reverses histidine and tyrosine but not lysine or cysteine modifications, this data suggests that either histidine or tyrosine modification is responsible for the slow phase of inactivation. Both the rapid reversal by hydroxylamine treatment and the pH dependence of the slow phase of inactivation are more consistent with histidine modification. ATP partially protects the Mg^{2+}-ATPase from inhibition by DEPC.

The above results suggest that the transverse tubule Mg^{2+}-ATPase is not closely related to the SR Ca^{2+}-ATPase or (Na,K)-ATPase. Supported by NIH grants T32 HL 07382 and PO1 HL 22619.

W-Pos321 INVESTIGATION OF NUCLEOTIDE BINDING SITES OF PLASMA MEMBRANE H^+-ATPase FROM YEAST S. POMBE. M. RONJAT*, P.C. CARVALHO ALVES*, Y. DUPONT*, J.P. DUFOUR** and A. GOFFEAU**
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The plasma membrane of yeast contains an ATPase responsible for generation of an H^+ electrochemical gradient across the cell membrane. This translocating ATPase shares several common properties with mammalian transport ATPases. A reaction scheme has been proposed wich include two isomeric forms of the enzyme, one being phosphorylated by ATP and the other by inorganic phosphate. However the present knowledge on the mechanism of the H^+ATPase is, by far, much less advanced than that of other plasma membrane ATPases. This report concerns a study of the nucleotide binding site of yeast H^+ATPase.

Two types of fluorescents probes have been used which are : 1) formycin triphosphate-terbium (ATP-Tb) as an analog of ATP-Mg and 2) 2' 3'-O-(2,4,6-trinitrophenyl) ATP (TNP-ATP). We have found that these two complexes are recognized by the active site of H^+ATPase. Fluorescence recording and direct binding of labelled nucleotides have highlighted the existence of two types of nucleotide binding sites both of hight affinity for the analogs (Kd = 5 μM). In addition we have found that the binding of nucleotide is regulated by a metallic ion binding site distinct from the catalytic center of the enzyme.

W-Pos322 MONOMERS OF THE NEUROSPORA PLASMA MEMBRANE H^+-ATPASE CATALYZE EFFICIENT PROTON TRANSLOCATION. Erik Goormaghtigh and Gene A. Scarborough, Department of Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, N.C. 27514

Liposomes prepared by sonication of asolectin were fractionated by glycerol density gradient centrifugation, and the small liposomes contained in the upper region of the gradients were used for reconstitution of purified, radiolabeled, Neurospora plasma membrane H^+-ATPase molecules by our previously published procedures. The reconstituted preparation was then subjected to two additional rounds of glycerol density gradient centrifugation, which separates the H^+-ATPase-bearing proteoliposomes from the other liposomes by virtue of their greater density. The isolated H^+-ATPase-bearing proteoliposomes exhibit a specific H^+-ATPase activity of approximately 10 μmole of Pi liberated/mg protein/min which is increased to about 20 in the presence of nigericin plus K^+, indicating that a large percentage of the H^+-ATPase molecules in the preparation are capable of generating a transmembrane protonic potential difference sufficient to impede further proton translocation. Importantly, quantitation of the number of 105,000 dalton ATPase monomers and liposomes in the final preparation by radioactivity determination and counting of negatively stained images in the electron microscope indicates an ATPase to liposome ratio of 1.12 ± 0.13. Because every liposome in the preparation must have at least one ATPase monomer, this ratio indicates that very few liposomes have more than one. The results thus clearly demonstrate that 105,000 dalton monomers of the Neurospora plasma membrane H^+-ATPase can catalyze efficient ATP hydrolysis-driven proton translocation. (Supported by NIH Grant GM24784.)

W-Pos323 EFFECTS OF BILAYER CHOLESTEROL ON CONVERSION OF THE PHOSPHORYLATED NAK-ATPASE PROTEOLIPOSOMES. Shizuko Yoda and Atsunobu Yoda, Department of Pharmacology, University of Wisconsin Medical School, 1300 University Avenue, Madison, Wisconsin 53706

In the absence of K^+, the NaK-ATPase forms the phosphorylated form (EP) by MgATP in the presence of Na^+, and this EP changes from the ADP-sensitive K^+-insensitive form (E_1P) to the ADP-insensitive K^+-sensitive form (E_2P) via the ADP- and K^+-sensitive form (E*P). This conversion in the proteoliposomal enzyme was much slower than that in the fragmental enzyme, and the EP of the proteoliposomes contained more E_1P or E*P than that of the fragmental enzyme under the same phosphorylating condition. The E_1P content in the phosphorylated proteoliposomes increased, and the E_2P content decreased with the increase of the cholesterol contents in the lipid bilayer of the proteoliposomes. When the bilayer cholesterol was less than 30 mole %, the E_1P content decreased and the E_2P content increased with the elevation of temperature; however, the E*P content did not change significantly. In the high cholesterol proteoliposomes (more than 35 mole % cholesterol in the lipid bilayer), the E*P content increased with the elevation of temperature and the E_2P content was less than 10% in the presence of 10 mM Na^+ on both sides at 3 to 20°C. This interference of the E*P to E_2P conversion in the high cholesterol proteoliposomes was counteracted with monensin (Na^+-ionophore), and the results of this ionophore effect seemed to suggest the participation of Na^+ on this conversion step. (Supported by NIH grant HL16549)

W-Pos324 ARE MY CELLS DEAD? HOW TO TELL DURING AN ESR EXPERIMENT Philip D. Morse II, Marjeta Sentjurc, and Harold M. Swartz, University of Illinois College of Medicine, Urbana, IL and Jozef Stefan Institute, E. Kardelj University, Ljubljana, Yugoslavia.

It is difficult to assess sample viability during spin-label EPR experiments on living biological materials. We demonstrate that the spin label broadening agent potassium trioxalatochromiate (III) and potassium ferricyanide (CrOX and FeCN, respectively) can be used to determine cell viability during such experiments.

Mouse Thymus-Bone (TB) cells ($1x10^8$/ml) in McCoy's 5A medium, supplemented with 10% fetal calf serum, were made 0.1 mM in TEMPONE (2,2,6,6-tetramethylpiperidine-N-oxyl-4-one) and 55 mM in CrOX or 60 mM in FeCN, and were placed in a gas-permeable Teflon tube (Zeus Industries). The intensity of the mid-field line of TEMPONE was followed with time. N-ethyl maleimide (NEM) kills TB cells over 30 minutes as shown by loss of Trypan Blue exclusion. When 5 mM NEM was added to the cell sample, the intensity of the midfield line remained at its initial value for the first 5 minutes, then dropped to the baseline value over the next 20 minutes. Cell viability measured with Trypan Blue followed the same time course. In the absence of NEM, the mid-field line intensity stayed at the starting value. Control experiments showed that these data are not a result of cellular metabolism of TEMPONE by the cells with subsequent loss of signal. When $NiCl_3$ was used, signal intensity remained high in the presence or absence of NEM.

We conclude that CrOX and FeCN enter the cells as they die, and that the loss of signal intensity results from broadening of the TEMPONE signal by these agents. However, $NiCl_3$ does not enter the cell at death, and when using $NiCl_3$ as a broadening agent other methods must be used to determine cell viability.

W-Pos325 MEMBRANE CONDUCTANCE MECHANISMS IN NEONATAL AND EMBRYONIC MOUSE OLFACTORY NEURONS
R.A. Maue and V.E. Dionne, University of California, San Diego; La Jolla, California 92093

In parallel with patch clamp studies of adult mouse olfactory neurons [Biophys. Soc. Abstr. (1984) 41:266a] we have begun similar studies on cells from embryonic and neonatal mice. Timed pregnant mice were sacrificed by cervical dislocation, the embryos quickly removed, and the olfactory epithelium from 4-6 embryos subjected to the same dissociation procedures used for the studies of the adult neurons. Although these procedures yielded significantly fewer cells than from the adults, the same isolated cell types were observed, including neurons with dendritic processes and cilia. Patch-clamp recordings of single channel activity have been made from neurons as early as E19. One channel type observed was a Ca-activated K channel with similar single channel conductance (approximately 130 pS in elevated K saline) and kinetic behavior as that observed in receptor neurons from adult mice. A second type of ion channel found in embryonic neurons had approximately 48 pS conductance in normal saline and appeared to be voltage-activated. In cell-attached patches, this channel was not activated by hyperpolarization or depolarizing steps less than 30 mV. As depolarizations were made larger, the first latency for opening decreased and the percentage of time spent open during the voltage pulse increased. Mammalian olfactory receptor neurons develop selectivity in their response to odorants just prior to birth of the animal. Comparing channel activity in adult and embryonic neurons may provide insight into how this occurs. Embryonic neurons may also provide a useful preparation for studying odorant responses because they lack the selectivity of adult cells but retain adult-like sensitivity. [Supported by NIH:NS 20962]

W-Pos326 INTRACELLULAR RECORDINGS FROM OLFACTORY RECEPTOR NEURONS ISOLATED FROM THE SALAMANDER.
Peter A. V. Anderson and Kathryn A. Hamilton. C. V. Whitney Laboratory and Depts. of Physiology and Neuroscience, Univ. of Florida, St. Augustine, FL 32086 and Dept. of Neurosurgery, Tufts New England Medical Center, Boston, MA 02111.

Enzymatic dissociation of nasal sacs from the land-phase tiger salamander Ambystoma tigrinum yields suspensions of isolated receptor cells. The isolated cells are morphologically similar to those of intact tissue, as revealed by dye injection (Masukawa et al., 1985. J. Neurosci. 5: 128-135). They have an ovoid soma, a dendrite of variable length which terminates in a cilia-bearing dendritic knob, and an axon, also of variable length. Intracellular recordings were obtained using patch pipettes. Seal resistances were in the range 10-20 Gohms. Good recordings were characterized by resting potentials of -40 mV, high input impedances in the range 400-800 Mohms and the occurance of fast overshooting action potentials upon depolarization or rebound excitation. The action potential parameters were similar to those obtained from cells in situ using microelectrodes (Musakawa et al., 1985). With one cell, stimulation with an aqueous mixture of volatile chemicals evoked a large depolarization and from 1-3 action potentials. The reversal potential of the depolarization was +2.7 mV. The fact that the isolated cells were electrically and, in one case, chemically excitable indicates that this is a promising preparation for examining the mechanisms of olfactory transduction. (Supported by grant BNS 82-09849 to P.A.V.A.).

W-Pos327 AN EXOCYTOSIS-SENSITIVE PHOSPHOPROTEIN IS RELEASED FROM THE CYTOPLASM OF PARAMECIUM BY DECILIATION.
T. Hamasaki, T.J. Murtaugh, P. Satir and B.H. Satir (Intr. by S. Seifter) Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, NY.

It has previously been shown that upon incubation of living Paramecium tetraurelia with ^{32}P inorganic phosphate, one of the major labelled phosphoproteins is at M_r 65Kd. This 65Kd phosphoprotein is dephosphorylated upon stimulation of exocytosis (Gilligan and Satir, J. Biol. Chem. 97: 224. 1982). We have labelled the exocytosis sensitive 65Kd phosphorprotein in vivo and then examined its fate after Ca^{2+}-induced deciliation in these cells. After labelling, the cells in 5mM Pipes pH 7, 20mM $MgCl_2$ and 1mM KCl are cooled, diluted 1:1 with a sucrose-containing buffer and subjected to a Ca^{2+} shock treatment. Deciliation is followed microscopically. At the time of deciliation, the cells slowly become leaky as judged by trypan blue penetration. Autoradiography demonstrates that the 65Kd phosphoprotein remains labelled throughout this procedure. Centrifugation is used to fractionate the sample into cell bodies, cilia and a high speed cell and cilia-free supernate (17,000xg supernate). The labelled 65Kd phosphoprotein is generally almost absent in the cell body and cilia fractions, but is retained in the 17,000xg supernate. However, if 1mM IBMX and 0.1mM dibutyryl cAMP is added when the cells are being labelled, after deciliation the labelled species is found in both the cell body and the supernate. From these observations, we confirm the previous suggestion that, in its phosphorylated state, the 65Kd phosphoprotein is probably located in the cytosol just beneath the cell membrane and is freely diffusable, and we hypothesize that the same protein remains with the cell body fraction (bound to the cell membrane?) after deciliation but is normally dephosphorylated. Supported by NIH and NCI.

W-Pos328 VOLTAGE- AND CURRENT-CLAMP RECORDING OF THE RECEPTOR POTENTIAL IN OLFACTORY RECEPTOR CELLS. I. Schmiedel-Jakob, P. A. V. Anderson and B. W. Ache. C. V. Whitney laboratory, Univ. of Florida, St. Augustine, FL 32086

The olfactory organ (antennule) of crustaceans such as crabs and lobsters consists of a tuft of sensilla (aesthetascs). Each sensillum is innervated by dendrites of several hundred primary bipolar receptor cells. Intracellular recording from these neurons, both as isolated cells and in situ, is possible using patch pipettes in the whole-cell configuration. All cells show high input impedance with pronounced outward rectification and have long time constants. When depolarized to above -20 to -25 mV, they produce fast, overshooting action potentials. Receptor cells in situ exhibit a prolonged depolarizing receptor potential and spike in a dose-dependent manner to chemical stimulation (extract of invertebrate muscle tissue). The magnitude of the receptor potential is dependent on the resting potential, with one cell exhibiting a positive calculated reversal potential, suggesting that an inward positive current underlies the receptor potential. This view is verified by the finding that chemical stimulation evokes a slow inward current in cells voltage-clamped to a holding potential of -60 mV, and that the amplitude of the inward current varies with the holding potential and reverses at +40 mV (Brain Res. 338: 273-280). These cells are being used for a detailed ionic and pharmacological analysis of the ionic mechanism(s) that underlie the receptor potential of a primary chemosensory neuron. Supported by a grant from the Whitehall Foundation.

W-Pos329 IMPLICATION OF PROTEIN KINASE C IN CONTROL OF EFFERENT TRANSMISSION IN THE SACCULAR HAIR CELL. A.Steinacker & L. Rojas*, Dept. of Otolaryngology, Washington Univ. Sch. of Med. St. Louis, Mo. 63110 * Lab. of Neurobiology, Univ. of Puerto Rico Sch. of Med. San Juan, P.R. 00901.

Transmitter release from the vestibular hair cell is modulated by an efferent symapse on the hair cell. I had previously shown that ACh, the efferent transmitter modulates the opening of a K^+ channel in the hair cell. The receptor is muscarinic and requires an intracellular mediator since bath applied oxotremorine produces an increase in the open time of the K^+ channel under the pipette(A. Steinacker, Biophys. J. 47:144a, 1984). The nature of the intracellular mediator was investigated with the use of the phorbol ester, phorbol 12-myristate13-acetate (PMA). PMA mimics the physiological activation of protein kinase C by diacylglycerol (Nishizuka, Y. Nature, 308: 693, 1984). The cell preparation used was a cell attached patch clamp recording from an enzymatically isolated toadfish (Opsanus tau) saccular hair cell. The cell was bathed in normal salt water Ringer while the recording pipette contained 200 mM KCl. Under these conditions,application of ACh, oxotremorine (10^{-6}M) or PMA (0.1 ug/ml) to the cell surface all produce an increase in the opening rate and open time of a high conductance K^+ channel (250 pS.). The response to PMA is not dependent on extracellular Ca^{++} since it occurs in Ca^{++} free media. The data indiates that the mechanism of action of the efferent transmitter at this synapse may involve a protein kinase C system.(Supported by NSF grant BNS 82-18429. Part of this work was carried out at the Marine Biological Laboratory, Woods Hole, Mass.

W-Pos330 A FLUORESCENCE METHOD FOR STUDYING THE BINDING OF DEFINED BIVALENT LIGANDS TO IgE ON THE CELL SURFACE. **J. Erickson, P. Kane, B. Goldstein, D. Holowka and B. Baird. Department of Chemistry, Cornell University, Ithaca, NY 14853 and Los Alamos National Laboratory, Los Alamos, NM 87501 (Intr. by H.A. Scheraga).** **Crosslinking of IgE-receptor complexes on the surface of mast cells and basophils by multivalent antigens triggers cellular degranulation. We have developed an experimental system for investigating the factors that control the binding and crosslinking of cell-bound IgE by bivalent ligands which will help us determine the essential requirements for the initiation of the transmembrane signal. This system employs monoclonal anti-2,4-dinitrophenyl (anti-DNP) IgE that is labeled with fluorescein-5-isothiocyanate (FITC), and ligand binding is measured continuously and sensitively by the FITC quenching that accompanies DNP occupation of the antibody combining site. The FITC quenching method has been used in a detailed study of the equilibrium binding of monovalent DNP-aminocaproyl-L-tyrosine (DCT) and bivalent $(DCT)_2$-L-cystine $[(DCT)_2$-cys] to FITC-IgE and its Fab fragments in solution and to cell-bound FITC-IgE. In all cases DCT binding is well described by a single intrinsic affinity constant (K_1) of $2x10^9$ M^{-1}, while a satisfactory description of $(DCT)_2$-cys binding to either anti-DNP or its Fab fragments in solution requires two affinity constants: the same K_1 as for DCT and a crosslinking constant (K_2) that is about 50 times smaller. Our equilibrium binding studies indicate that crosslinking of cell-bound anti-DNP IgE by $(DCT)_2$-cys is very efficient even though this ligand does not stimulate degranulation nearly as well as multivalent antigen $(DNP)_{16}$BGG. Currently we are exploiting our ability to carry out time dependent measurements with the FITC quenching method to determine kinetic parameters for the binding events and how these are related to cell triggering.** **Supported by grants from NIH (AI18306, AI22449, GM13292, and GM35556).**

W-Pos331 Wilson Radding, Dept of Biochem., P&S, Columbia U., Intr. by Wayne Hendrickson
LIPIDS AND THE UR-RECEPTOR: Receptors for molecules get about 10kcal/m from the free energy of binding. However, pheromone receptors are often sensitive to one molecule per several seconds with detection rates as good as that of rhodopsin. Using an allosteric model, thermal noise may be 1 to 100 times the signal. To overcome this noise the receptor should have a built-in signal filter-amplifier. The binding site must be very close to or identical with the amplifier, so that thermal activity cannot interfere with signal propagation. Many pheromones are monoenes, and the simplest monene, ethylene, can be active in both plants and animals. Postulate 1: In the ur-receptor, that receptor from which all others have sprung, a monoene is an integral part of the amplifier. When the effects of polyenes incuding retinoic acid, retinol and leukotrienes are considered together, it is clear that lipids with an odd number of conjugated bonds affect nearly every cellular system. Postulate 2: The ur-receptor, when not light sensitive, utilizes polyenes with an odd number of conjugated bonds in the signal detection process. Hypothesis: The amplification step in the ur-receptor utilizes both a polyene and a monoene. When these are juxtaposed correctly, a temporary exothermic cyclization occurs, which accomplishes two things, alteration of the protein shape, and the release of up to 36kcal/mol for signal propagation. Supporting points: 1) A charge transport band at 34kcal/mole. 2) Woodward-Hoffman rules give the same reaction symmetry for retinal with light as odd numbered polyenes without light, 3) 30kcal/m available from autophosphorylation to reset system.

W-Pos332 ADENINE NUCLOETIDES STIMULATE OXIDATION INDUCED RAPID CALCIUM EFFLUX FROM SARCOPLASMIC RETICULUM VESICLES. Janice Stuart, Guy Salama*, Jonathan J. Abramson**, Portland State University, Department of Chemistry, **Department of Physics, P.O. Box 751, Portland, Oregon 97207, and *Department of Physiology, University of Pittsburgh, Pittsburgh, PA 15261.

We have previously shown that addition of Cu^{2+} and Cysteine induces a rapid efflux of Ca^{2+} from sarcoplasmic reticulum (SR) vesicles via oxidation of a critical sulfhydryl group on the release protein. The data presented here indicates that adenine containing compounds synergistically stimulate the oxidation induced efflux of Ca^{2+} from SR vesicles. When vesicles were actively loaded using ATP the oxidation induced maximal efflux rate was 3-4 times as large as in passively loaded vesicles. The efflux rates observed for vesicles actively loaded using acetyl phosphate were comparable to those from passively loaded vesicles. When adenine containing compounds were added simultaneously with Cu^{2+} and Cysteine to vesicles loaded either passively or actively, using acetyl phosphate, the efflux rate was potentiated. The order of potentiating effectiveness was AMP-PCP ∿ cAMP > AMP > adenine ∿ NAD ∿ NADH. Vesicles actively loaded using non-ATP nucleotides (GTP, CTP, UTP, ITP) showed oxidation induced efflux rates approximately equal to vesicles loaded using acetyl phosphate. Caffeine also had a slight potentiating effect on oxidation induced efflux rates. Our data suggest that the binding of adenine nucleotides to the release protein results in a greater accessibility of a critical sulfhydryl group whose oxidation causes Ca^{2+} to be released from the SR. Both Ca^{2+} induced Ca^{2+} release and oxidation induced Ca^{2+} release are stimulated by adenine nucleotides suggesting similar mechanisms are involved. Supported by AHA, AHA Alaska Affiliate and PHS RO1-NS18590.

W-Pos333 ARRHENIUS ACTIVATION ENERGIES FOR LIPID FLUIDITY, PROTEIN MOBILITY, AND ENZYMATIC ACTIVITY ARE THE SAME IN SARCOPLASMIC RETICULUM. D.J. Bigelow, T.C. Squier, and *D.D. Thomas Dept. of Biological Chemistry, University of Maryland School of Medicine, Baltimore, MD; *University of Minnesota Medical School, Minneapolis, MN.

We have developed a quantitative measure of lipid fluidity, using electron paramagnetic resonance (EPR), and have applied this method to compare the temperature dependence of lipid fluidity, protein mobility, and Ca-ATPase activity in sarcoplasmic reticulum (SR). We have defined lipid fluidity to be the inverse of the viscosity of a reference solvent in which a fatty acid spin label gives the same conventional EPR spectrum as observed for the same probe in the membrane. The reference solvents chosen are ordered long-chain hydrocarbons, in which the type of molecular motion is similar to that in membranes. EPR order parameters (S), of probe motions in these solvents, are plotted against log (T/η), where T is temperature and $1/\eta$ is fluidity. The resulting calibration plots are independent of the solvent, position of the nitroxide group along the fatty acid chain, and the charge on the probe, supporting the model independence of (T/η) using these calibration plots. We argue that this empirical measurement of fluidity, defined in analogy to the macroscopic fluidity ($1/\eta$) of a bulk solvent, is more likely to be directly related to protein mobility than is the rotational motion of the lipid hydrocarbon chains. This prediction is supported by the application of this method to SR. Arrhenius analysis shows that the activation energies for lipid fluidity, protein mobility, and enzymatic activity (above 20°) in SR are the same (11-12 kcal/mol deg), suggesting that mobility of the enzyme (directly modulated by lipid fluidity) may be rate limiting.

W-Pos334 CALCIUM RELEASE FROM THE SARCOPLASMIC RETICULUM OF SKINNED SKELETAL MUSCLE: EFFECT OF DECREASED K CONDUCTANCE. Carla W. Abramcheck and Philip M. Best, Department of Physiology and Biophysics, University of Illinois, Urbana, IL 61801.

The effect of decreasing K conductance of the sarcoplasmic reticulum (SR) membrane on Ca release rate in single skinned (sarcolemma removed) skeletal muscle fibers was determined. Ca release was measured optically using 0.5 mM Antipyrylazo III (APIII). Solutions contained approximately 100 mM monovalent cations (Na and K), 2 mM MgATP, 1 mM Mg, 15 mM creatine phosphate, 2 mM EGTA (pCa=8) and about 35 mM MOPS buffer (pH=7.0 at 10°C), I=0.15. The concentration of highly permeable monovalent anions was limited (7 mM total Cl) by using propionate salts. Following a standard loading procedure SR calcium release was stimulated by exposing fibers to 5 mM caffeine. Changes in dye absorption (720 nm-790 nm) were monitored and analyzed by computer. The initial rate of absorbance change was determined for three successive releases (control, test, control) from individual fibers. The test release rate was compared to the average of the two control rates. Exposure to 0.1 mM bisG10 (1,10-bis-guanidino-n-decane), a SR K channel blocker, produced an 18% decrease in the calculated Ca release rate (n=5). The calculated rates from bisG10 treated fibers were corrected for bisG10-APIII binding (K_D=2.35 mM). Substitution of impermeant choline for 34% of the total monovalent cations present produced a 33% decrease in release rate (n=5). Thus both methods of lowering K conductance of the SR caused a substantial decrease in the rate of Ca release. These results indicate the importance of K ion conductance in maintaining Ca release presumably by acting as a counterion. Supported by NIH AM32062 and PHS training grant GM7143.

W-Pos335 **L-CYSTEINE TRIGGERS CA^{2+} RELEASE FROM THE SARCOPLASMIC RETICULUM (SR) OF CHEMICALLY SKINNED SKELETAL FIBERS.** G. Salama*, G. Pike, F. Fuchs and J. Abramson†. Dept. of Physiology, Univ. of Pittsburgh, School of Medicine, Pittsburgh, PA 15261 and †Dept. of Physics, Portland State Univ., Portland, OR 97207.

Heavy metals, sulfhydryl reagents and cysteine (5-50 μM) were previously shown to trigger the rapid release of Ca^{2+} from SR vesicles. Cysteine acted by forming a disulfide bond with an SR protein and the formation of this bond required catalytic concentrations of Cu^{2+} (1-5 μM). Such a sulfhydryl interaction is now shown to occur in muscle fibers. Rabbit psoas fibers were chemically skinned in a Cl^--free medium containing 5 mM EGTA and 50% glycerol, then 4-6 fibers were transferred for tension recordings to a relaxing, chelexed solution containing (in mM): 170 K gluconate, 2.5 Mg-ATP, 10 MOPS at pH 7.0 and 23°C. Free Mg^{2+} and Ca^{2+} were calculated to be ~10^{-5} and 10^{-8} M respectively. A Ca^{2+} addition (1 μM total = 0.18 μM free) produced a contraction via Ca^{2+}-induced Ca^{2+}-release (CICR) which required pre-loading of the SR with 5-8 successive additions of Ca^{2+} (0.18 μM free) in the presence of ATP. Cysteine (5-50 μM; total Ca^{2+} contamination < 10^{-8} M by atomic absorption), like CICR produced contractions which reached peak tension within 30 s followed by relaxations back to baseline tension at similar rates. High Mg^{2+} (1 mM free) inhibited cysteine and CICR contractions but unlike CICR, prior addition of Cu^{2+} (5 μM) reinstated cysteine contractions. Both CICR and cysteine contractions could be repeatedly obtained whereas Hg^{2+}(10 μM) elicited a maximal contraction with considerably weaker contractions henceforth. The results indicate that sulfhydryl oxidation or formation of disulfide bonds at the Ca^{2+} release site on the SR may represent a physiological trigger for muscle activation. Furthermore, Ca^{2+}-release from the SR by cysteine in the absence of Cu^{2+} implies the existence of an endogenous catalyst. Supported by AHA 82 1231, NIH NS 18590 and K04 NS00909 to G.S., J.A. is an Established Investigator of the AHA.

W-Pos336 **CHARACTERIZATION OF CARDIAC SARCOPLASMIC RETICULUM (Ca^{2+} + Mg^{2+})ATPase USING LASER EXCITED EU^{3+} LUMINESCENCE.** Nanda B. Joshi and Adil E. Shamoo, Membrane Biochemistry Research Laboratory, Department of Biological Chemistry, University of Maryland School of Medicine, Baltimore, MD 21201.

The calcium binding sites of cardiac sarcoplasmic reticulum (Ca^{2+} + Mg^{2+})ATPase were characterized using laser excited luminescence of Eu^{3+} ion. The excitation spectrum of bound Eu^{3+} showed a maximum at 579.3 nm as compared to 578.8 nm in 20 mM MOPS pH 6.8. Europium ion, upon binding with SR (Ca^{2+} + Mg^{2+})ATPase showed an increase in fluorescence intensity as well as lifetimes. The fluorescence decay of bound Eu^{3+} was non-exponential and showed two lifetimes. In the presence of ATP we observed an increase in the fluorescence lifetimes. The lifetimes showed a further increase when c-AMP + PK were present in addition to ATP. Upon binding of Eu^{3+} with SR (Ca^{2+} + Mg^{2+})ATPase we observed an inhibition of ATPase activity and Ca^{2+} uptake. The concentration of Eu^{3+} required for 50% inhibition was 1.8 μM for both ATPase and Ca^{2+} uptake. It is worthwhile to mention that we did not observe any change in c-AMP dependent phosphorylation. The above studies show the existence of two calcium binding sites in cardiac SR (Ca^{2+} + Mg^{2+})ATPase and give information on the molecular environments of these sites upon phospholamban phosphorylation and ATP hydrolysis.

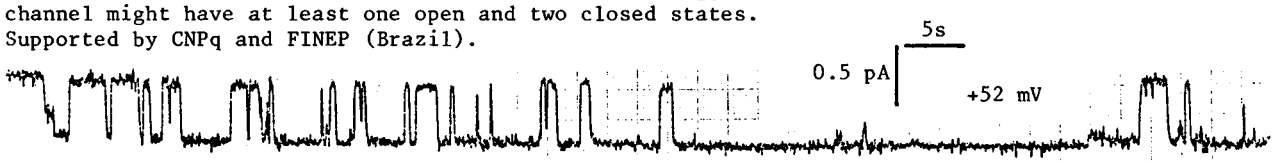
W-Pos337 **OCCLUSION OF Ca^{2+} IN SOLUBLE MONOMERIC SARCOPLASMIC RETICULUM Ca^{2+}-ATPase** Bente Vilsen and Jens Peter Andersen, Institute of Physiology, University of Aarhus, 8000 Aarhus C, Denmark.

To answer the question, whether Ca^{2+}-occlusion in sarcoplasmic reticulum Ca^{2+}-ATPase requires cooperation between two polypeptide chains in a dimer, we incubated soluble monomeric enzyme with $^{45}Ca^{2+}$ in the presence of CrATP as phosphorylating substrate. A stable complex was formed, which was subjected to High Performance Liquid Chromatography (cf. Andersen and Vilsen, FEBS Lett. 189, 13-17), in the presence of non-radioactive Ca^{2+}. Two Ca^{2+}-ions per phosphorylation site were unable to exchange with medium Ca^{2+} within the time required for chromatography (15 min). The Ca^{2+}-ATPase protein containing the occluded $^{45}Ca^{2+}$ eluted corresponding to monomer. We propose that a single Ca^{2+}-ATPase polypeptide chain provides the full structural basis for Ca^{2+}-occlusion.

W-Pos338 Low-conductance channel of purified rabbit skeletal Sarcoplasmic Reticulum. S.Cukierman, A.C.Campos de Carvalho and S.Verjovski-Almeida. Instituto de Biofísica and Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro, 21910 Rio de Janeiro, RJ, BRAZIL.

Sarcoplasmic reticulum (SR) vesicles prepared from rabbit skeletal muscle were fused to asolectin painted membranes (30-50 mg/ml in decane). The membrane separated two compartments containing 50 mM K_2SO_4 and 10 mM HEPES (pH 7.5). SR vesicles (Miller and Rosenberg, 1979, Biochemistry 18:1138 (ref. 1) and purified vesicles (Meissner et al., 1973, Biochim. Biophys. Acta 298:246 (ref. 2) were used yielding distinct electroforetic patterns with the content of 115,000 MW ATPase increasing markedly from preps (1) to (2). The nonpurified prep (1) exhibited the high conductance 200 pS channel described by (1) and a low conductance channel of 10 pS. In contrast, when purified sarcoplasmic vesicles were used only the low conductance channel was observed; the high conductance was only seen once in more than 30 trials performed. The low conductance channel of purified SR has a linear current-voltage relationship over the range -80 to +80 mV; it is virtually impermeable to $SO_4^=$; it has a weak voltage dependence (probability of the open configuration increases from 0.08 at -73 mV to 0.38 at +76 mV). Preliminary observations suggest that ATP has no effect and that the channel might have at least one open and two closed states.

Supported by CNPq and FINEP (Brazil).



W-Pos339 IDENTIFICATION OF COMMON STRUCTURAL DOMAINS BETWEEN THE SR Ca-ATPase AND THE TT Mg-ATPase.

E. Damiani, A. Margreth, P.J. Yazaki and R.A. Sabbadini, NRC Unit for Muscle Biology and Physiopathology, Via Loredan 16, 35100 Padova, Italy, and Department of Biology, San Diego State University, San Diego, CA 92182, USA.

The structural relationship between the sarcoplasmic reticulum (SR) Ca-ATPase and the 100 KDa glycoprotein of transverse tubule (TT), previously tentatively identified as the Mg-ATPase (Okamoto et al., ABB 237,43, 1985), were studied with membranes derived chicken skeletal muscle. Anti-(chicken) SR Ca-ATPase antibody cross-reacted specifically with the TT 100 KDa protein in Western immunoblots of TT proteins, and the extent of cross-reactivity between the SR Ca-ATPase and TT 100 KDa protein was found to be virtually the same, as quantitated by a competitive ELISA. Peptide maps of SR Ca-ATPase and TT 100 KDa protein were obtained by the technique of Cleveland et al., (JBC, 1977), using S. aureus V8 protease and chymotrypsin. Both the proteases yielded similar fragmentations patterns for the two proteins. Further, immunoblots of the peptide maps showed that the antigenic determinants were located in the same proteolytic fragments. Polyclonal antibodies raised against the TT 100 KDa protein, purified by electrophoretic elution, inhibited the Mg-ATPase activity of TT. Furthermore, the binding of anti-(chicken) 100 KDa protein antibody to TT membranes was prevented by preincubation of TT with Con-A, which selectively labeled the TT and, Con-A prevented the inhibitory effect of anti-(chicken) TT 100 KDa protein antibody on the Mg-ATPase activity. We conclude from these results that the 100 KDa protein of TT is the Mg-ATPase and that a high degree of structural homology exists between the SR Ca-ATPase and the TT Mg-ATPase. Supported by Italian National Research Council and MDA and NSF. E.D.is a Fellow of the American Heart Assoc.

W-Pos340 ALTERATION OF SKELETAL JUNCTIONAL SARCOPLASMIC RETICULUM CALCIUM PERMEABILITY BY RYANODINE, DI- AND TRIVALENT CATIONS AND IONIC STRENGTH. F.A. Lattanzio, R.G. Schlatterer & J.L. Sutko. Departments of Physiology and Internal Medicine. University of Texas Health Science Center, Dallas, TX 75235.

Ryanodine either increases or decreases the Ca permeability of rabbit fast twitch skeletal muscle junctional sarcoplasmic reticulum (JSR) membranes depending on the conditions used (Lattanzio et al. Biophys. J. 47:450a, 1985; G. Meissner, personal communication). These effects are not seen with either longitudinal SR or t-tubular membranes and do not require intact triads. We have investigated the actions of ryanodine further by measuring the efflux of Ca from passively loaded JSR vesicles over 1-10 sec intervals. In the presence of intra- (IV) and extravesicular (EV) KCl (100 mM), Ca efflux is rapid and is blocked by Mg or La (5 mM). Ryanodine (1 μM) increased a rapid Ca efflux (<1 sec), which is not blocked by either Mg or La. Hg (1-500 μM) also increased a rapid Ca efflux in both the presence and absence of ryanodine by altering -SH groups (Abramson et al. PNAS 80:1526, 1983). In the presence of only IV KCl (sucrose substitution), ryanodine, but not Hg alone, increased Ca efflux and this was blocked by La (50 μM) or Hg (500 μM), but not by Co, Ni, Cd, Mn or Sr. In the presence of only EV KCl, Hg alone, but not ryanodine, increased Ca efflux. In the absence of both IV and EV KCl, the effects of ryanodine and Hg were absent and reduced, respectively. Both La and Hg decreased [^{3}H]ryanodine binding, but this effect did not occur over the 1-10 sec intervals used in the present studies. These results suggest that the Ca permeability of JSR membranes and the extent to which it is altered by either ryanodine or di- and trivalent cations are influenced by the ionic strength of the medium present at both the IV and EV aspects of the membrane. Supported by PCM 8402100 and HL 17669.

W-Pos341 RAPID KINETIC STUDIES OF CA^{2+}-INDUCED CONFORMATIONAL CHANGES OF THE SARCOPLASMIC RETICULUM (SR) CA^{2+} ATPASE Magotoshi Morii and Noriaki Ikemoto Dept. Muscle Res., Boston Biomed. Res. Inst., and Dept. Neurol., Harvard Med. Sch., Boston, Mass. 02114

Extrinsic fluorescence probes are useful for the studies of conformational changes especially when the probes are attached to a specific site of the enzyme. One of 12 reactive thiols per 10^5 g of the SR Ca^{2+} ATPase (SH_1) has a significantly high reactivity with maleimide derivatives than the others, and its chemical modification produces no effect on the enzyme activity. We have found that the fluorescence intensity of the maleimide derivative, N-(7-dimethylamino-4-methyl-3-coumarinyl)maleimide (DACM), attached to SH_1 increases upon removal of the enzyme-bound Ca^{2+} by EGTA (k_-=4.1-5.0 s^{-1}), and decreases upon a Ca^{2+} jump from 0 to several uM (k_+=3.4-4.1 s^{-1}). The time course of both forward and backward reactions are approximately parallel to those monitored by the intrinsic tryptophan fluorescence. Addition of 0.5 mM AMPPNP during the Ca^{2+} dissociation and binding reactions led to a considerable increase of the rate constants (e.g. k_- up to 15.9-20.5 s^{-1}). Only $\sim$0.5 mol SH_1 per mol enzyme reacts rapidly with DACM at pCa 5 (SH_1-Ca^{2+}), whereas the other 0.5 mol SH_1 becomes reactive by addition of EGTA (SH_1-EGTA). SH_1-Ca^{2+} or SH_1-EGTA was selectively labeled with DACM, and changes in the DACM fluorescence coupled with the Ca^{2+}-isomerization reaction were monitored. The amplitude of the fluorescence change coupled with both forward and backward Ca^{2+} isomerization reactions was 4-5 times larger when DACM was attached to SH_1-EGTA than to SH_1-Ca^{2+}. Supported by grants from NIH(AM 16922) and MDA.

W-Pos342 THE $E_1 \rightarrow E_2$ TRANSITION OF Ca^{2+}-ATPase IN SARCOPLASMIC RETICULUM (SR) OCCURS WITHOUT MAJOR NET CHANGES IN SECONDARY STRUCTURE. P. Csermely[1], C. Katopis[2], S. Papp[1], B. A. Wallace[2] and A. Martonosi[1]. [1]Dept. Biochemistry, SUNY Upstate Med. Ctr., Syracuse, NY 13210, [2]Dept. Biochemistry and Molecular Biophysics, Columbia University, New York, NY 10032.

The transport of Ca^{2+} by SR involves transition between two major conformations (E_1 and E_2) of the Ca^{2+}-ATPase. Circular dichroism (CD) spectroscopy was used to investigate the structure of Ca^{2+}-ATPase in the E_1 and E_2 states in native, in fluorescein isothiocyanate (FITC)-labeled and in solubilized sarcoplasmic reticulum preparations. The two conformational states are characterized by distinct crystal forms and proteolysis patterns (Dux et al., J. Biol. Chem. 260, 11730, 1985) in addition to differences in kinetic properties and fluorescence (Pick and Karlish, J. Biol. Chem. 257, 6120, 1982). For circular dichroism measurements the E_1 state was stabilized by 100 µM Ca and the E_2 state by 0.5 mM Na_3VO_4 and 0.1 mM EGTA. The CD data yield a secondary structure of 46% α helix, 7% β sheet, 12% turn and 35% random coil. There were no significant differences detected in the CD spectra and the calculated secondary structures between the E_1 and E_2 states on any of the three types of SR preparations. The FITC-labeled sarcoplasmic reticulum did show the characteristic changes in FITC fluorescence upon addition of Ca^{2+} or vanadate, indicating that the preparations were competent for $E_1 \rightarrow E_2$ transitions. The absence of changes in the CD spectra implies that the $E_1 \rightarrow E_2$ transition in the Ca^{2+}-ATPase occurs without major net rearrangement of the polypeptide backbone conformation and may involve hinge-type or sliding motion of domains. (Supported by the NIH, NSF and the Muscular Dystrophy Association.)

W-Pos343 $[CA^{2+}]$-DEPENDENCE OF CA^{2+} RELEASE CHARACTERISTIC FOR CARDIAC SARCOPLASMIC RETICULUM (SR). Do Han Kim, Hae Won Kim, Evangelia G. Kranias and Noriaki Ikemoto. (Intr. by S. Sarkar) Dept. of Muscle Res., Boston Biomed. Res. Inst.; Dept. of Neurol., Harvard Med. School, Boston, Ma ; Dept. of Pharmacol. and Cell Biophys., Univ. of Cincinnati, Coll. of Med., Cincinnati, Ohio

Fragmented SR was prepared from canine cardiac muscles and passively loaded with Ca^{2+} by incubating in 5 mM $CaCl_2$ for 6 hrs at 0-4°. $[Ca^{2+}]$-dependence of Ca^{2+}-induced Ca^{2+} release was measured by mixing the loaded SR with various $[Ca^{2+}]$ solutions by means of fluorometry or filtration method. Plots of the initial rate of Ca^{2+} release from cardiac SR versus pCa_0 were compared with those of skeletal muscle SR. The $[Ca^{2+}]_0$ for half maximal activation of Ca^{2+} release is approximately 0.1 uM and 0.5 uM in cardiac and skeletal muscle SR, respectively. On the other hand, Ca^{2+} release from cardiac SR was inhibited in a much higher $[Ca^{2+}]_0$ range than that from skeletal muscle SR. Occasionally, the $[Ca^{2+}]$-dependence pattern appears to consist of two peaks (approximately at 0.2 uM and 1 uM), suggesting that two components may be involved in the regulation of the cardiac Ca^{2+} release. In an actively loaded SR, addition of 2 mM caffeine or 19 uM inositol trisphosphate induced Ca^{2+} release only at low $[Ca^{2+}]_0$. The finding that Ca^{2+} release from cardiac SR is activated at much lower $[Ca^{2+}]_0$ than that from skeletal muscle SR is consistent with the view that Ca^{2+}-induced Ca^{2+} release plays an important physiological role in cardiac muscle. Supported by grants from NIH (AM 16922, HL 26057 and HL 22619) and MDA. D.H.K. was supported by an NIH postdoctoral fellowship.

W-Pos344 PROPERTIES OF CALCIUM UPTAKE BY SARCOPLASMIC RETICULUM OF SAPONIN SKINNED VASCULAR SMOOTH MUSCLE. M.A. Stout* and K.M. Sullivan* (Intro. G.B. Weiss). Dept. Physiology, UMDNJ-New Jersey Medical School, Newark, NJ 07103.

^{45}Ca uptake by sarcoplasmic reticulum (SR) was investigated in strips of rat caudal artery skinned with saponin. The free Ca concentration of the loading medium was varied from 10^{-8} to 10^{-4}M. Ca uptake in skinned strips consists of two major components. The largest requires MgATP and is significantly enhanced by creatine phosphate. The energy-dependent uptake exhibits multimolecular kinetics and saturates at approximately 100 uM/kg wet tissue. This fraction can be released with 10^{-6}M ionophore A-23187. Ca sequestration and Ca release are abolished by skinning in Triton X-100, which disrupts the SR. At free Ca concentrations greater than 3 x 10^{-6}M, total uptake is inhibited by azide and appears to represent Ca sequestered by the mitochondria. Oxalate and higher temperature stimulate the energy-dependent uptake several fold. Lesser increases are caused by phosphate and ruthenium red, an inhibitor of the Ca-sensitive-Ca release. Ca uptake is not affected by 50 uM trifluoperazine (TFP) or by 5 mg/l verapamil. The second component of uptake is ATP-independent and increases as a linear function of the free Ca concentration. It can be released by A-23187 but is not affected by TFP or verapamil. The results indicate that the largest component of Ca sequestration by SR is energy-dependent with a lesser amount proceeding through energy independent pathways. The passive process does not appear to be blocked by compounds which are effective at the plasma membrane. [Supported by NIH grant HL-31152 and AHA, NJ Affiliate grant #84-36].

W-Pos345 CALCIUM REGULATION BY SKELETAL MUSCLE SARCOPLASMIC RETICULUM AND SARCOLEMMA VESICLES ISOLATED FROM MALIGNANT HYPERTHERMIA SUSCEPTIBLE PIGS. James R. Mickelson, Julie A. Ross, Brian K. Reed, Robert J. Hyslop, and Charles F. Louis. Dept. of Veterinary Biology, University of Minnesota, St. Paul, MN 55108.

There is now considerable agreement that sarcoplasmic Ca-regulation is abnormal in the skeletal muscle of malignant hyperthermia (MH) susceptible individuals. We have investigated Ca-transport by sarcoplasmic reticulum (SR) and sarcolemma (SL) vesicles isolated from normal (Yorkshire, Y) and MH-susceptible (Pietrain, P) pigs. Ca accumulation by a terminal cisternae-derived SR fraction (G. Meissner, 1984; JBC 259, 2365) was similar in both Y and P membranes (0.29±.05 vs. 0.25±.03 μmole Ca/mg/min at 22°C, n=5), as was Ca^{2+}-ATPase (0.97±.08 vs. 0.99±.22 μmole P_i/mg/min). However, the extent of Ca-induced Ca release (5 sec) from Ca-loaded SR was significantly greater in P (68%±3) compared to Y (48%±4), $p<.05$. In both cases Ca release was maximal at a Ca^{2+} of 1 μM and half-maximal at a Ca^{2+} of 0.04μM. ATP-dependent Ca transport by Y and P SL vesicles, in the presence of cAMP-PK and calmodulin, had a similar $K_{1/2}$ for Ca^{2+} (0.64-0.68 μM) and V_{max} (20-25 nmole Ca/mg/min at 37°C, n=5). The total Ca-accumulating capacity of P SL, however, was significantly less than that of Y SL (82±9 vs. 112±11 nmole Ca/mg, n=14, $p<.05$). Although cAMP-PK or calmodulin stimulated Y and P SL Ca transport to a similar extent, the Ca capacity of P SL was still 25% reduced. Thus, our results support other findings that while SR Ca uptake is unaffected in MH, SR Ca release is altered. Furthermore, an apparent defect in SL Ca permeability indicates an additional site of abnormal Ca regulation in MH-susceptible muscle. (Supported by The Muscular Dystrophy Association and NIH GM-31382.)

W-Pos346 REGULATION OF THE RYANODINE-SENSITIVE CALCIUM CHANNEL OF CARDIAC SARCOPLASMIC RETICULUM BY pH, CALCIUM AND MAGNESIUM. Grayson B. Lipford and Joseph J. Feher, Department of Physiology and Biophysics, Medical College of Virginia, Richmond VA 23298

Ryanodine is a neutral plant alkaloid which is postulated to close a calcium channel in cardiac sarcoplasmic reticulum vesicles. The effect of pH, calcium and magnesium on the sensitivity of the vesicles to ryanodine was investigated to determine if these ions could open or close the calcium channel. Unambiguous interpretation of the results requires that the pre-incubation conditions allow for maximal stimulation of calcium uptake by ryanodine when assayed under optimal conditions. We have found that the degree of stimulation of calcium uptake by ryanodine depends on the time of pre-incubation, and the temperature, ryanodine concentration and calcium concentration during pre-incubation. Following optimal pre-incubation, loss of ryanodine sensitivity when assayed under different conditions permits the conclusion that the assay conditions closed the channel rather than interfered with the action of ryanodine. The results indicate that calcium opens the channel with an apparent Km near 0.3 x 10^{-6}M, while magnesium and acidic pH both close the channel. The channel was closed about 50% at pH 6.0 or at 4 x 10^{-3}M magnesium. The calcium requirement for opening the channel was very similar to the calcium pre-incubation requirement for ryanodine action. This suggests that the calcium which opens the channel also allows ryanodine to bind and close the channel. The affinity for calcium is about ten times higher for the channel than for the pump, suggesting the channel is distinct from the pump.

This work was supported by NIH Grant HL34681 and a grant-in-aid from the American Heart Association, Virginia Affiliate.

W-Pos347 VOLATILE ANESTHETICS ALTER THE PH DEPENDENCE OF CALCIUM UPTAKE
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A previous study (Tate, et al., J Biol Chem 256:2934,1981) showed that oxalate-supported, ATP-dependent Ca++ uptake by cardiac and skeletal muscle sarcoplasmic reticulum (SR) had a maximal uptake at pH 6.6-6.8 and was markedly inhibited (90-95%) at pH 7.4-7.6. This inhibition was prevented by added Ca++ in the preincubation medium or by changing the alkaline pH in the incubation medium to pH 6.8. We examined the effect of the volatile anesthetics, halothane (H), enflurane (E), and isoflurane (I) on this pH-dependent inhibition. H has been shown to decrease Ca++ uptake in SR at pH 6.9, but had little effect at pH 7.3 (Anesth Analg 60:390,1981). Experiments were carried out on rabbit cardiac SR at 37°C in the control state (no anesthetic present) and in the presence of each of the anesthetics from pH 6.6-7.6 in increments of 0.2 pH units. Three separate preps were used and each experiment was done in triplicate. The control exhibited maximum Ca++ uptake rate at pH 6.8 (406±45 nmoles/mg/min) and showed marked inhibition (82%) of Ca++ uptake at pH 7.6 (71±22 nmoles/mg/min). Ca++ uptake was enhanced by all three anesthetics at pH 7.2, 7.4 and 7.6 and was decreased at pH 6.6 and 6.8. For example, in the presence of H, the maximum Ca++ uptake rate at pH 7.6 was 279±49 nmoles/mg/min and at pH 6.8 was 312±17 nmoles/mg/min, compared to the above controls. The anesthetics had little effect at pH 7.0 (control: 340±24 nmoles/mg/min; H: 353±36 nmoles/mg/min). These results show that volatile anesthetics alter pH control of Ca++ uptake in rabbit cardiac sarcoplasmic reticulum.

W-Pos348 TERBIUM BOUND TO THE HIGH-AFFINITY CALCIUM BINDING SITES OF THE SARCOPLASMIC RETICULUM ATPase IS LESS THAN 10A FROM THE AQUEOUS INTERFACE.
Christopher D. Sprowl, Richard O. Leder, and David D. Thomas, Intr. by E. Ackerman, Department of Biochemistry, University of Minnesota Medical School, Mpls., MN 55455.

The location of the high-affinity calcium binding sites in the three-dimensional structure of the Ca-ATPase of skeletal muscle sarcoplasmic reticulum is essential to an understanding of the ATP-driven calcium transport process. In order to assess the exposure of these sites to the external aqueous interface, we have measured fluorescence energy acceptors outside the SR vesicles, including methylene blue (+1), rhodamine B (0) and Co(III)EDTA (-1). For all acceptors tested the distance of closest approach to the bound terbium site is less than 10 angstroms using either standard dipolar or non-dipolar energy transfer assumptions. Therefore, we propose that the sites to which terbium is bound are collisionally accessible to the external aqueous surface of the vesicles. The electrostatic repulsion expected from a negatively charged membrane surface should make the collisional approach of a negative Co(III)EDTA to the bound Tb particularly unfavorable unless the site is exposed. If these sites are buried in the membrane, they must be in a pore that communicates with the aqueous extravesicular environment. The effect of acceptor charge on the energy transfer efficiency suggests that the region containing these sites has a net negative charge. The application of this approach to study the catalytic intermediates in the Ca-ATPase cycle will be discussed.

W-Pos349 ESTIMATION OF THE DISTANCE BETWEEN Ca^{2+}-BINDING SITES OF SARCOPLASMIC RETICULUM (Ca^{2+} + Mg^{2+})-ATPase.
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Lanthanide ions bind to the high-affinity Ca^{2+}-binding sites of SR ATPase. The luminescent lanthanides Eu^{3+} and Tb^{3+} were bound to the ATPase and excited directly by pulsed laser light. In the absence of energy transfer acceptors, we observe luminescence decays with at least two exponential components. In each case, the decay components correspond to water hydration numbers of 4 and 1.5. These may reflect the environments of the two high-affinity Ca^{2+} binding sites. Using lanthanide donor-acceptor pairs of Eu^{3+} to Nd^{3+} or Pr^{3+} and Tb^{3+} to Nd^{3+}, Ho^{3+}, or Er^{3+}, we estimate an inter-site distance of 8 to 9 Å.

We have also used the stable complex Cr·ATP as an acceptor for energy transfer from Eu^{3+}. In this case, energy transfer is 100% complete. The R_0 value calculated for this pair is 20 Å, implying an upper limit of 10 Å ($R_0/2$) for the Ca^{2+} site to hydrolytic site distance.

W-Pos350 Phospholipid Alterations Induced By Acid-Active Phospholipase C Associated With Cardiac Sarcoplasmic Reticulum. Daniel A. Gamache, Michael L. Hess, and R. C. Franson (Spon:J.F. Chlebowski) Dept. of Biochemistry, Medical College of Virginia, Richmond, VA 23298

Phospholipid composition and calcium transport function were assayed in a preparation of canine cardiac sarcoplasmic reticulum (SR). Acute global ischemia of 30 minutes duration depressed calcium uptake and calcium-stimulated ATPase activities by SR compared to sham-operated controls. Similarly, calcium transport dysfunction was produced in normal SR by incubation at acid pH but not at neutral pH. Using an exogenous substrate, ^{14}C-sphingomyelin, we have demonstrated acid-active phospholipase C (PLC) activity associated with the SR. The specific activity of this sphingomyelinase is 0.51 nmole/min·mg protein at pH 5. Activity is barely detectable at neutral pH. Incubation of normal SR at pH 5 results in a decrease in total lipid phosphorus which is maximal (10%) by 30 minutes whereas incubation at neutral pH results in little to no change in total lipid phosphorus. Lipid analyses revealed lower sphingomyelin-, phosphatidylcholine-, phosphatidylethanolamine-, and phosphatidylserine-associated phosphorus following incubation of SR at pH 5 (30 min.). Also, incubation of SR at pH 5 resulted in an increase (23%) in phosphatidic acid-associated phosphorus compared to control. The accumulation of lysophospholipids was not detected in SR incubated at pH 5. These results demonstrate a non-specific, acid-active, PLC associated with this preparation of cardiac SR. Sodium bisulfite (1mM), an inhibitor of acid-active lysosomal phospholipases, prevented the changes in lipid phosphorus during incubation at pH 5. These results suggest a relationship between a reduced pH during ischemia, lysosomal PLC, and SR calcium transport dysfunction. Supported by a Fellowship from the American Heart Association/Virginia Affiliate

W-Pos351 GTP HYDROLYSIS BY CANINE CARDIAC SARCOPLASMIC RETICULUM: SENSITIVITY TO DETERGENT TREATMENT. C.A. Tate, R.J. Bick, N.M. Scherer, and M.L. Entman. Section of Cardiovascular Sciences, Baylor College of Medicine, Houston, Texas 77030.

We previously demonstrated that the SR isolated from canine cardiac muscle hydrolyzes GTP by a mechanism different than for ATP in that GTP hydrolysis is not sensitive to Ca^{2+} and does not involve the formation of a stable Ca^{2+}-sensitive acylphosphate. GTP and ATP may be hydrolyzed by the same enzyme, the ATPase, because: (1) GTPase activity is equal to the total ATPase activity; (2) the non-linear kinetics and kinetic constants are similar; and (3) ATP inhibits the GTPase in a non-competitive fashion. Thus, the GTPase may represent an alternative enzyme cycle of the ATPase. In contrast to the native enzyme, purification of the ATPase with detergent solubilization results in no apparent GTPase activity. In the present study, GTPase activity was followed throughout the purification procedure. Unlike the native enzyme, the purification procedure did result in a GTPase which was totally sensitive to Ca^{2+}; however, the Ca^{2+}-GTPase was very unstable and was inactivated rapidly with time. This sensitivity of the Ca^{2+}-GTPase to detergent treatment was explored by adding different detergents directly to the reaction mixture. With $C_{12}E_8$, the Ca^{2+}-GTPase was fully expressed at a 1:1 ratio (20 ug/ml; below the CMC) and was rapidly inactivated beyond this ratio. In contrast, Ca^{2+}-ATPase activity was activated and was stable with $C_{12}E_8$ treatment up to a 3:1 ratio (at the CMC). Other detergents showed similar effects. The GTPase was not sensitive to vanadate, unlike the Ca^{2+}-ATPase, until $C_{12}E_8$ was added to express the Ca^{2+}-GTPase. This suggests that the membrane lipids play an important role in the expression of the alternative enzyme cycle. Supported by NIH (HL 13870) and the American Heart Association (Texas Affiliate).

W-Pos352 THE EFFECTS OF VANADATE ON PROTEIN AND LIPID DYNAMICS IN SARCOPLASMIC RETICULUM.

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We have studied the effects of vanadate, a phosphate analog, on the Ca-ATPase from sarcoplasmic reticulum (SR), using saturation transfer electron paramagnetic resonance (ST-EPR). Since ST-EPR provides a sensitive measure of microsecond protein rotational mobility, and hence of protein-protein association, these studies allowed us to ask whether changes in protein association occur under conditions sufficient for the enzyme inhibition but too low for the production of the large vanadate-induced arrays detectable by electron microscopy (EM). Under conditions that crystallize the ATPase on greater than 90% of the membrane surface area in EM (40 mg/ml protein, 5mM decavanadate, 0.1M KCl, 10mM imidazole, 5mM $MgCl_2$, 0.5mM EGTA, pH 7.4 at 4°C), ST-EPR shows substantial immobilization of the spin-labeled protein, indicating protein-protein association in the unstained vesicles. Subsequent EM of the ST-EPR samples shows that spin labeling has no effect on crystallization. Conventional EPR spectra of lipid probes shows that lipid hydrocarbon chain mobility is unaffected by protein crystallization in SR, indicating that the protein-protein interaction is external to the bilayer. At 5mM monovanadate, a concentration sufficient to inhibit the ATPase but not to form crystals detectable by EM, no changes were observed in either the protein or lipid spectra, thus indicating no change in oligomeric state. The relationship between the enzyme-vanadate state and the phosphoenzyme will be discussed and related to the role of protein-protein interactions in molecular models of calcium transport.

W-Pos353 DIFFERENT SENSITIVITY OF FAST, SLOW, AND CARDIAC SARCOPLASMIC RETICULUM TO DOXORUBICIN

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Doxorubicin causes massive Ca2+ release from terminal cisternae of sarcoplasmic reticulum (SR) from rabbit fast muscle (JBC 260:7349,1985) at concentrations comparable to those reached in cardiac myocytes during therapeutical trials (BBA 779:274,1984). However cardiac toxicity of doxorubicin has been related mainly to peroxidative injury (PNAS 76:954,1979), but not to disturbances of calcium homeostasis. The possibility was therefore tested that SR from different muscles has different sensitivity to doxorubicin. Chemically skinned fibers from rabbit fast, slow, and cardiac muscle were challenged with stepwise increasing concentrations of doxorubicin, after loading the SR with calcium at three different pCa (7.0, 6.8, and 6.6). SR Ca2+ efflux was monitored by following tension development. The concentration of doxorubin that induced tension larger than 10% of Po (tension induced by 20mM caffeine) was determined as the threshold. The results show that, after loading the SR at pCa 7.0, doxorubicin threshold was 4uM, 13uM, and 50uM for fast, slow, and cardiac fibers, respectively. The ratio between tension developed by doxorubicin at the threshold and tension elicited by 20uM caffeine was higher in fast than in slow fibers (0.5±0.05 and 0.39±0.05, respectively). The differential pattern of responses was unchanged after loading the SR at higher Ca2+ concentrations. The results show that slow and cardiac SR are much less sensitive to doxorubicin than fast SR and suggest that the mechanisms controlling Ca2+ release by doxorubicin might be different in SR from different types of muscle. Supported by institutional funds from CNR and by grants from MPI.

W-Pos354 PATTERNS OF PROTEOLYTIC DIGESTION AND DICYCLOHEXYLCARBODIIMIDE (DCCD) LABEL DISTRIBUTION IN SARCOPLASMIC RETICULUM (SR) ATPase. Jorge Garcia de Ancos and Giuseppe Inesi, Department of Biological Chemistry, University of Maryland School of Medicine, 660 West Redwood Street, Baltimore, Maryland 21201.

SR vesicles were subjected to proteolytic digestion with trypsin and chymotrypsin before and after incubation with DCCD. No DCCD label was ever found in the ATPase tryptic fragment B that includes the FITC label, the nucleotide binding site and several of the transmembrane segments. On the contrary, the DCCD label was found in the A1 tryptic fragment that includes the phosphorylation site and several acidic residues in the stalk segment, and in the A2 tryptic fragment that also includes several acidic residues in the stalk segment, and in the connection between the first two transmembrane helices. Digestion of SR ATPase with chymotrypsin yields two main fragments similar in size to the main tryptic fragments, suggesting that the two enzymes act on an identical fold of the ATPase molecule. However, the aminoacid specificity of the two proteolytic enzymes results in a slightly larger B fragment (and smaller A fragment) in the case of chymotrypsin. The FITC labeled lysine 514 remains in the B fragment; DCCD labels in the A fragment and subfragments thereof following digestion with chymotrypsin. Our findings indicate that calcium binding and phosphorylation catalysis, can be blocked by derivatization of aminoacid residues in a region including segments of tryptic fragments A1 and A2, without involving the nucleotide binding site and the membrane spanning region of tryptic fragment B. This work was supported by NIH grant HL 27867 and the MDA.

W-Pos355 PREPARATION AND CHARACTERIZATION OF SITE-SPECIFIC CALMODULIN DERIVATIVES.

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Wheat germ calmodulin (CaM), which contains a single cysteine (Cys 27), was derivatized with either the fluorescent reagent 1,5-IAEDANS or the photoactivatable crosslinker benzophenone-4-maleimide (Bz). Comparison of the native and derivatized wheat germ CaMs with native bovine testes CaM indicated that the concentration of these proteins required for half-maximal stimulation of either erythrocyte membrane Ca-ATPase or cardiac sarcoplasmic reticulum (CSR) phosphorylation were very similar. While there was little change in fluorescence intensity due to Ca^{2+}-binding to the target protein troponin I (TnI), fluorescence anisotropy experiments indicate AEDANS-CaM binds TnI with $K_a \cong 10^8$. Preliminary experiments with CSR vesicles indicate that AEDANS-CaM is suitable for fluorescence anisotropy studies of CaM binding to these membranes. Crosslinking experiments using Bz-CaM that was labeled with ^{125}I at the single tyrosine residue (with no loss in biological activity) demonstrate CaM-binding to TnI and TnT in binary complexes, as well as to both subunits in CaM-TnI-TnT ternary complex. This would suggest that both subunits are within 10Å of Cys 27 of CaM. Affinity-labeling of CSR with Bz-CaM exhibits Ca^{2+}-dependent labeling of phospholamban as shown previously with bovine CaM (Louis and Jarvis, J. Biol. Chem., 257:15187 (1982)). Thus, it is possible to chemically modify Cys 27 of CaM without altering its biological activity. Site-specific CaM derivatives such as these will prove useful in further defining the interactions that CaM makes with its receptor proteins. (Supported by NIH HL 25880).

W-Pos356 **RETENTION OF ELLIPTICITY BETWEEN ENZYMATIC STATES OF THE CA^{2+}-ATPase OF SARCOPLASMIC RETICULUM.** Robert K. Nakamoto and Giuseppe Inesi, Department of Biological Chemistry, University of Maryland School of Medicine, 660 W. Redwood St., Baltimore, MD 21201.

Circular dichroism spectra in the peptide region were obtained from the Ca^{2+}-ATPase of sarcoplasmic reticulum, in order to establish whether transitions of intermediate states of the enzyme cycle are accompanied by large changes of secondary structure. Since membrane-bound ATPase was used to avoid denaturation, absolute estimates of secondary structural content could not be obtained, due to light scattering interference. Nevertheless, it was possible to demonstrate unambiguously that nearly constant ellipticity is retained by the enzyme following enzyme transitions produced by calcium binding or phosphorylation even though conformational changes are revealed by other structural probes in the presence of the Ca^{2+}-ATPase under the same conditions. We conclude that the conformational changes involved in the long range reciprocal influence of calcium and phosphorylation sites, are related to ligand induced displacements of amino acid residues which in turn produce reorientation of whole peptide segments of the ATPase protein. This is contrasted by the behavior of calmodulin which undergoes a definite change in ellipticity upon calcium binding.

W-Pos357 **A STRUCTURAL ORIGIN FOR THE LOW-TEMPERATURE TRAPPING OF E~P IN ISOLATED SARCOPLASMIC RETICULUM.** D. Pascolini[1],L.G. Herbette[2], V. Skita[1], F. Itshak[1], A. Scarpa[1] and J.K.Blasie[1],[1]Depts. of Chemistry and Biochemistry/Biophysics, U. of Pennsylvania, Philadelphia, PA 19104 -[2] Depts. of Medicine and Biochemistry, U. of Connecticut, Farmington, CT 06032.

The calcium uptake reaction kinetics of isolated SR, as determined utilizing flash-photolysis of caged ATP and double-beam spectrophotometric techniques, is at least biphasic over a range of temperatures. A fast phase is identified with the formation of E~P and the Ca^{2+} "occlusion" phenomenon and a slow phase with the Ca^{2+} translocation across the membrane profile and turnover of the Ca^{2+} ATPase ensemble. At temperatures of 0°C or lower, only the fast phase is apparent in the absence of turnover over a time interval of several seconds, indicative of the transient trapping of the first phosphorylated intermediate of the Ca^{2+} ATPase for a few seconds within this time interval. The analysis of time-resolved X-ray diffraction of oriented multilayers of SR membranes performed at 7°C and 0°C via model refinement calculations have indicated that at both temperatures a redistribution of protein mass from the extravesicular surface to the interior regions of the membrane profile occcurs with the formation under turnover conditions or the trapping of E~P. Since at the two temperatures this redistribution is only qualitatively similar and since the profile structures in the resting state prior to enzyme phosphorylation are also significantly different, the origin of the low temperature trapping of E~P appears to be structural in nature. We have investigated at relatively high resolution the nature of the X-ray diffraction and the membrane profile structure over a range of temperatures from 7°C to -2°C. The membrane appears to undergo a reversible structural transition in a narrow range of temperatures centered at 2°C, which involves the protein and possibly the lipid components. Support: NIH HL-18708,-32588.

W-Pos358 PURIFICATION OF CARDIAC JUNCTIONAL SARCOPLASMIC RETICULUM HIGH MOLECULAR WEIGHT (HMW) PROTEINS Seiler, S.*, Mitchell, R.D. and Jones, L.R. *Dept. of Cardiovascular Biol., Bristol-Myers Pharm. R. & D. Div., Evansville, IN, and Krannert Inst. of Cardiol., Dept. of Pharm. and Dept. of Med., Indiana Univ. School of Med., Indianapolis, IN.

A set of high molecular weight (HMW) proteins uniquely located in junctional sarcoplasmic reticulum (JSR) isolated from both cardiac and skeletal muscle has previously been identified (Seiler et al. JBC 259, 8550 (1984)). These proteins bind iodinated-calmodulin, are phosphorylated by an endogenous Ca^{2+} plus calmodulin dependent protein kinase, and are probably components of the junctional process between transverse-tubule and JSR. We now report the purification of the HMW proteins. Cardiac JSR HMW proteins were solubilized in high ionic strength medium (0.5 M NaCl) containing 1% Zwittergent 3-14 and protease inhibitors. Solubilized proteins were purified by gel exclusion chromatography (using Fractogel TSK-HW 65F), followed by selective precipitation using low concentrations of polyethylene glycol. Purity of the proteins was based on SDS-PAGE which indicated a protein doublet with molecular weight approximately 320,000. The cardiac JSR HMW proteins were phosphorylated by the endogenous Ca^{2+} plus calmodulin dependent protein kinase while in the intact membrane and then isolated in the phosphorylated state. The JSR HMW proteins were phosphorylated to between 1 and 2 sites per 320,000 subunit. Analysis of the amino acid(s) phosphorylated indicated the presence of only phospho-serine. The JSR HMW proteins were also isolated after labelling with ^{125}I-calmodulin. Comparison of the cardiac JSR HMW proteins with microtubule-associated proteins II (from brain) demonstrated that the proteins were different by a variety of criteria. This work was supported by grants from the NIH, by the MDA, by the AHA and by the Herman C. Krannert Fund.

W-Pos359 ISOLATION OF SARCOPLASMIC RETICULUM CaATPase FRACTIONS BY DETERGENT TREATMENT AND ISOELECTRIC FOCUSING. R.J.Bick, G.M.Albright, C.A.Tate, M.L.Entman and W.B. Van Winkle. Depts., of Medicine and Biochemistry, Baylor College of Medicine and The Methodist Hospital, Houston, Texas 77030.

Isoelectric focusing of skeletal muscle and cardiac SR in 1% Agarose gels, following solubilization in the detergent $C_{12}E_8$, showed different band patterns for the two fractions and different pI points for the major bands in these fractions (skeletal 5.6 and 5.8, cardiac 4.8 and 5.1). Further separation of these solubilized fractions by gel filtration through a BioGel TSK3000 SW column, using an LKB GTI system, and subsequent isoelectric focusing, showed one or two major peaks for the skeletal muscle sample and always two major peaks for the cardiac sample. As stated above, the pI points of these fractions are different. Protein from these peaks was phosphorylated by $AT^{32}P$ in the presence of calcium, to an extent found by rapid chemical quenching, acyl phosphate experiments. The fractions obtained from the column exhibited only "basal" ATPase activity. SDS polyacrylamide gel electrophoresis of these major bands showed them all to have approximately the same molecular weight of 96,000. We believe that these different bands shown via isoelectric focusing are possibly different isomeric subunits of the calcium pump protein, and that the ATPase from the skeletal muscle SR differs to a greater extent from the cardiac enzyme than originally thought.

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W-Pos360 INACTIVATION OF THE SARCPLASMIC RETICULUM Ca-ATPase BY FITC OR CROSS-LINKING: EVIDENCE FOR PROTEIN-PROTEIN INTERACTIONS IN THE ENZYME MECHANISM. Stephanie E. Hughes, Thomas C. Squier and David D. Thomas, Dept. of Biochemistry, University of Minnesota, Minneapolis, MN. 55455.

We have used two modifications of the Ca-ATPase to investigate the need for protein mobility and the role of protein-protein interactions in the sarcoplasmic reticulum (SR). One method involved intermolecular cross-linking with the cleavable homobifunctional cross-linker dithiobis succinimidyl propionate (DSP). Cross-linking forms covalently bound oligomers, as seen in gel electrophoresis, and inhibits protein rotational mobility, as measured by saturation transfer EPR. The second method involved an intramolecular reaction using fluorescein isothiocyanate (FITC). FITC is known to be a site-specific inhibitor of the Ca-ATPase and prevents ATP binding to the high affinity site on the enzyme. This is fundamentally different from cross-linking, which involves modification of two polypeptide chains. Inactivation of the Ca-ATPase with either the specific inhibitor FITC or the cross-linker DSP is second-order with respect to the monomeric unit of the Ca-ATPase, indicating that polypeptide interactions occur. While these inactivation studies demonstrate that protein-protein interactions are important, cross-linking studies show no evidence for the presence of stable oligomers of a specific size. Therefore, we propose that protein associations and dissociations are essential steps in the reaction mechanism. In this model, overall rotational mobility is important in the formation and dissociation of productive complexes.

W-Pos361 PHOSPHORYLATION-DEPENDENT RELEASE OF CALCIUM FROM THE TRANSPORT SITES OF PURIFIED SARCOPLASMIC RETICULUM ATPase: A TIME-RESOLVED FILTRATION STUDY.
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When the catalytic cycle is initiated by addition of MgATP to a calcium-saturated SR ATPase preparation, a conformational transition of the enzyme in the phosphorylated state, associated with a diminution of the affinity for calcium of the transport sites, leads to release of the bound ions on the luminal side of the transport enzyme. Using leaky preparations, we have investigated the kinetics of these calcium movements from the ATPase sites towards the vesicle internal medium by Millipore filtration techniques with a newly-designed rapid filtration system (Biologic, Anal. Biochem. 142, 504-510).

At 20°C, magnesium, pH, KCl or DMSO modified the rate and extent of calcium release in a way consistent with what has been learnt (mainly from work performed at 0-4°C) about the effect of these agents on the transition between ADP-sensitive (Ca_2E_1P) and ADP-insensitive (E_2P) phosphoenzymes. In addition, we give here direct demonstration that ATP accelerated the rate of calcium release, with a poor affinity (0.5 mM at 20°C, pH 6, in the presence of 0.1 mM Ca, 20 mM Mg, 150 mM Mes-Tris). Under conditions where this transition from Ca_2E_1P to E_2P is rate limiting (for instance in the presence of potassium at neutral pH and room temperature), we suggest that such an accelerating effect will be respnsible for the well-known activation of overall ATP hydrolysis by mM concentrations of ATP.

W-Pos362 PRESTATIONARY KINETICS OF THE SARCOPLASMIC RETICULUM ATPase WITH Ca-ATP AS SUBSTRATE. ASSOCIATION OF RAPID FILTRATION AND MULTIMIXING TECHNIQUES. J.J. LACAPERE, Y. DUPONT and F. GUILLAIN. Service de Biophysique, Département de Biologie, CEN Saclay, 91191 Gif s/Yvette cedex, FRANCE

In the absence of magnesium and KCl, at 5°C, pH 7, and at low ATP concentration, SR vesicles slowly hydrolyse ATP and accumulate Ca (Ca/ATP=1.4). When SR vesicles preincubated with Ca (uM) are layered on a Millipore filter and then perfused during various times with a medium containing Ca and a mixture of ATP.γ-^{32}P and ATP.U-^{14}C, the ^{32}P and ^{14}C radioactivities bound by the vesicles increase in a very similar manner during the first 0-200 ms ($t_{1/2} \simeq 50$ ms). Then the ^{32}P radioactivity still increases but very slowly, whereas the ^{14}C radioactivity decreases ($t_{1/2} \simeq 1$s). In multimixing experiments carried out under very similar conditions, the pool of the different covalent phosphoenzymes appears slower than the rapid increase in ^{32}P and ^{14}C but faster than the slow decrease of the ^{14}C radioactivity signal. When taken together, the results of the rapid filtration and multimixing methods lead to the estimation of several intermediate species. Analysis of the amplitudes and the rates of appearance and desappearance of the measured species allows the following interpretations : i) Ca.ATP binding is a fast process whose rate increases with substrate concentration ($k=2.10^6 M^{-1} s^{-1}$). The rate constant of Ca.ATP dissociation has been estimated to amount 6 s^{-1}. ii) the E.Ca.ATP complex goes first through an overshoot and then decreases but does not fall down to zero. iii) the rate of ADP release is not very different from the one of the phosphorylation process. iv) the Ca release inside the vesicles during transport is a slow process, whose rate is very similar to the rate of the steady state ATP hydrolysis.

W-Pos363 ISOLATION OF THE SPANNING PROTEIN FROM SKELETAL MUSCLE TRIADS. R. M. Kawamoto, J-P Brunschwig and A. H. Caswell. Dept. of Pharmacology, Univ. of Miami School of Medicine, P.O. Box 016189, Miami, Fla. 33101.

We have purified a protein of approximately 300,000 daltons from preparations of terminal cisternae (TC)/triads. It has been shown earlier that this protein represents the junctional protein spanning the gap between transverse tubule and sarcoplasmic reticulum in rabbit skeletal muscle triads (Cadwell and Caswell, J. Cell Biol., 93:543, 1982). An enriched preparation of the 300,000 dalton (spanning) protein was used to immunize mice for the development of monoclonal antibodies. Membrane fragments were obtained by Triton X-100 treatment of TC/triads. The fragments were solubilized with detergent and NaCl and chromatographed using a Sephacryl S-400 column to fractionate spanning protein enriched fractions. The hybridomas positive for the spanning protein were cloned and used for the production of ascites fluid in mice. An affinity column was constructed by coupling the monoclonal antibodies purified by DEAE Affigel Blue chromatography to CNBr activated Sepharose. Preparations of TC/triads were dissolved with a combination of a zwitterionic detergent and NaCl, applied to the column and eluted with a solution of 4M NaSCN and detergent. A single major protein of approximately 300,000 daltons was obtained as determined by SDS-PAGE. The eluate was chromatographed on a Sephacryl S-300 molecular sieve column to separate the protein from the detergent and salt. Two protein peaks were detected. The first peak migrated with an apparent M_r of approximately 530,000 daltons suggesting a polymeric association. The second peak gave an apparent M_r of approximately 170,000daltons suggesting interaction with the column matrix. Supported by Muscular Dystrophy Fellowship (to RMK) and NIH grant AM-21601.

W-Pos364 EFFECTS OF RYANODINE ON SKINNED STRIATED MUSCLE FIBERS OF THE RABBIT. Judy Y. Su, Department of Anesthesiology, RN-10, University of Washington, Seattle, WA 98195

Ryanodine produces irreversible contracture in vertebrate intact skeletal muscle, and a biphasic effect (positive and negative inotropic effects) on isolated intact cardiac muscle. Evidence indicates that sarcoplasmic reticulum (SR) is the site of action. However, a direct demonstration of cause-effect relationship has not been established. We used skinned muscle fibers to study the effect of ryanodine on Ca^{2+} uptake or release from the SR at the same time tension was measured. Papillary muscle (PM), soleus (SL) and adductor magnus (AM) (slow- and fast-twitch skeletal muscle) of the rabbit were isolated and skinned by homogenization in relaxing solution (7 mM EGTA and no added Ca^{2+}). Fiber bundles from PM and single fibers from SL and AM were dissected from the homogenates and mounted on a photodiode force transducer. The SR of skinned fibers were then loaded with Ca^{2+} (uptake phase) and Ca^{2+} was released (release phase) from the SR with caffeine resulting in a tension transient (Pflügers Arch. 380:29, 1979). We found that ryanodine, up to 1 mM, did not significantly change Ca^{2+}-activated tension development of the contractile proteins. Ryanodine ($\geq$5 nM for PM, >μM for AM, and >10 μM for SL) irreversibly decreased, in a dose-dependent manner, subsequent control tension transient. Following first administration of ryanodine, the Ca^{2+} uptake by the SR was increased by ryanodine in PM (0.1 μM), but not in AM or SL. Ryanodine (0.01-5 μM for AM and 10-100 μM for SL) increased Ca^{2+} release (for both AM and SL) and Ca^{2+} uptake (for SL) by the SR. We conclude that ryanodine-induced increases in Ca^{2+} uptake by the SR and decreases in subsequent control tension transients in PM are responsible for positive and negative inotropic effects on cardiac muscle. Ryanodine-induced increases in Ca^{2+} release from the SR in AM and SL are responsible for contracture in skeletal muscle. Supported by grants #HL 20754 and #HL 01100 (RCDA) from NIH and a grant-in-aid from the American Heart Association of Washington.

W-Pos365 ALLOSTERIC REGULATION OF CARDIAC SARCOPLASMIC RETICULUM. Michael B. Cable and F. Norman Briggs, Medical College of Virginia, Richmond, Virginia 23298.

Four mechanisms for the allosteric regulation of the Ca,Mg-ATPase by ATP were investigated: i) negative cooperativity in substrate binding; ii) modulation of kinetics via occupancy of an effector site in a functional monomer; iii) modulation of kinetics via interaction of active sites in an oligomer; and, iv) modulation of kinetics via a branched pathway created by binding of nucleotide at the active site. Negative cooperativity was not observed in the binding of the ATP analog [^{3}H]AMPPCP. Cardiac sarcoplasmic reticulum had a single class of high affinity sites (K=6.42μM) which bound [^{3}H]AMPPCP] in a noncooperative manner. Binding of substrate to an allosteric site in addition to the active site was eliminated by the site stoichiometry of the AMPPCP sites, 1.4 nmol/mg protein or about 1 mole site per mole enzyme. Allosteric regulation via site-site interaction in enzyme oligomers was eliminated by the linear inactivation of ATPase by fluorescein isothiocynate incorporation. Thus three of the mechanisms are excluded by the experimental data. The proposed mechanism is a branched kinetic pathway in which the E* form of the enzyme binds ATP at a lower affinity than the E form and the E* to E transition is stimulated by bound ATP. This mechanism was supported by two lines of experimental evidence: The ratio of v/EP is constant at increasing ATP concentrations, and enzyme turnover decreases the apparent affinity of the enzyme for ATP. Computer simulation of the proposed kinetic scheme showed that a branched pathway on an enzyme monomer could account for all the kinetic effects observed with ATP and AMPPCP.

W-Pos366 COMPLEMENTARY FREEZE-FRACTURE PREPARATIONS OF Ca^{2+}-ATPase CRYSTALS FROM SARCOPLASMIC RETICULUM. H. P. Ting-Beall[1], L. Dux[2], and A. N. Martonosi[2]. [1]Dept. of Anatomy, Duke Univ. Med. Ctr., Durham, NC 27710, [2]Dept. of Biochemistry, SUNY Upstate Med. Ctr., Syracuse, NY 13210.

Two distinct forms of Ca^{2+}-ATPase crystals have been analyzed in SR membranes. The E_1 type crystals, induced by Ca^{2+} or lanthanide ions, consist of single chains of ATPase monomers and the E_2 type crystals, induced by vanadate ions, consist of dimer chains. Using improved freeze-fracture techniques we have obtained high resolution images of complementary surface replicas of SR membranes containing these 2 crystal forms. In E_1 crystals, the concave fracture (P) faces display obliquely oriented rows of intramembrane particles (IMPs) spaced at ≈60-70 Å along both crystal axes, while the convex fracture (E) faces show corresponding rows of pits. In E_2 crystals, regular arrays of oblique parallel ridges with spacing of ≈105-110 Å appear on the P faces and complementary grooves or furrows on the E faces. In many instances the ridges break up into elongated particles repeating every 55 Å. When the direction of the shadow is parallel to the axis of the ridges, the 95 Å particles can be resolved into 2 domains, which could represent intramembranous contacts between the 2 monomers within the same dimer chain or the two adjacent dimer chains. 3-D reconstruction of frozen hydrated samples of E_2 crystals seem to suggest the latter (see Ho, Taylor and Martonosi, abstract at this meeting). Complementary grooves on the E faces can also be resolved into rows of pits complementary to the particles of the ridges on the P faces. In the control SR membranes, randomly dispersed IMPs and corresponding pits are observed on the P and E faces, respectively. The data suggest that transport of Ca^{2+} involves significant structural changes of the enzyme molecule, reflected in the ATPase-ATPase interactions both on the cytoplasmic surface and in the lipid bilayer. (Supported by NIH grants GM 27804 and AM 26545).

W-Pos367 STRUCTURE OF FROZEN HYDRATED VANADATE INDUCED SARCOPLASMIC RETICULUM Ca^{2+}-ATPase CRYSTALS. M.-H. Ho[1], K. A. Taylor[1] and A. N. Martonosi[2]. [1]Anatomy Dept., Duke University Med. Center, Durham, NC 27710, [2]Dept. of Biochemistry, SUNY Upstate Med. Center, Syracuse, NY 13210.

Sarcoplasmic reticulum membranes, when incubated with 5 mM Na_3VO_4 and 0.5 mM EGTA, form crystalline tubules with diameters ranging from 60-100 nm. Because vanadate ion functions as the analog of inorganic phosphate, this crystalline form of Ca^{2+}-ATPase is believed to contain molecules in the E_2 conformation. Low dose electron microscopy has been used to obtain images of such crystals prepared in the frozen hydrated state. We have reconstructed the 3-dimensional density of 4 membrane tubules using Fourier-Bessel image reconstruction methods. Layer line data were extracted from 512 x 1024 point transforms followed by (1) averaging over the near and far side data and (2) imposing a 2-fold symmetry axis. The density maps obtained from these unstained membranes give the overall molecular structure, including the cytoplasmic portion of the molecule outside the lipid bilayer, the shape of which has been previously determined from negatively stained samples, and the intramembranous regions which have not been observed previously. The shape of the cytoplasmic region of the molecule obtained from both negatively stained and frozen hydrated samples match well and include both a protruding lobe and a bridge region which connect Ca^{2+}-ATPase molecules to form the dimer. The intramembranous peptide domains of the two protein molecules making up the dimer spread further apart as they span the bilayer towards the luminal side of the tubule. Contact is made with Ca^{2+}-ATPase molecules in neighboring dimer chains thereby determining the lateral aggregation of dimer chains to form the crystalline lattice. (Supported by NIH, NSF and MDA.)

W-Pos368 MEMBRANE CRYSTALS OF THE Ca^{2+}-ATPase OF SARCOPLASMIC RETICULUM (SR) AND THE Na^+,K^+-ATPase OF DOG KIDNEY. S. Varga, P. Csermely, N. Müllner and A. Martonosi, Dept. Biochemistry, SUNY Upstate Medical Center, Syracuse, New York 13210.

The Ca^{2+}-ATPase of SR forms monomer type (P1) crystals in the presence of Ca^{2+} (100 μM) or lanthanides (5-8 μM) reflecting the E_1 conformation, and dimer type (P2) crystals in the presence of vanadate (1-5 mM) or Pi, reflecting the E_2 conformation (Dux et al., J. Biol. Chem. 260, 11730-11743, 1985). The P1 and P2 type crystals do not coexist in significant amounts in SR under either of the two conditions. The vanadate-induced crystallization of Ca^{2+}-ATPase is inhibited by guanidino group reagents (2,3-butanedione and phenylglyoxal), suggesting the involvement of arginine residues in vanadate binding. SH group reagents (salyrgan, PCMB, N-ethylmaleimide), phospholipases C or A_2, and various detergents also inhibit crystallization, together with inhibition of ATPase activity. Amino group reagents (fluorescein 5'-isothiocyanate, pyridoxal-5'-phosphate. fluorescamine) inhibit ATPase activity but do not interfere with crystallization. The Na,K-ATPase of dog kidney forms primarily protomeric (P1) crystals upon treatment with Ca^{2+}, lanthanides, vanadate or inorganic phosphate, that may coexist with a small amount (< 20%) of diprotomeric (P2) form. The extent of crystallization was influenced by $[Na^+]$, $[K^+]$ or $[Mg^{2+}]$, but the relative amounts of P1 and P2 crystals did not show clear correlation with the expected conformation of Na^+,K^+-ATPase. Although fine structural analysis may eventually reveal subtle differences between the P1 crystals of Na^+,K^+-ATPase obtained under different conditions, it appears that in contrast to Ca^{2+}-ATPase, the crystal form of Na,K-ATPase is not a sensitive indicator of enzyme conformation. The attachment of the β-subunit on the extracellular surface of the Na,K-ATPase (Herbert et al., FEBS Lett. 187, 182-186, 1985) may be responsible for this difference. (Supported by the NIH, NSF and MDA).

W-Pos369 **PHOSPHORYLATION OF JUNCTIONAL-SPECIFIC SARCOPLASMIC RETICULUM PROTEINS.** Toshiaki Imagawa, Steven D. Kahl, Carol Reynolds Raab and Kevin P. Campbell, (Intr. by Andrew Yen), Dept. of Physiology and Biophysics, The University of Iowa, Iowa City, IA 52242.

We have discovered that the isolated triad fraction from rabbit skeletal muscle contains an intrinsic phosphorylation system. This phosphorylation system was neither Ca^{2+}-calmodulin dependent nor cAMP-dependent. The major protein substrates phosphorylated in the isolated triads by this intrinsic phosphorylation system exhibited apparent molecular weights of 300,000, 166,000, 90,000 and 57,000. Minor protein substrates phosphorylated exhibited molecular weights of 350,000, 220,000, 60,000 and 56,000. This phosphorylation system was not present in the light fraction of skeletal sarcoplasmic reticulum. Phosphorylation of the T-system fraction of skeletal muscle revealed protein substrates of molecular weights 220,000, 166,000 and 56,000. Thus, the phosphorylated substrates of molecular weight 350,000, 300,000, 90,000, 60,000 and 57,000 were specific for the sarcoplasmic reticulum. Identification of the phosphorylated substrates was performed by immunoprecipitation with monoclonal antibodies specific to the junctional sarcoplasmic reticulum proteins. The 90,000 and 60,000 dalton phosphoproteins were immunoprecipitated with monoclonal antibodies to the junctional-specific 90,000 and 60,000 membrane proteins. Results suggest that the junctional sarcoplasmic reticulum membrane contains an intrinsic phosphorylation system. We are currently using monoclonal antibodies to 90,000 and 60,000 proteins to investigate the possible function of the intrinsic phosphorylation system in regulation of Ca^{2+} release and/or inactivation of Ca^{2+} release. (T.I. is MDA Postdoct. Fellow. Supp. by NIH NS 18814 and MDA)