Tu-Pos383

ELECTROPHORESIS OF NATIVE F-ACTIN IN A SMALL CAPILLARY. J. Borejdo* and H. Ortega, CVRI, U.C. San Francisco CA 94143 (*Present addr: Baylor Research Foundation, Baylor University Medical Center, Dallas TX 75226)

Native F-actin was electrophoresed in a capillary tube filled with agarose. Small conductivity of the capillary allowed the use of large electric fields even in the presence of salts, i.e. under the conditions where actin was fully polymerized. Large surface-to-volume ratio of a capillary assured rapid dissipation of heat liberated during electrophoresis. In the presence of 20 mM KCl and 2 mM $MgCl_2$ at pH 8.8 F-actin migrated in the electric field of 35 V/cm in 1% agarose as a single band at a rate of 2.0 cm/hr. The width of the band reflected the distribution of the molecular sizes on the polymer. F-actin was specifically labeled with 5-iodoacetamidofluorescein. Linear dichroism of the absorption dipole of the dye, which is a measure of the orientation of F-actin, was monitored during the electrophoresis. Long F-actin polymers oriented best; short polymers did not orient at all. The extent of orientation increased linearly with the strength of the electric field. These results showed that native F-actin could be fractionated by electrophoresis and that it moved through the gel matrix by a process of "reptation." (Supported by NIH and AHA.)

Tu-Pos385

NONCOVALENT ASSOCIATIONS BETWEEN GLOBULAR PROTEINS AND F-ACTIN STUDIED BY TRACER SEDIMENTATION EQUILIBRIUM MEASUREMENTS. Susan Lakatos and Allen P. Minton, NIDDK-NIH, Bethesda, MD 20892.

Each of three globular proteins bearing chromophoric groups absorbing in the visible region (human cyanmethemoglobin, FITC-labeled rabbit muscle aldolase and FITC-labeled bovine serum albumin) were centrifuged to sedimentation equilibrium in solutions containing varying initial concentrations of F-actin, at pH 8.0, I = 0.15, 5°C. Analysis of the equilibrium gradients so obtained indicates that aldolase and BSA bind significantly to actin under the conditions of these experiments. The weight-average molecular weight of globular protein-actin complexes is significantly greater than that of the globular protein in the absence of actin and significantly less than that of actin in the absence of globular protein, suggesting that both aldolase and BSA partially depolymerize F-actin under these conditions. In contrast, mixtures of cyanmethemoglobin and F-actin appear to contain only free hemoglobin and intact F-actin. The results obtained for actin and aldolase confirm and strengthen conclusions derived from our previously reported studies of these mixtures via the techniques of sedimentation velocity, size exclusion chromatography and electron microscopy.

Tu-Pos384

ACCESSIBILITY OF THIOLS IN ACTIN—A KINETIC STUDY WITH FLUORESCENT MALEIMIDE PROBES. T.-I Lin*, M. Kim*, and R.M. Dowben*+. *Baylor Research Foundation, Baylor Univ. Med. Ctr., Dallas, TX 75246. +Dept. Neurology, Univ. of Texas Southwestern Medical School, Dallas, TX 75253.

Fluorescent probes, 7-dimethylamino-4-methyl-3-*N*-maleimido-coumarin (DACM), 7-dimethylamino-3-(4'-*N*-maleimidylphenyl)-4-methyl-coumarin (CPM),4-dimethylamino-4'-*N*-maleimido-stilbene (DMAMS), 2-(4'-*N*-maleimidylanilino) naphthalene-6-sodium-sulfonate (MIANS), and 3-*N*-maleimido-pyrene were used to investigate the accessibility of sulfhydryls in actin under various conditions. The reaction kinetic curves for the various maleimido probes were obtained by monitoring the very large fluorescence increases over time following mixing. The kinetic data clearly show that all five probes are capable of reacting with more than one thiol in both G- and F-actin under non-denaturing conditions. Each probe exhibits its own characteristic reaction kinetic curve and is very different from other probes; also, G-actin behaves very differently from F-actin. After the labeling reactions were completed, DACM-labeled actin (labeled in G- and F-forms, or denaturing conditions) were cleaved with CNBr to 17 fragments. The fragments were separated on 5% polyacrylamide gel using isoelectric focusing (IEF) technique. Five fluorescent bands were identified for the labeling of actin denatured in 8M urea with pI of 4.6, 4.7, 5.0, 5.7, and 6.9, respectively. The fluorescent cysteine-containing fragments were assigned according to Sutoh (*Biochemistry* 21, 3654 (1982)). The IEF results clearly suggest that in G-actin, both cys-374 and cys-285 are readily reactive to DACM, while in F-actin, cys-374 is most reactive. However, other thiols also get lightly labeled depending on the probe, reaction time, and condition. These results are consistent with studies using HPLC to separate the CNBr fragments on a C_{18} reversed phase column (20 mM phosphate, pH 7.5 with 0–100% CH_3CN gradient) in which more than two fluorescent fragments were detected. This work is supported in part by U.S. Contract SDIO-84-88-C-0031.

Tu-Pos386

ACTIN FILAMENT REANNEALING AFTER DISRUPTION BY SONICATION. H.J.Kinosian, L.A. Selden, J.E.Estes, L.C.Gershman. Depts. of Physiology and Medicine, Albany Medical College, and Research and Medical Services, VA Medical Center, Albany, NY 12208.

Phalloidin-stabilized actin polymers at physiologic ionic strength reanneal after disruption by sonication with an apparent second order rate constant of reannealing of $10^6\ M^{-1}\ sec^{-1}$ and an apparent rate constant for fragmentation of $10^{-7}\ sec^{-1}$. The rate of reannealing was approximately 10-fold faster at physiologic ionic strength (0.1) than at low ionic strength (0.01). Phalloidin-stabilized actin polymer was shown to incorporate very little actin monomer during recovery from sonication under conditions where the number of polymer ends is decreasing at a substantial rate. This suggests that the major mechanism governing the length redistribution of phalloidin stabilized actin polymers is annealing rather than monomer cycling. In other work, we found that during nucleated polymerizations of ADP-actin, the number of polymer ends decreases and that annealing of filaments can account for the deviation from the predicted single exponential time course. Supported by the Veterans Administration and NIH Grant 32007.

Tu-Pos387

MEASUREMENT OF NUCLEOTIDE EXCHANGE WITH FLUORESCENT LABELED MONOMERIC ACTIN J.E. Estes, L.A. Selden, H. J. Kinosian, L. C. Gershman, Research and Medical Services, Veterans Administration Medical Center, and Depts of Physiology and Medicine, Albany Medical Center, Albany, N.Y. 12208.

We have determined the effect of the actin bound nucleotide on the fluorescence characteristics of monomeric actin labeled with 4-chloro-7-nitrobenz-2-oxa-1,3-diazole (NBD) or n-(1-pyrene) iodoacetamide (pyrene). Mg-NBD-actin exhibits a 14% increase in fluorescence intensity when ATP replaces ADP. This higher fluorescence intensity appears to require both Mg++ at the high affinity cation binding site and ATP at the nucleotide binding site. The fluorescence intensity of monomeric Mg-pyrene-actin exhibits a 50% increase in intensity on replacement of ATP with ADP and a smaller (10%) increase when Ca++ replaces Mg++ at the high affinity cation binding site. The fluorescence changes for NBD and pyrene-actin parallel nucleotide exchange kinetics measured directly on native actin, suggesting that these changes in fluorescence reflect nucleotide exchange. Use of these labeled actins allows measurement of nucleotide exchange kinetics at very low actin concentrations, making it possible to monitor nucleotide exchange on monomeric actin at physiological salt concentrations. Supported by the Veterans Administration and NIH grant 32007.

Tu-Pos388

Mg++ BOUND AT THE HIGH AFFINITY SITE ON ACTIN IS A COFACTOR FOR ACTIN ATPase ACTIVITY L.A. Selden, L.C. Gershman, H. J. Kinosian, J. E. Estes, Research and Medical Services, Veterans Administration Medical Center, and Depts of Physiology and Medicine, Albany Medical Center, Albany, N.Y. 12208.

It is well known that solutions of actin below the critical concentration hydrolyze ATP in the presence of Mg++. We have investigated the role of Mg++ in this hydrolysis, using Mg-EDTA buffers to adjust the free Mg++ concentration. Increases in the Mg++ concentration in the micromolar range have no effect on the ATP hydrolysis rates until the Mg++ concentration is high enough to result in polymerization of the actin. Mg-actin when complexed with DNase I to prevent polymerization exhibits an ATPase which is relatively unaffected by millimolar concentrations of Mg++. Experiments using cytochalasin D to accelerate ATP hydrolysis suggest that complete removal of divalent cation from actin inhibits the ATPase activity. These experiments indicate that it is not Mg++ bound to the lower affinity sites on actin or independently to the substrate ATP, but rather the Mg++ bound to the high affinity site on actin which is a cofactor in actin ATPase. Supported by the Veterans Administration and NIH grant 32007.

Tu-Pos389

PROBE DIFFUSION IN F-ACTIN SOLUTIONS. **Jay Newman, Kenneth L. Schick, Richard Arnold, Peter Lambert and Michael Migliozzi, Physics Department, Union College, Schenectady, N.Y. 12308.**

We have extended previous dynamic light scattering studies on the diffusion of uniform polystyrene latex spheres (PLS) in solutions of polymerized actin (Biopolymers 28:655-666, and 28:1969-1980, 1989) to higher, more physiological, concentrations of actin and to lower scattering angles. At each concentration of Spudich-Watt Ca-actin (0.4 mg/ml to 5 mg/ml) the diffusion coefficient of added PLS (either 0.06 or 0.1 μm diameter) and the amplitude of the correlogram were determined as functions of the magnitude of the inverse scattering vector probing distance (40 to 420 nm). From these results both the fraction of mobile PLS (from 100% at low actin concentration and short probing distances to less than 0.1% at the highest concentration and longer probing distances) and the local microviscosity within the actin network (as high as 40 times that of water) were determined. Mobile fractions decrease uniformly with both increasing actin concentration and probing distances. Estimates are made of the mean pore size within the actin network of from 150 nm at 0.4 mg/ml actin to less than 10 nm at an actin concentration of 5 mg/ml . We thank J.E. Estes and L.A. Selden for purified actin. Supported by NSF grants DMB-8607031 and DMB-8905906.

Tu-Pos390

MODULATION OF THE LOCALIZATION OF HEXOKINASE WITH MITOCHONDRIA IN VIVO. R.M. LYNCH AND F.S. FAY, Dept. Physiology, Univ. Mass. Med. Ctr, Worcester, MA 01655.

Hexokinase (HK) is co-localized with mitochondria in a variety of cells, and this specific localization is proposed to be influenced by the cells metabolic state. To test this hypothesis we have made a fluorophore labeled HK which maintains its native enzymic and binding activities. Immediately after microinjection, the fluorescent HK is distributed uniformly throughout A7R5 smooth muscle cells, as observed by digital imaging microscopy. However, within minutes HK localized into structures identified as mitochondria by colabeling with rhodamine 123. Incubation of HK with an antibody to a domain needed for binding to mitochondria blocks HK localization. In an attempt to alter HK distribution, glucose metabolism was inhibited with 2-deoxyglucose. Within 5 min, a significant amount of HK moved off of mitochondria. Our findings demonstrate that HK is associated with mitochondria in A7R5 cells, and inhibiton of metabolism alters this localization. Current studies are designed to determine if modulation of HK distribution can occur under other conditions. Funded by NIDDK DK32520 and a Charles A. King Fellowship (RML).

Tu-Pos391

EFFECT OF COMBINED CYTOCHALASINS ON ACTIN SELF-ASSEMBLY. Fei Wang, J.L. Arauz-Lara, and Bennie R. Ware, Department of Chemistry and Graduate Biophysics Program, Syracuse University, Syracuse, NY 13244

The effect of combined cytochalasins on the self-assembly of column-purified actin was studied using fluorescence photobleaching recovery and fluorescence enhancement techniques. The objective of our experiments is to investigate the separability of cytochalasin activities, including cleavage of actin filaments and "capping" the filaments by binding to the barbed end. Actin critical concentrations and filament diffusion coefficients in the presence of various combinations of cytochalasins (CB, CD and CE) were determined at different actin to cytochalasin ratios. We found that CB does not affect the critical concentration of actin either in the presence or absence of CD or CE. Our measurements also reveal an intermediate effect in diffusion coefficients when cytochalasins are combined.

Tu-Pos392

C-TERMINAL MODIFICATION OF ACTIN AFFECTS FILAMENT INTERACTION. S. I. O'Donoghue, M. Miki and C. dos Remedios, Anatomy Dept, Sydney Univ., NSW 2006, Australia. Using proteolysis we removed residues 374-375 of actin leaving 1-373 (A_{-2}). Under the electron microscope, A_{-2} filaments form into bundles contacting every monomer. Thus removing 374-375 produces an extra actin-actin contact site. Previous investigators removed 374-375 from actin-DNase I (Suck et al., 1981 Proc. Natl. Acad. Sci. USA 78, 4319); crystals formed from this complex had an extra actin-actin contact distinct from those in F-actin. It is possible that the extra site in the crystals is the same as the site which causes bundling of F-A_{-2}. F-A_{-2} polymerizes to the same extent as unmodified actin, but its viscosity is reduced to half that of unmodified actin. This is consistent with the observed bundle formation. By further digestion of A_{-2} we removed residues 372-373 leaving 1-371. This caused the viscosity to return to that of unmodified actin suggesting that Arg-372 & Lys-373 are involved in the extra contact site. We also found that Cu^{2+} binding (known to occur at actin C-terminus) promotes gel formation. We conclude that the C-terminus of actin seems to be important in modulating the assembly of actin filaments into higher order structures. Support: NH&MRC, Australia.

Tu-Pos393

PHALLOIDIN (PH) AND CYTOCHALASIN D (CYD) RELAX SKINNED MESENTERIC ARTERIOLES (MA). Piet J Boels and Gabriele Pfitzer (Intro. by N.M. De Clerck)
Physiology II, University of Heidelberg,FRG.
Isolated guinea-pig MA (internal in situ diameter, 50-100 µm) were treated with 1% Triton for 4 hrs. Electron microscopy showed absence of membranes and presence of microfilaments. MA were mounted to a transducer in relaxing solution (free Ca^{2+} 1nM) to measure circumferential force. Ca^{2+} (1-40µM) and Calmodulin (0.1-0.3µM) induced contractions were potentiated by the phosphatase inhibitor Okadaic acid (1µM). 1-100µM PH, after a transient increase in force, caused slow (1 hr) irreversible relaxations (70 - 90%) of half-maximal contractions (1.5-2µM Ca^{2+}) and of subsequent contractions at maximal Ca^{2+} (40µM). PH-application under relaxed conditions did not inhibit subsequent maximal force. PH inhibited neither MA-MLCK nor force in skinned psoas fibres. 20 µM CyD relaxed half-maximal force quickly (10') and irreversibly. Thus, in addition to phosphorylation dependent regulation of contraction, tension maintenance in skinned MA may depend on a dynamic equilibrium between G- and F-actin, although a direct interference of PH and CyD with active force generating actomyosin interactions cannot be excluded.

Tu-Pos394

INVESTIGATION OF ACTIN BINDING SITES USING PROTEIN ENGINEERING.
D.R.Drummond, E.S.Hennessey, J.C.Sparrow & D.C.S.White, Department of Biology, University of York, YORK, U.K.

We have investigated the binding sites of actin by in vitro mutagenesis of the flight muscle specific Actin88F gene of Drosophila melanogaster. Effects of C-terminal aminoacid changes have been studied by in vitro expression of the mutant genes or by P-element transfection of Drosophila embryos. In no case was the ability of the mutant actins to bind DNase-I and profilin, or to co-polymerise with rabbit actin totally abolished. The mutants with the greatest decrease in DNase-I affinity have the greatest increase in profilin binding. In addition, these same mutants have the greatest decrease in ability to co-polymerise with rabbit actin in vitro but still disrupt myogenesis and induce 'heat shock' in the fly. As these binding sites occur at different locations in actin, these results suggest long-range effects of the aminoacid changes.

Supported by SERC.

Tu-Pos395

PHOTOLABELING OF GIZZARD MYOSIN WITH 3'(2')-O-(4-BENZOYL)BENZOYL ATP (Bz_2ATP): $PROLINE_{324}$ IS AT THE ACTIVE SITE.

Douglas G. Cole and Ralph G. Yount. Biochemistry/Biophysics Program, Washingtom State University, Pullman WA 99164-4660.

[^{14}C]Bz_2ADP was stably trapped at the active site of gizzard myosin in nearly stoichiometric amounts by complexation with vanadate and Co^{2+}. Irradiation of this complex results in covalent attachment of [^{14}C]Bz_2ADP to the 50-kDa tryptic fragment of subfragment one (S1) (Cole and Yount, *Biophys. J.* **53**, 464a, 1988). S1 isolated from photolabeled myosin was exhaustively digested with trypsin and then subtilisin. Two radioactive peptides were purified by HPLC and sequenced by an automated gas phase sequencer:

Peptide IIA: S-N-G-H-V-P-I-X_{324}-A-Q-Q-D_{328}

Peptide IIB: G-H-V-P-I-X_{324}-A-Q-Q-D_{328}

A new amino acid was detected at the position of Pro_{324} which contained all the radioactivity, indicating this amino acid was covalently labeled by the [^{14}C]Bz_2ADP. Experiments are in progress to determine if [^{14}C]Bz_2ADP labels the same or different amino acids when gizzard myosin is photolabeled while in the 10S folded conformation. Supported by NIH (DK 05195).

Tu-Pos396

DIRECT PHOTOAFFINITY LABELING OF GIZZARD MYOSIN WITH UDP

Todd E. Garabedian and Ralph G. Yount
Biochemistry/Biophysics Program, Washington State University, Pullman, WA 99164-4660

The active site of chicken gizzard myosin is being investigated by direct photoaffinity labeling with [^{3}H]UDP. UDP can be stably trapped at the active site by addition of vanadate (V_i) and Co^{2+} ($t_{1/2}$ = >4 days). The presence of Co^{2+} prevents the photoreaction of V_i with active site residues. The stability of the myosin-UDP-V_i complex allows it to be purified free of extraneous label before photolysis is initiated. Upon UV irradiation, 50% of the trapped UDP photoincorporates into the active site. Only the 200K heavy chain was labeled; there was no apparent labeling of either LC_{20} or LC_{17}, confirming earlier results of Maruta and Korn [*J. Biol. Chem.* **256**, 499-502 (1981)] using untrapped UDP. Limited trypsin digestion of labeled S1 indicates that the NH2-terminal 29K peptide contains >90% of the photolabel. Since UDP is a zero-length crosslinker, it presumably identifies amino acids at the active site of myosin. The location and characterization of the specifically labeled amino acids are in progress. Supported by NIH (DK 05195)

Tu-Pos397

CHARACTERIZATION OF A MONOCLONAL ANTIBODY (IgM) THAT REACTS WITH HEAD AND TAIL PORTIONS OF MYOSIN.

K. Mabuchi and J. Gergely, Dept. of Muscle Res., Boston Biomedical Research Institute; Dept. of Neurology, Mass. General Hosp.; Dept. of Biol. Chem. and Mol. Pharmacol., Boston MA; 02114.

Among a battery of monoclonal antibodies (McAbs) raised against rabbit skeletal myosin, one was found to react with the N-terminal, 25K, tryptic fragment of subfragment 1 (S1) and with light meromyosin (LMM). Rotary-shadowed images of McAb-myosin complexes show two molecules of this McAb bound to the same myosin molecule. The affinity to LMM seems to be higher than to S1 and if the reaction is carried out in the presence of powdered milk (10%), the McAb reacts only with LMM. Binding to LMM is preferred for one isoform of myosin (type 2A) while binding to S1 shows much less selectivity among isoforms. The pentameric structure of IgM and the well-known heterogeneity with respect to the light chains may explain the dual reaction of our McAb. On the other hand the possibility exists that some sequence/-structure in the N-terminal 25K fragment is similar to one in LMM of type 2A myosin. The latter view is supported by the lack of changes in the ratio of doubly reacting McAbs after the pretreatment with S1. (Supported by grants from NIH (R37-HL-5949) and MDA).

Tu-Pos398

MYOSIN INTERACTIONS WITH DETERGENTS

by S. Highsmith (Intro. by Joel Cohen, U. Pacific, San Francisco)

The concentration dependences of the activation of myosin subfragment-1 (S1) Mg-ATPase by the detergents CHAPS (a cholate derivative) and $C_{12}E_8$ (a dodecanol derivative) were determined at 23°C. At [detergent]'s expected to bind hydrophobic S1 surface areas equally, $C_{12}E_8$ caused an 8.5-fold greater increase in activity than CHAPS. A series of n-alcohols (which unlike $C_{12}E_8$ and CHAPS do not form micelles) with from three to ten carbons all increased S1 Mg-ATPase activity as much or more than $C_{12}E_8$ or its analog $C_{12}E_9$ (Highsmith 1989 Biochem 28, 6745). The largest increase (5.7-fold) was caused by n-hexanol. The more hydrophobic alcohols activated S1 at lower alcohol concentrations. The apparent free energy of binding per CH_2-group of the alcohols was -0.60±0.03 kcal/mole. The intrinsic fluorescence intensity increase of S1 during steady-state activity was reduced from 17.5 to 12.8 percent by n-hexanol. The data suggest activation by $C_{12}E_8$ or by n-alcohols is due to binding to S1 at non-surface sites, which are not accessible to CHAPS. (NIH AR37499)

Tu-Pos399

VANADATE MEDIATED PHOTOCLEAVAGE OF MYOSIN S1 AT THE V2 SITE. Christine R. Cremo & Jeff Wilcott, Chem Dept, Colorado College, Colorado Springs, CO 80903. S1 can be specifically photocleaved at two sites (V1 and V2) by irradiation with UV light in the presence of mM concentrations of vanadate (G. Mocz (1989) Eur. J. Biochem. **179**, 373; Cremo et al.(1989) Biophysical J. **55**, 81a). It has been shown by ^{51}V-NMR that tetravanadate binds S1 at these sites, whereas mono and divanadate do not (Cremo et al., 1989), suggesting that tetravanadate is responsible for cleavage. In addition, lowering the pH during irradiation, increases V2 cleavage, which is expected as the tetramer is in higher concentrations at lower pH's. If the S1-CoADP-V_i complex is irradiated with excess vanadate, only the V2 site (just NH_2-terminal to the 50-20 kDa tryptic site) is cleaved. With this specificity, the effects of cleavage upon the actin binding and ATPase activities of S1 have been examined. Cleavage strongly disrupts the interaction of S1 with actin. As the V2 site is within the region on S1 that has been implicated as a polyphosphate recognition site that controls actin-20 kDa interactions, the effects of various polyphosphates on the efficiency of cleavage has been examined. These results indicate that tetravanadate may be interacting at the same polyphosphate site, which may explain its photocleaving specificity. Various buffers also inhibit cleavage by a different mechanism from the one described above for polyphosphates.

Tu-Pos400

THE INTERACTION BETWEEN MYOSIN S-1 AND G-ACTIN AND ACTIN POLYMERIZATION, T. Chen and E. Reisler, Dept. Chem. & Biochem. and the Mol. Biol. Inst., Univ. of Calif., Los Angeles, CA 90024.

The polymerization of G-actin by S-1 (A1), S-1(A2), and various forms of tryptically cleaved S-1 was monitored by light scattering and pyrene fluorescence measurements. It was found that S-1(A1) polymerized G-actin at least six-fold faster than S-1(A2) and that this difference depended on the intact form of the N-terminal segment of A1. The initial binding of G-actin and S-1 could be detected through rapid increases in pyrene fluorescence which were approximately equal for the two isozymes. The binding of S-1 and the polymerization of G-actin were greatly reduced by a tryptic cut at the 75/20 kDa junction on S-1. It is concluded that the contributions of S-1 sites to acto-S-1 interaction can be probed in actin polymerization experiments.

Tu-Pos401

DISTRIBUTION OF DEVELOPMENTAL MYOSIN ISOFORMS IN ISOLATED A-SEGMENTS. D.A. Gordon and S. Lowey. Rosenstiel Research Center, Brandeis University, Waltham, MA 02254.

During transitions in myosin isoform expression, neonatal myosin has been reported to be preferentially located in the bare zones of heterogeneous filaments composed of neonatal and embryonic or adult myosins (Taylor & Bandman, JCB 108:533, 1989). This result suggests that neonatal myosins prefer antiparallel interactions and act to nucleate thick filament formation. To test this hypothesis at the sarcomere level, we have developed a method to isolate single A-segments (arrays of myosin filaments) from various aged posthatch chicken pectoralis muscles. A-segments were first labeled with monoclonal antibodies specific for developmental myosins, and then with gold-conjugated secondary antibodies prior to negative staining. At days 7 and 12, a high degree of labeling with neonatal-specific antibody was obtained while there was no labeling with adult-specific antibodies. Conversely, A-segments from >60-day tissue, reacted only with adult-specific antibody. Experiments to determine the spatial distribution of isoforms at ages where two myosins are coexpressed are underway. Supported by grants from NIH, MDA and NSF.

Tu-Pos402

THE FLEXIBILITY OF STRIATED MUSCLE MYSOSIN James F. Curry, Sonja Krause, Dept. of Chemistry, Rensselaer Polytechnic Institute, Troy, NY 12180-3590

Rabbit striated muscle myosin was extracted from the same rabbit muscles by two methods and purified by either Sephadex or hydroxylapatite chromatography. Transient electric birefringence studies in pyrophosphate solutions indicate that there are only minor differences in the rotational diffusion of myosin isolated by the different methods. The importance of this study is that, independent of preparation methods, myosin shows a variable degree of flexibility in solution. The field free birefringence decays have been analyzed using a Fortran program, DISCRETE [S. W. Provencher, Biophys. J. 1976, 16,27.]. This curve fitting analysis has shown that our birefringence decay curves can be satisfactorily fitted by two relaxation times of 9-15μs and 38-50μs. Other workers have reported either the shorter or longer of these relaxation times. The shorter relaxation times may be interpreted as a bent myosin molecule, while the longer relaxation times are due to an extended molecule and/or aggregates.

Tu-Pos403

PROPERTIES OF CYSTEINE MUTANTS OF CHICKEN MYOSIN LIGHT CHAINS. L.D. Saraswat and S. Lowey. Rosenstiel Research Center, Brandeis University, Waltham, MA 02254.

Site-directed mutagenesis has been used to insert cysteine residues at strategic positions in the regulatory light chain (LC2) sequence, with the aim of studying conformational changes in this myosin subunit. Native LC2 has two cysteines located at positions 125 and 154; a third sulfhydryl was added by replacing either Pro2, Ser73 or Pro94 with cysteine. By oxidizing the endogenous cysteines to an intramolecular disulfide bond (Katoh and Lowey, JCB 109:1549, 1989), the new cysteine could be labeled with 5-(iodoacetamido) fluorescein, and a singly labeled species was isolated by FPLC. Limited proteolytic digestion of the Pro2→Cys mutant showed that labeling was at a single site. By exchanging the fluorescein-labeled light chains into myosin, we can trace the location of the chain in the myosin head by immunoelectron microscopy, using anti-fluorescyl antibodies as probes. Furthermore, by reacting Cys125 with a donor fluorophore, we will be able to measure the distance between pairs of thiols in the free and bound light chain. Supported by grants from NIH, NSF and MDA.

Tu-Pos404

MODIFICATION OF THIOL GROUPS OF GIZZARD MYOSIN HEAVY CHAINS WITH 7-CHLORO-4-NITROBENZO-2-OXA-1,3-DIAZOLE. Gary Bailin

Chicken gizzard myosin modified with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole, NBD-Cl, resulted in a 65% inhibition of the K^+-ATPase activity when 3.5 mol of the reagent were bound per 4.7×10^5 g protein. The labeling was located in the heavy chain region and none of the light chains was lost. Treatment of NBD-myosin with dithiothreitol restored the K^+-ATPase activity and one mol of the NBD group was removed from thiols of tryptic fragments of Mr 17,000 mainly and some Mr 27,000 whose origins were in the heavy chain region. Cysteine residues were modified in NBD-myosin at sites other than the active site as MgATP did not protect against the inactivation or labeling. NBD-Cl reacted differentially with thiols of gizzard myosin at 0.6 M KCl (6S elongated state) compared to those at 0.15 M KCl (10S folded state). There was more labeling of the chymotryptic heavy meromyosin and the Mr 17,000 tryptic fragment when the myosin or phosphorylated myosin was in the 6S form. Reactive thiol groups of the heavy chains are involved in maintaining the structure of gizzard myosin. Supported by NIH AR38829 and AHA (N.J. Affiliate).

Tu-Pos405

TRAPPING SPIN-LABELED ADP ON MYOSIN HEADS USING VANADATE. G. Wilson, J. Paige & R. Cooke. Dept. Biochem./Biophys., UC San Francisco, Ca., 94143-0524.

The reason why myosin has two heads and whether they interact during contraction is unclear. Vanadate (Vi) was used to trap stable myosin-ADP-Vi complexes, which produce no force, in rabbit slow-twitch skeletal muscle fibers. Mechanical properties of fibers activated in the presence of Vi were determined. Although force declined as [Vi] increased, fiber velocity remained high, showing that trapped heads did not interact with actin. The proportion of trapped myosin heads was determined from EPR spectra of spin-labeled (SL) analogs of ADP. These complexes stayed trapped in relaxing solution for at least 15 minutes. In Ca^{2+}-activating solution the trapped SL-ADP was released with a half life of approximately 8 minutes. The amount trapped increased with increasing [Vi]. Preliminary results suggest that the [Vi] (1mM) for achieving half maximal trapping of SL-ADP is approximately the same as that for inhibiting force production in the presence of SL-ATP by half. This would support the hypothesis that the heads interact independently in force generation. Supported by USPHS AM30868.

Tu-Pos406

IDENTIFICATION OF A NEW TYPE OF MYOSIN HEAVY CHAIN IN ACANTHAMOEBA. J.A. Horowitz and J.A. Hammer III, LCB, NHLBI, NIH, Bethesda, MD 20892. The complete amino acid sequence of myosin III (MIII) has been deduced from its gene sequence. The N-terminal ~90 kDa of the ~177 kDa MIII HC is highly similar to the globular head sequences of myosin I (MI) and myosin II (MII). In contrast, the C-terminal ~87 kDa of the MIII HC shows no significant similarity to the tail sequences of either MI or MII. The only exception to this is the C-terminal ~50 amino acid region of MIII, which is homologous to the C-termini of the protozoan MIs. The tail of MIII appears to be incapable of forming a coiled-coil rod structure characteristic of MII. Consequently, MIII (like the MIs) is probably monomeric and nonfilamentous. Antibodies generated against a bacterially expressed fusion peptide containing most of the MIII tail domain recognize a single ~177 kDa polypeptide in whole cell extracts (but not the ~171 kDa MII HC). MIII is precipitated from crude extracts using F-actin and is quantitatively released from the pellet by ATP. Fractions enriched in MIII (and devoid of MI and MII) have significant Ca^{2+} and K^+EDTA ATPase activities. These results support the conclusion that this protein is a new member of the myosin family.

Tu-Pos407

MODIFICATION OF SKELETAL AND SMOOTH MUSCLE MYOSIN ATPASE BY BIFUNCTIONAL PHOTOREACTIVE ATP ANALOGUE

Shinsaku Maruta, Morris Burke*, and Mitsuo Ikebe Department of physiology & biophysics, *Department of biology, Case Western Reserve University, Cleveland, OH 44106

A bifunctional photoreactive ATP analogue, azidonitrobenzoic-8-azido-ATP (ANB-8-N_3-ATP) was synthesized to study the active site conformation of skeletal and smooth muscle myosin ATPase. This analogue carries photoreactive azido groups on 8-position of adenine ring and 3'-position of ribose. When skeletal myosin subfragment-1 (S-1) was photomodified with this analogue, a new extra 120kDa band was observed just above the intact S-1 heavy chain on SDS-PAGE. The formation of this band was reduced significantly when the photoreaction was carried out in the presence of excess ATP. The tryptic digestion of the photomodified S-1 by ANB-8-N_3-ATP produced 45kDa peptide in addition to the 25,50 and 20kDa tryptic fragments of S-1 heavy chain. The photomodification was also carries out using fluorescence labelled S-1 in which SH_1 was specificaly labelled and was found that the 45kDa peptide contained the labelled SH_1. It was also found that the 45kDa peptide was reacted with the antibody which reconizes N-terminus 1-23 amino acid residues of skeletal myosin heavy chain. Therefore, it was concluded that the 45kDa peptide was the cross-linked product of 20kDa and 25kDa tryptic fragments. In the case of smooth muscle myosin, similar cross-linked product was not obseved under the same experimental conditions. These results indicate that the 25kDa and 20kDa fragments of skeletal myosin are near the ATP binding site and the ATPase site conformation of smooth myosin is different from skeletal myosin. (supported by NIH,syntex and AHA)

Tu-Pos408

CHANGE IN THE FLEXIBILITY OF THE HEAD-ROD JUNCTION OF SMOOTH MUSCLE MYOSIN BY PHOSPHORYLATION.

M. Ikebe and R. Takashi, Dept. of Physiol. and Biophys., Case Western Reserve Univ., Cleveland, Ohio 44106 and Baylor Res. Fdn., Baylor Univ. Med. Ctr., Houston, Texas 77030

Smooth muscle myosin forms two distinct conformations, referred to as 6S and 10S myosin. We have proposed that a conformational change at the head-rod junction is important for dictating the ATPase activity and the conformation (10S-6S) of myosin. In this study, the change in the conformation at head-rod junction was examined by measuring fluorescence anisotropy of myosin molecule. Phosphorylated and dephosphorylated myosins were mixed with -ADP in the presence of Vanadate (V_i) and the formed myosin . -ADP.V_i complex was separated from free nucleotides. The rotational relaxation times of the complex were estimated from the polarization fluorescence measurement. The relaxation time of dephosphorylated myosin at 0.18 M KCl (10S myosin) was markedly higher than that of S-1 while the relaxation times of phosphorylated myosin at 0.18 M KCl and dephosphorylated myosin at 0.4 M KCl (6S myosin) were comparable to that of S-1. These results suggest that the flexibility of head-rod junction is significantly altered by phosphorylation. (Supported by NIH, Syntex, and AHA).

Tu-Pos409

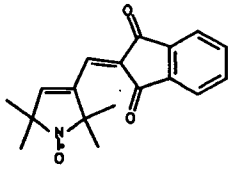
PROXIMITY RELATIONSHIPS BETWEEN SITES ON MYOSIN REGULATORY LIGHT CHAIN, HEAVY CHAIN AND ACTIN. H.-S. Park, T. Tao & P. D. Chantler (Intro. by W.F. Stafford III). Dept. of Muscle Research, Boston Biomed. Res. Inst., Boston, MA 02114, & Dept. of Anatomy, Med. College of Penn., Philadelphia, PA 19129.

In order to understand molecular aspects of myosin-linked regulation we have used resonance energy transfer to explore proximity relationships between specific sites on the myosin regulatory light chain, myosin heavy chain, and actin. Using 1,5-IAEDANS and DABMI as the donor-acceptor pair, and taking the critical transfer distance and the orientation factor to be 40Å and 2/3, respectively, we obtained a distance of ~60Å between Cys-50 of the *Mercenaria* regulatory light chain (mRLC) and the reactive sulfhydryl (SH1) in the heavy chain of scallop myosin. This distance was invariant with calcium-, MgATP- and actin-binding, and switching of the donor-acceptor locations. Using the same donor-acceptor pair the distance between Cys-50 of mRLC and Cys-374 of actin was found to >80Å, while that between SH1 and Cys-374 of actin was measured to be ~68Å. These distances are consistent with the localization of Cys-50 of mRLC near the head-neck junction of myosin, SH1 in the middle portion of the myosin head and the actin-binding site at the tip of the myosin head.

Tu-Pos410

A NOVEL BIFUNCTIONAL NITROXIDE SPIN LABEL FOR PROBING MYOSIN ROTATIONAL DYNAMICS . Osha Roopnarine, Kalman Hideg and David D. Thomas. Dept. of Biochemistry, Univ. of Minnesota Medical School, Minneapolis, MN 55455.

We have prepared a new spin-labeled myosin derivative using a bifunctional probe, kindly provided by Professor Kalman Hideg (Univ. of Pecs, Hungary). The spin label is synthesized from the condensation of a five-membered nitroxide aldehyde and a methylene group to yield a highly reactive diene that reacts specifically with SH groups. Further, the spin label contains an aromatic group that is designed to anchor the probe in a hydrophobic pocket. This increases the probability that the label will be strongly immobilized, thus making it appropiate for ST-EPR measurements of global protein motion. K-EDTA ATPase assays indicate that this probe reacts selectively with SH1 on the myosin head. Conventional EPR spectra of spin labeled myosin monomers and filaments indicates that the spin label was strongly immobilized on the myosin head. ST-EPR experiments clearly differentiated the slower and faster motions of the spin labeled heads in myosin filaments and monomers, respectively. Actin binding to the spin labeled myosin filaments greatly decreased the motion of the heads as indicated by ST-EPR. Finally, the effect of nucleotides (Mg.ATP and Mg.ADP.VO_4) and ligands (K_4PP_i and $MgCl_2$) was negligible (less than 0.5 Gauss change) indicating that this new derivative is ideal for measuring the overall motion of myosin heads.



Tu-Pos411

IDENTIFICATION OF THE SITE PHOSPHORYLATED BY PROTEIN KINASE C IN THE HEAVY CHAIN OF VERTEBRATE CELLULAR MYOSINS

Mary Anne Conti, Marshall Elzinga*, Peter Mc Phie, James R. Sellers and Robert S. Adelstein, *Inst. Basic Research, Staten Island, NY and NHLBI, NIH, Bethesda, MD 20892

The vertebrate cellular myosin heavy chain (MHC) is a substrate for protein kinase C both in situ and in vitro. In vitro protein kinase C phosphorylates the MHC of human platelet and chicken intestinal epithelial (CIE) cell myosin to a stoichiometry of 0.2-0.4 mol PO_4/mol MHC. In contrast, a higher stoichiometry has been achieved in situ: 0.7 mol PO_4/mol MHC in human platelets (Kawamoto et al., J. Biol. Chem. 264: 2258, 1989) and 1 mol PO_4/mol MHC in RBL-2H3 cells (Ludowyke et al., J. Biol. Chem. 264: 12492, 1989). Of note is that 2-dimensional peptide mapping indicates that the same phosphorylated MHC peptide is released by tryptic hydrolysis of human platelet, RBL-2H3 cell and CIE cell MHC. The sequence of the platelet MHC phosphopeptide, -E-V-S-S*-L-K-, indicates that the phosphorylated residue corresponds to serine-1915 in the CIE cell MHC (Shohet et al., PNAS 86: 7726, 1989). The serine is located in the alpha-helical rod portion of the MHC, 11 residues amino-terminal to the proline residue which initiates the uncoiled tail sequence. Previous work demonstrated that aggregation of the receptors for IgE on RBL-2H3 cells results in stoichiometric phosphorylation of the MHC and 20 kDa light chain as well as release of histamine (Ludowyke et al., op. cit.). The location of the MHC site is consistent with its putative role in unraveling the cortical myosin filaments and thus permitting histamine release.

Tu-Pos412

DEVELOPMENTAL CHANGES IN ACTIN AND MYOSIN HEAVY CHAIN ISOTYPE EXPRESSION IN SMOOTH MUSCLE.

T.J. Eddinger and R.A. Murphy, Department of Physiology, Health Sciences Center, University of Virginia, Charlottesville, VA 22908.

In newborn rats and swine the non-muscle (NM) actin and myosin heavy chain (MHC) isotypes present in smooth muscle can exceed 40% of the total. These values decreased to about 15-18% by the time the animals reached maturity. The time course of these changes was very similar for both the actin and MHC isotypes. There were also changes in the relative contents of the two smooth muscle MHC's (SM1 & SM2) with development. These changes appeared to be species specific. In young rat aorta the SM1:SM2 ratio was 0.5 while it was about 3 in young swine carotid. Both of these tissues showed a SM1:SM2 ratio of 1.2 in mature animals. Other smooth muscle tissues exhibited still different changes with development. Thus, there are distinct developmental changes in the actin and MHC isotypes present in smooth muscle that are species and tissue specific. The functional significance remains unclear. Supported by NIH grant 5-PO1-HL19242 and fellowship 1-F32-HL07528.

Tu-Pos413

Thermodynamic Studies Between Two States of Myosin Subfragment-1 in the Presence of ADP and Actin

Shau-Hua Lin and Herbert C. Cheung, Graduate Program in Biophysical Science and Department of Biochemistry, University of Alabama at Birmingham, Birmingham, AL 35294.

We previously reported that S1•εADP exists in two isomers based on temperature dependence of the fluorescence decay of 1,N^6-ethenoadenosine diphosphate (εADP) (Aguirre et al. 1989, Biochemistry 28, 799). We have confirmed the thermodynamic parameters for the equilibrium between the two isomers, $S1_H$•ADP ⇌ $S1_L$•ADP by using a fluorescently labeled myosin S1 (S1•AF). In S1 alkylated with IAF, the decay of the label was biexponential in the presence and absence of ADP or actin. In the presence of ADP, the two decay times were 4.43 ns (A_1 = 0.62) and 1.29 ns (A_2 = 0.38) at 14.5 °C and in 60 mM KCl, 30 mM TES, 2 mM $MgCl_2$, pH 7.5. The steady-state fluorescence intensities of S1•AF, (S1•AF)•ADP, and acto•(S1•AF) were dependent on temperature over the range 3 °C to 30 °C. By combining the lifetime and steady state data, we obtained for the two state transition $(S1•AF)_H$•ADP ⇌ $(S1•AF)_L$•ADP the following parameters: ΔH° = 14.4 kcal/mol and ΔS° = 55 cal/deg/mol, in agreement with previous results obtained with εADP.

Tu-Pos414

PHOSPHORYLATION OF THE HEAVY CHAIN OF ADRENAL MEDULLA MYOSIN.

Barbara Barylko and Joseph P.Albanesi (Introduced by Stylianos P.Scordilis). Dept.of Pharmacology, U.T. Southwestern Med. Center, Dallas, TX 75235. Heavy chain phosphorylation has been demonstrated in several muscle and non-muscle myosins. We have shown that bovine adrenal medulla myosin can be also phosphorylated on the heavy chain by casein kinase II. Limited proteolytic digestion of adrenal myosin with papain yields major phosphorylated fragments of 140 kDa and 120 kDa, the latter corresponding to the rod portion of the myosin. Further digestion with papain removed the phosphorylated peptide without causing a detectable change in the electrophoretic mobility of the rod. By analogy with other myosins phosphorylated on the heavy chain, it is likely that the adrenal myosin heavy chain phosphorylation site is located at the C-terminus of the molecule.

Tu-Pos415

THE EFFECT OF DIVALENT IONS ON THE THERMAL STABILITY OF THE 50 kDa DOMAIN OF MYOSIN HEAVY MEROMYOSIN. Renné C. Lu & Anna Wong, Dept. of Muscle Research, Boston Biomed. Res. Inst., Boston, MA 02114

Previous studies have shown that the 50 kDa region, but not the 25 & 20 kDa regions, becomes susceptible to tryptic cleavage after S1 has been thermally denatured and then renatured (Setton & Muhlrad, ABB 235, 411, 1984; Burke et al., Biochemistry 26, 1492, 1987), suggesting that the 50 kDa region behaves as an independent domain in terms of thermal denaturation. Using the photocrosslinking and resonance energy transfer techniques we have shown that thermal unfolding and degradation of the 50 kDa region do not disturb the part of S1 structure involving the 25 & 20 kDa regions and a crosslink between the 20 and the 50 kDa regions stablized the latter (Lu et al., Biophys. J. 53, 176a, 1988). Recently we found that the 50 kDa region in HMM remained resistant to trypsin after thermal treatment unless EDTA was present during incubation at 39°. The protection of the 50 kDa region by divalent ions was not found in S1, suggesting that divalent ions affect the stability of the 50 kDa domain via light chains or S2/neck region in HMM. Support by NIH AR28401.

Tu-Pos416

SCANNING TUNNELING MICROSCOPY (STM) AND IMAGE RECONSTRUCTIONS OF THE STRUCTURE OF MYOSIN ROD. A. Bertazzon & T. Y. Tsong. Dept of Biochem, Univ of Minnesota, St. Paul, MN 55108

The helical tail of myosin is composed of two helical strands in a double-coiled coil 2 nm in diameter and 140 nm in length, as revealed by the transmission electron microscopy. STM measured an average length of 142±4 nm and a width of 1.72±0.08 nm. The best resolution of the helix was obtained using fragments of myosin (Rod, LMM and S2). Aggregation patterns varied with salt concentrations and the conditions in which samples were prepared. Fragments aggregated to form thick helical fibers in Rod and short tactoids in LMM and S2. However, individual molecules showed a periodicity, with a pitch of 3.28 nm, within each period are two asymmetric masses, or domains, the peak distance between the two masses being 2.4 nm. A computer model has been constructed from the published amino acid sequence of LMM with constraints from the STM data.

Tu-Pos417

ANTIBODIES TO A SYNTHETIC PEPTIDE HAVING A SEQUENCE FOUND NEAR THE CARBOXYL TERMINUS OF BRAIN MYOSIN HEAVY CHAIN Noriko Murakami, Gregory Healy-Louie, and Marshall Elzinga, Lab. of Neurobiochemistry, Institute for Basic Research, Staten Island, NY 10314

Studies on the casein kinase II-phosphorylated peptide from bovine brain myosin heavy chain gave the sequence: L-E-L-S*-D-D-D-D-E-S-K-A-S-( )-I-N-E-T-Q-P-P-Q. The S* is phosphorylated, and the peptide is probably part of a non-helical "tail-piece". We synthesized a peptide corresponding to the carboxyl-terminal portion of the above sequence, and raised antibodies against it in rabbits. The antibodies reacted with beef brain myosin in ELISA tests, and recognized both the intact heavy chain and the COOH-terminal 6 kDa chymotryptic fragment, but not the head region of the molecule, in Western blots. The antibodies seemed to be specific for cellular myosins in that they reacted with beef brain myosin, and with myosin from two cell lines of neuronal origin, PC-12 cells (rat) and SY5Y cells (human). However the antibodies do not react with smooth muscle myosin from chicken gizzard or with skeletal muscle myosin from rabbit. Studies on the tissue specificity of the antibodies, as well as an assessment of the effects of antibody binding on filament formation and the stability of brain myosin filaments, are in progress.

Tu-Pos418

TRYPTOPHAN DYNAMICS IN SKELETAL MYOSIN ROD

Yoke-chen Chang & Richard D. Ludescher, Department of Food Science, Rutgers University, New Brunswick, NJ 08903-0231

Rabbit skeletal myosin rod, generated by chymotryptic digestion, contains two tryptophan residues per heavy chain (located in the LMM region at equivalent hydrophobic coiled-coil sites). We are investigating the fluorescence depolarization of these residues as a probe of the rotational dynamics of the alpha-helical coiled-coil. The residues are exposed to solvent in monomeric rod (based on the emission maximum and fluorescence quenching constants). At 20°C the steady-state anisotropy (r) is 0.13 in high salt (500 mM KCl, pH 7.0). Perrin plots of the influence of sucrose on r are biphasic and support a dynamic model of rapid but restricted tryptophan motion through a cone semi-angle of 35°. r increases to 0.24 in filaments (formed by dilution to 125 mM KCl) and increases further in 5mM $MgCl_2$. Mg^{++} binding to the low affinity sites in rod filaments may thus restrict myosin filament dynamics locally, within LMM, as well as globally, throughout the cross-bridge.

Tu-Pos419

DIFFERENTIAL EFFECTS OF INHALATIONAL ANESTHETICS ON CALCIUM SENSITIVITY

Zeljko J. Bosnjak, Anil Aggarwal, Lawrence A. Turner, Jure Marijic, and John P. Kampine. The Medical College of Wisconsin, Milwaukee, WI 53226

Alteration of calcium sensitivity at the myofilaments may be an important mechanism by which inhalational anesthetics exert their negative inotropic effects. To directly examine the effects of halothane (0.6 mM), enflurane (1.1 mM), and isoflurane (0.8 mM) on the relationship between intracellular [Ca++] and tension development in the papillary muscle of the guinea pig, we simultaneously measured Ca^{++} transients with bioluminescent protein aequorin, and isometric contractile force. Myofibrillar responsiveness to Ca^{++} was assessed by increasing the extracellular Ca^{++} from 0.5 to 15 mM. Our studies indicate that isoflurane shifts the $[Ca^{++}]$/isometric tension curve toward higher intracellular calcium concentrations, while no differences were observed between calcium sensitivity curves obtained in the absence and the presence of equipotent concentrations of halothane and enflurane. In contrast to their differential effects on Ca^{++} sensitivity, these doses of halothane, enflurane and isoflurane produce similar dose-dependent depression of inward calcium current as determined with a whole-cell voltage clamp method in isolated ventricular cells (*Anesthesiology* 69: A452, 1988). These results suggest that the subcellular effects of these volatile agents are different. The negative inotropic effect of isoflurane but not halothane and enflurane may be in part due to a decrease in the responsiveness of contractile proteins to calcium.

Supported by NIH grants HL 34708 and HL 01901 (ZJB).

Tu-Pos420

PROFILIN-ACTIN INTERACTION INVOLVES MORE THAN SIMPLE SEQUESTRATION OF ACTIN MONOMERS.

P.J. Goldschmidt-Clermont, L.M. Machesky, S.K. Doberstein and T.D. Pollard. Intro. by R. Weihing. Department of Cell Biology and Anatomy, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205.

Substoichiometric amounts of human platelet profilin act catalytically to increase the rate of exchange of ATP and of Ca^{++} at the high affinity site on monomeric platelet actin. The inhibiton of the elongation of platelet actin filaments by platelet profilin is consistent with a 1:1 complex with a K_d of 1-5uM. Similar values were obtained with direct binding assays involving separation of free profilin and actin from complexes by ultrafiltration or by binding to immobilized poly-L-proline. On the other hand, profilin inhibits the spontaneous polymerization of actin at lower concentrations than expected from a simple monomer sequestering mechanism with a K_d in the 1-5uM range. This difference suggests that profilin has additional effects on actin such as interaction with oligomeric species along the nucleation pathway or some sort of uncharacterized but long lasting effect on monomers similar to its catalytic effect on the bound ligands.

Tu-Pos421

SPECIFIC BINDING OF PROFILIN TO PHOSPHOINOSITIDES

L.M. Machesky, P.J. Goldschmidt-Clermont, and T.D. Pollard. Intro. by R. Dintzis. Department of Cell Biology and Anatomy, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205.

Profilins -I & -II from *Acanthamoeba* and human platelets (P-I, P-II, and HPP) all bind to PIP_2 (phosphatidyl inositol-4,5-bisphosphate) and to PIP (phosphatidyl inositol-4-monophosphate) with stoichiometries of approximately 1 profilin molecule per pentamer of phospholipid. Their affinities for PIP_2 pentamers differ widely. The dissociation constants are 0.1-1 uM for HPP, 1-5 uM for P-II and 40-50 uM for P-I. The affinities of these profilins for PIP follow a similar pattern. None of these profilins binds to PI (phosphatidyl inositol). We have compared the interactions of these profilins with phosphoinositides in micelles, small vesicles and large unilamellar vesicles with various phospholipids and conclude that the binding is specific for PIP and PIP_2. Based on sequence comparisons, we hypothesize that basic residues in the region of 50-81 (where 11 of the 19 differences between P-I & P-II occur) may be important in binding phospholipids. However, the interactions are more specific than we can account for by pure electrostatics and are likely to involve the protein as well as lipid conformation.

Tu-Pos422

NMR STUDIES OF THE SOLUTION STRUCTURE OF ACANTHAMOEBA PROFILIN-I.

R. A. Atkinson, W.J. Fairbrother, D.A. Kaiser*, L. Machesky*, T.D. Pollard* and B.A. Levine. Intro. by H. Dintzis. Oxford University, UK and *Johns Hopkins Medical School, Baltimore, MD.

Profilin, an abundant actin-binding protein in most cells, shares structural and functional characteristics with at least 2 classes of larger actin-binding proteins. Consequently determination of its structure will provide a wealth of information about the regulation of actin assembly. Using proton magnetic resonance spectroscopy, we are studying the solution conformation of the 13kDa Acanthamoeba profilin-I. Two dimensional correlated spectroscopic data derived from a combination of COSY, RELAY, HOHAHA and NOESY experiments is being complemented by extrinsic paramagnetic probe data obtained using site specific reagents to define the tertiary fold of the protein. Comparisons with a closely related isoform, profilin-II, provide independent spectral assignments that act as reporter groups for different regions of the primary structure. Definition of the mutual sites of interaction of the profilin-actin complex is being sought using the native molecules as well as specific synthetic peptides of each protein. The derived data is being integrated with independent progress on the x-ray crystallographic structure of profilin-I being done by K. Magnus, E. Lattman and T. Pollard.

Tu-Pos423

PHOSPHORYLATION OF MYOSIN 20 kD LIGHT CHAIN ISOFORMS AFFECTS CELL SHAPE IN CULTURED RAT AORTIC SMOOTH MUSCLE. P. Monical and R.A. Murphy, Dept. of Physiology, University of Virginia, Charlottesville, VA 22908.

Actin and myosin form stress fibers that terminate in transmembrane attachment plaques in cultured vascular smooth muscle cells. In confluent cultures, 42 ± 2% of the LC-20 was the smooth muscle type. Basal LC-20 phosphorylation was 20 ± 3% in both the smooth and non-muscle isoforms. This value of phosphorylation in intact tissues causes cross-bridge cycling and significant force generation. 1μ M Forskolin or 0.1 mM IBMX induced dephosphorylation of both LC-20 isoforms within 5 minutes. Scanning EM revealed cells rounded into stellate structures attached to the substrate via thin cytoplasmic processes by 30 minutes. Removal of the drugs was followed by rephosphorylation to basal levels and resumption of a normal oblate morphology. We conclude that significant myosin LC-20 phosphorylation is necessary to maintain cell shape in cultured cells. There was no evidence for differential isoform regulation. Supported by USPHS 5T32 HL07355 and 5P01 HL29242.

Tu-Pos424

TIME-DOMAIN FLUORESCENCE MICROSCOPIC STUDIES OF MONOBROMOBIMANE LABELED SPINACH CALMODULIN BINDING INTERACTIONS. Albert H. Gough and D. L. Taylor, Dept. of Biological Sciences/Center for Fluorescence Research in the Biomedical Sciences, Carnegie Mellon University, Pittsburgh, PA 15213

The single cysteine residue of spinach calmodulin has been covalently modified with monobromobimane to produce a fluorescent analog for use in time-resolved fluorescence anisotropy studies. The fluorescence of bimane calmodulin is not sensitive to Ca^{++} and the 7.5 nsec fluorescence lifetime is very close to the mean rotational correlation time. Steady state anisotropy measurements indicate that the rotational correlation time of this analog is viscosity dependent, is similar to published values for other calmodulin analogs, and increases upon binding Ca^{++}. Data will be presented demonstrating the myosin light chain kinase binding activity and the effect of myosin light chains on that interaction. A time-domain fluorescence microscope system will be described, and the results of both *in vitro* and *in vivo* studies of bimane calmodulin distribution and rotational mobility will be presented.

Tu-Pos425

ACANTHAMOEBA MYOSIN I HEAVY CHAIN KINASE IS ACTIVATED BY PHOSPHATIDYLSERINE-ENHANCED PHOSPHORYLATION. H. Brzeska, T.J. Lynch, B. Martin*, A. Murphy and E.D. Korn, NHLBI, and *NIMH, NIH, Bethesda, MD 20892. The actin-activated Mg^{2+}-ATPase activities of myosins I from Acanthamoeba castellanii are fully expressed only when a single amino acid on their heavy chain is phosphorylated by myosin I heavy chain kinase. We have found that kinase purified by a procedure designed to minimize its phosphorylation during purification can incorporate up to 7.5 mol of phosphate per mole of enzyme, when incubated with ATP, possibly by autophosphorylation. The rate of phosphorylation is enhanced about 20-40 fold by phosphatidylserine, but is unaffected by calcium ions. Phosphorylation increases the rate at which kinase phosphorylates the regulatory site of myosin I (and synthetic peptides corresponding to this site) by about 50-fold and changes the kinase mobility in SDS-PAGE. Phosphatidylserine does not enhance the activity of fully phosphorylated kinase. The above regulatory cascade is unique among myosins. We are in the process of studying the effect of factors other than phosphatidylserine on kinase activity and defining in more detail the sequence requirements of potential phosphorylation sites.

Tu-Pos426

THE RESPONSE OF CARDIAC FORCE GENERATORS TO THE COMBINATION OF ALPHA AND BETA ADRENERGIC STIMULATION.

G. McClellan, A. Weisberg, S. Winegrad, Dept. of Physiology, Sch. of Medicine, Univ. of Pennsylvania, Philadelphia, PA

Contractile activity of crossbridges in mammalian cardiac muscle has been assayed by measurement of both Ca and actin activated ATPase activity in cryostatic sections of quickly frozen rat ventricles. Alpha, beta and combined alpha and beta adrenergic stimulation has been examined by comparing the response of serial sections (thickness equals 30% of cell diameter) to different combinations of agonists and antagonists. Dose-response curves were generated for isoproterenol, cAMP and norepinephrine with and without antagonists. Isoproterenol produces maximum increase in ATPase activity at 10^{-7}M and very small reduction in the inotropic response occurs at 10^{-6}M. Propranolol produces a 100-1000 fold increase in the optimal concentration of isoproterenol. Norepinephrine produces an increase in ATPase activity with a sharp peak in the inotropic response at 10^{-6}M. At 10^{-5}M, the increase in ATPase activity has totally disappeared. Either beta antagonist (propranolol) or alpha antagonist (prazosin) completely inhibits the increase in ATPase produced by 10^{-6}M norepinephrine without significantly altering the value at 10^{-5}M (where no effect of norepinephrine alone was seen). cAMP increases ATPase activity with a peak at 10^{-6} and a plateau in the response with 10 fold higher concentration. Cryostatic sections normally carry some alpha and beta adrenergic stimulation produced by the catecholamine release during sacrifice of the animal. We interpret these data as indicating: 1)a role for both alpha and beta adrenergically regulated reactions in regulating cardiac myosin; the two responses are interactive; 2)two separate, dose-dependent effects of alpha adrenergic stimulation on myosin: one to increase and another to decrease ATPase activity; 3)the negative effect of alpha stimulation does not occur by reducing the concentration of cAMP. These observations can be explained by the existance of two different but interactive modifications of myosin with respectively up and down regulatory consequences.

Tu-Pos427

TWO DIFFERENT PHOSPHORYLATIONS OF REGULATORY LIGHT OF MYOSIN REGULATE CROSS BRIDGE FUNCTION IN CARDIAC MUSCLE.

S. Winegrad, G. McClellan, A. Weisberg, Dept. of Physiology, Sch. of Medicine, Univ. of Penna., Phila., PA

Force generators in cardiac muscle exist in multiple states in which ability to generate force and shorten differ even under the same degree of activation by Ca. The molecular basis for these states has not been explained. Evidence for a role of phosphorylation of the regulatory light of myosin (LC_2) by myosin light chain kinase (MLCK) has been tantalizing but inconsistant. With 2-dimensional gel electrophoresis we have observed two different patterns of phosphorylation of LC_2 that appear to have different but interdependent effects on contractile function. These studies have been conducted on rat hearts with either exclusively alpha myosin heavy chain or a mixture including beta myosin heavy chains. Contractile activity has been assayed by measuring maximum Ca activated force and maximum velocity of unloaded shortening in detergent skinned fibers and both Ca and actin activated ATPase activity with quantitative histochemistry in cryostatic sections of quickly frozen hearts. Pattern I for phosphorylation of LC_2 is produced by MLCK and is associated with upregulation of contractility that accompanies an increase in cellular Ca from elevated extracellular Ca, increased rate of electrical stimulation and beta adrenergic agonists. Pattern II is produced by increase in resting tension or metabolic deprivation, conditions that down regulate contractility of V_1 and up regulate V_3. In the presence of pattern II, the upregulatory response of V_1 force generators to beta adrenergic agonists is markedly reduced. The two different patterns of phosphorylation of LC_2 appear to be associated with regulation of two different steps in the cross bridge cycle: hydrolysis of ATP and cross bridge attachment to actin. These results are strikingly similar to those of Adelstein and coworkers in their studies of smooth muscle regulatory light chain and the response of actomyosin ATPase of turkey gizzard to phosphorylation by MLCK or protein kinase C. We propose that a similar mechanism operates in mammalian heart and contributes to the changes in contractile performance.

Tu-Pos428

KINETIC STUDIES OF MYOFIBRILS

E.W. Taylor, The Univ. of Chicago

The kinetic mechanism of rabbit white muscle myofibrils was investigated by stop flow and quench flow experiments. The rate of dissociation of myosin heads by ATP was determined by change in fluorescence of myofibrils in which actin was preferentially labeled with pyrene iodoacetamide. The rate constant was 400 S^{-1} at 8° and too fast to measure at 20°. The phosphate burst in the absence of calcium was 0.7 to 0.8 moles per myosin head with a rate constant of 40 to 50 S^{-1}. In the presence of calcium there was a small phosphate burst with rate constant greater than 100 S^{-1}. Fitting the data to a three step model, $AM + T \underset{}{\overset{(1)}{\rightleftharpoons}} AMT \overset{(2)}{\rightleftharpoons} AMDP \overset{(3)}{\rightleftharpoons} AM + D + P$ with corresponding steps for dissociated states, yielded rate constants similar to values obtained in solution (Rosenfeld and Taylor, J Biol Chem, 259, 11908, 1984 and 262, 9984, 1987). It is proposed that the regulation mechanism is similar to the mechanism in solution and that the rate of step 3 is the primary parameter in determining the rate of unloaded shortening.

Tu-Pos429

ACTOMYOSIN ATPASE AND THE COMPOSITION OF MYOSIN FILAMENTS

P. Cheung and E. Reisler, Dept. of Chem. & Biochem. and the Mol. Biol. Inst., Univ. of Calif., Los Angeles, CA 90024

In order to detect catalytically significant intermolecular interactions in assembled myosin, copolymers of myosin and myosin rod minifilaments were formed by dialysis of these particles into 5mM citrate-Tris (pH 8.0) buffer and exchange reactions between myosin and rod minifilaments. The formation of copolymers was verified by sedimentation velocity and electron microscopy experiments. The observed inhibition of actomyosin ATPase at 25 C by rod particles was related to the decrease in the K_m values for the actin activated ATPase reactions with an increase in rod: myosin ratios. Similar amounts of rod minifilaments did not affect the acto-S-1 ATPase activities. When the rod particles were replaced in these minifilaments by SH_1-modified myosin, the ATPase of these copolymers was increased over that of the combined ATPases of the individual minifilaments. These results demonstrate that the catalytic function of myosin heads in the filaments is affected by the interactions of vicinal myosin molecules with actin.

Tu-Pos430

ESTIMATE OF MYOSIN STEP SIZE FROM SLOW SLIDING MOVEMENT AT LOW HEAD DENSITIES IN AN *IN VITRO* ASSAY. T. Q. P. Uyeda, S. J. Kron, and J. A. Spudich, Department of Cell Biology, Stanford University School of Medicine, Stanford, CA 94305.

The swinging cross-bridge theory of muscle contraction predicts that the step size (d: displacement per ATP hydrolysis) should be <40 nm. However, reported values of d range up to >280 nm/ATP. We have estimated d from the velocity, v, of slowly moving actin filaments in the presence of 0.8% methylcellulose on surfaces coated with low densities of heads. In this regime, filaments are predicted to move at $v=v_o\times\{1-(1-t_s/t_c)^n\}$, where v_o is the maximum sliding velocity, t_s is the stroke time, t_c is the cycle time, and n is the number of heads that can interact with the filament. We estimated t_s by fitting velocity data with this formula. From $d=v_o\times t_s$, we obtained a value for d of 11-28 nm/ATP, which is consistent with force generation by swinging cross-bridges. At limiting head densities, filaments showed both slow sliding and diffusive movements. We are studying this movement to abstract the characteristics of the movement of individual heads.

Tu-Pos431

PHOSPHATE DEPRESSES THE TENSION AND THE EXTENT OF STRUCTURAL CHANGE IN TNC INDUCED BY CROSS-BRIDGES IN DEMEMBRANATED FIBERS OF RABBIT PSOAS MUSCLE T.StC. Allen and A.M. Gordon (Intr. by M. Bosma) Dept of Physiology, UWa, Seattle, Wa. 98195

By measuring the fluorescence from troponin C labelled at Met-25 with dansylaziridine (TnC-DANZ), exchanged for endogenous TnC in skinned single fibers, we are studying the influence upon TnC structure exerted by Ca^{2+} binding to TnC-DANZ and by cycling cross-bridges. At 2.4μm sarcomere length, in the presence of 3mM MgATP, titration of the fiber with Ca^{2+} elicits two transitions in the fluorescence from TnC-DANZ: a small one attributable to Ca^{2+} binding at the Ca-Mg-binding sites, and a large one, occurring approximately concomitantly with the development of tension, attributable to Ca^{2+} binding at the Ca-specific sites and to actomyosin interaction. That the large transition is reduced in magnitude when the titration is performed on a fiber set to > 4.0μm corroborates that actomyosin interaction produces a structural change in TnC. The presence of 20mM inorganic phosphate during the titration reduces peak isometric tension 29% (s.d.9%,n=5) and the total change in fluorescence only 15%(s.d.15%,n=5). (Support: NS08384,HL21962,&GM07270)

Tu-Pos432

DIRECT MEASUREMENT OF ENHANCED CALCIUM BINDING INDUCED BY RIGOR CROSSBRIDGES IN THE OVERLAP REGION OF SKINNED SKELETAL MUSCLE FIBERS. M. Cantino, T. Allen, D. Johnson and A. Gordon. (spon. by L.Huntsman) Ctr for Bioeng. and Dept. of Physiol. and Biophys., U. of Wa., Seattle, WA 98195

As described previously (Biophys.J. 53:589a) we have used electron probe x-ray microanalysis to measure directly the distributions of Ca binding along the sarcomere in glycerinated rabbit psoas fibers. Data have been obtained both from matched spectra collected over H, I and overlap regions of the same sarcomeres, and from similar areas of digital x-ray images. The level of Ca measured in the I band of fibers in pCa 4.8 rigor solution is most consistent with binding of three Ca ions per TnC. Calcium levels measured in the overlap region of the same sarcomeres were, on average, 25% higher than those in the I band when corrected for Ca bound to myosin as estimated from the Ca levels in the H zone. At pCa 6.3, Ca levels in the overlap region also exceeded those in the I band. These data provide direct evidence of enhanced Ca binding in sarcomeric regions with rigor crossbridge attachment. (Supported by NS 08384, HL 31962 and GM 07270).

Tu-Pos433

PHOSPHATE-WATER OXYGEN EXCHANGE PROBES OF INSECT FLIGHT MUSCLE FIBERS FROM VESPULA VULGARIS. *M. Peckham, @Martin R. Webb, *J.E. Molloy & *D.C.S. White. *University of York, YO1 5DD, U.K. @N.I.M.R., London, NW7 1AA, U.K.

Oxygen exchange between phosphate and [^{18}O]water during ATP hydrolysis by Ca^{2+}-activated, skinned flight muscle (IFM) fibers of the wasp Vespula vulgaris was investigated for unstrained, strained or oscillating (maximally activated) conditions. Some of the product Pi, contained more than one oxygen derived from water. Analysis of the distribution of ^{18}O in Pi indicated that there are two pathways for formation of Pi in unstrained fibres but a single pathway in strained or oscillated fibres. Although the extent of exchange was much less, these results are qualitatively similar to those for IFM fibres from Lethocerus indicus (Lund et al, J.Biol. Chem., 1988, 263,5505) which has a much lower wingbeat frequency. In oscillated fibres, the ATPase rate and the rate of Pi release as indicated by the pattern of oxygen exchange were both five times faster in V. vulgaris than in L. indicus, compatible with Pi release being rate limiting in both species.
Supported by the MRC and the SERC

Tu-Pos434

THE INEQUIVALENCE OF IONIC STRENGTH AND IONIC EQUIVALENCE: A STRONG CASE FOR CONTROLLING IONIC STRENGTH IN SKINNED MUSCLE FIBER BATHING SOLUTIONS.

M.A. Andrews, T.M. Nosek and R.E. Godt. Dept. Physiol. and Endo., Med. College GA, Augusta GA 30912.

In solutions containing polyvalent ions, we have attempted to determined which measure of the ionic environment, ionic strength ($\mu_s = 1/2\ \Sigma\ c_i\ z^2_i$) or ionic equivalence ($\mu_e = 1/2\ \Sigma\ c_i\ |z_i|$), is most appropriate to assess and control. Using detergent skinned single rabbit psoas fibers with maximal Ca^{2+}-activated force (F_{max}) as the dependent variable, we have studied a variety of uniunivalent salts over a range of μ_s (Andrews et al., *Biophys. J. 53*:570a, 1988). Tetramethylammonium (TMA^+) and methanesulfonate ($MeSO_3^-$) were found to minimally affect F_{max}. Similarly, we now find hexamethylenediamine N,N,N',N'-tetracetic acid (H_2HDTA^{2-}) to be preferred over creatine phosphate^{2-}, sulfate^{2-}, succinate^{2-} and phosphoenolpyruvate^{3-}. To investigate μ_s and μ_e, TMA^+ salts of $MeSO_3^-$ and H_2HDTA^{2-} were added to bathing solutions in a step-like fashion to increase: 1) μ_e from 69 to 369 mM ($TMAMeSO_3$) and from 69 to 269 mM (TMA_2H_2 HDTA), and 2) μ_s from 90 to 390 mM (with each salt). Results indicate that as μ_e was increased, $TMA_2H_2HDTA > TMAMeSO_3$ in decreasing F_{max}. Alternatively, as μ_s was increased, both salts were equally benign on F_{max}. We conclude that μ_s is the appropriate parameter for assessing the ionic environment, contradicting Fink et al. (*J. Physiol. 370*:317-337, 1986.) who may have been misled by the limited number of ions they used. (Support: NIH AR31636)

Tu-Pos435

REGULATION OF FORCE PRODUCTION IN ISOLATED RAT MYOCARDIAL BUNDLES.

Lin-Er Lin, G. B. McClellan, and S. Winegrad. Department of Pharmacology and Toxicology, University of Mississippi Medical Center, Jackson, MS 39216 and Department of Physiology, University of Pennsylvania, Philadelphia, PA 19104.

Maximum Ca-activated force (F_{max}) produced by thin papillary muscles that were chemically skinned with 1% Triton is closely related to maximum twitch force generated before skinning. F_{max} averaged 60mN/mm^2 in thin bundles skinned immediately after isolation and dropped to 23 mN/mm^2 when the bundles were superfused for 40 min without contracting. Decline in the force production was partially reversed by a brief period of stimulation at 1Hz or by beta-adrenergic stimulation prior to detergent treatment. Raising [Ca^{2+}] to 7.5mM in the superfusing solution before skinning produced a high level of force development in the skinned bundles. The decline in contractility with superfusion and its reversal by stimulation, high Ca^{2+} or beta agonists follows total cell calcium. The data suggest a role for Ca^{2+} in regulation of contractile proteins distinct from the tropomyosin-troponin system. (Supported by NIH grants HL 16010 and HL 33294)

Tu-Pos436

EFFECT OF LIMITED TRYPTIC DIGESTION ON THE KINETICS OF SKELETAL ACTOS-1

V.A.HARWALKAR, M.P.WHITE, D.T.ANNIS, F. ZERVOU, and L. A. STEIN; LMC, SUNY AT STONY BROOK MEDICAL CENTER

We have investigated the effect of limited tryptic digestion of chymotryptic S-1 on its kinetic properties. We find that the apparent activation constant, K_{ATPase}, is significantly weakened by tryptic digestion, but that the Vmax observed remains approximately constant. However, we also find that the apparent dissociation constant of actin to myosin during steady state hydrolysis of ATP, $K_{binding}$, is also reduced leaving the ratio of the apparent binding constant to the apparent activation constant approximately constant, independent of the degree of digestion. These studies strongly support the notion that the binding of S-1 to actin in the presence of ATP is <u>specific</u> to the activation process.

In addition, we have investigated the presteady state kinetics of the digested proteins. These studies show that tryptic digestion decreases the magnitude but has little effect on the rate of the Pi burst.

Tu-Pos437

PRODUCT INHIBITION OF CARDIAC ACTOS1

L.A. STEIN AND V.A. HARWALKAR; LMC, SUNY AT STONY BROOK MEDICAL CENTER

Product inhibition was studied by measuring the ATPase activity, and the concentrations of ATP, ADP and Pi as a function of time. The initial substrate concentrations and buffer conditions were such that no change in pH occured during the time course. We find that significant inhibition occurs after half of the ATP is split and that the inhibition observed is not due to the rising Pi nor the falling ATP concentration, and is therefore due to ADP binding. To analyze the data we have used a simplified scheme for the binding of nucleotide:

$$AM^{*}T \underset{k_T[T]}{\overset{k_{-T}}{\longleftrightarrow}} AM \underset{k_{-D}}{\overset{k_D[D]}{\longleftrightarrow}} AMD$$

with solution:

$$1/Vss([A]) = a([A]) + (1/k_{-T} + b([A]))((K_D[D]+1)/K_T[T])$$

When this equation is fit to the inhibition data, k_{-T} is determined using quench flow techniques, and a([A]) and b([A]) are determined from the initial ATPase activity, we find that $K_D \doteq K_T$, suggesting the importance of ADP bound crossbridges in cardiac muscle.

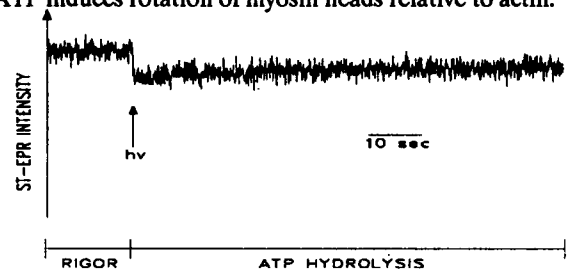
Tu-Pos438

TIME-RESOLVED PHOSPHORESCENCE ANISOTROPY OF EOSIN-S1 BOUND TO ACTIN DURING STEADY-STATE ATP HYDROLYSIS. **Richard A. Stein, Christopher L. Berger, John M. Menezes, David D. Thomas. Dept. of Biochem., Univ. of Minn., Mpls, MN 55455.**

To determine whether S1 is rotationally mobile while bound to actin, we measured the time-resolved phosphorescence anisotropy (TPA) decay of eosin covalently bound to S1 during steady-state actin-activated ATP hydrolysis at 4°C. TPA allows for the quantitative resolution of multiple components present during this complex enzymatic cycle. The TPA decay for S1 in solution has a submicrosecond correlation time and an r_∞ of 0.005 ± 0.004, indicating isotropic rotational motion. The TPA decay for Actin+S1 (A-S1) has a non-zero r_∞ indicating restriction of motion. The addition of ATP to A-S1 causes a decrease in r_∞ to an intermediate level between those of free S1 and A-S1. Since sedimentation experiments showed that 60% of S1 is bound, 40% of the free S1 TPA decay was subtracted, yielding a decay corresponding to bound S1. This decay differs from rigor, indicating that bound heads undergo additional microsecond motions from those seen in rigor. These results constitute direct evidence for microsecond motions of myosin heads while attached to actin during the ATPase cycle.

Tu-Pos439

TIME-RESOLVED ST-EPR DETECTION OF F-ACTIN ROTATIONAL DYNAMICS DURING ACTIVATION OF MYOSIN S1/HMM ATPase USING CAGED-ATP.
E. Michael Ostap and David D. Thomas. Dept. of Biochemistry, Univ. of Minnesota Medical School, Mnpls., MN 55455.
Berger et al. (PNAS 1989) demonstrated that the photolysis of caged-ATP induces microsecond rotational motions of myosin heads bound to actin. To determine whether the observed rotational motions of the bound myosin heads are independent of actin motions, we have used ST-EPR and laser induced photolysis of caged-ATP to monitor changes in the rotational dynamics of spin-labeled F-actin in the presence of S1 and HMM. At physiological ionic strength (μ = 186 mM),a decrease in ST-EPR intensity (increase in F-actin mobility) was clearly indicated upon photolysis of 1mM caged-ATP with a 200 msec, 351 nm laser pulse. This increase in mobility is due to the complete dissociation of the myosin heads from the actin filament. At low ionic strength (μ = 36 mM), when about half of the heads remain bound during ATP hydrolysis, a smaller increase in the actin mobility is apparent. However, this actin motion is at least 10 times slower than the motions of myosin heads attached to actin. Therefore, we conclude that the active interaction of myosin, actin, and ATP induces rotation of myosin heads relative to actin.



Tu-Pos440

ORIENTATION OF SPIN LABELED LC2 EXCHANGED INTO MYOSIN S1 BOUND TO GLYCERINATED MUSCLE FIBERS. B.D. Hambly#, K. Franks and R. Cooke. Biochemistry Department & CVRI, University of California, San Francisco CA 94143 and #Anatomy Department, Sydney University, NSW 2006, Australia.
We examined the orientation and motion of a spin label bound to myosin subfragment-1 (S-1) close to the head-rod junction. Spin labeled myosin light chain-2 (LC2) was exchanged into papain S-1 and the complex bound to glycerinated rigor muscle fibers. Electron paramagnetic resonance (EPR) spectra showed the spin label diffused rapidly around a centroid at 74° to the fiber axis within a "cone" of 40°. SL-LC2 S-1 fiber spectra were indistinguishable from spectra of SL-LC2 exchanged directly into rigor fibers. Binding of the SL-LC2 S-1 complex to ion exchange beads significantly slowed the spin probe, suggesting that the probe was immobilised on S-1. Rigor cross-bridge models propose that the region close to the head-rod junction (where LC2 binds) may adopt many orientations due to the constraints of the filament lattice. This study suggests that the region of the cross-bridge proximal to the head-rod junction undergoes considerable motion in rigor and that this motion is independent of any constraints from the S-2 region of myosin. Support: USPHS AM30868 & NH&MRC of Australia.

Tu-Pos441

PRESTEADY STATE BINDING OF CARDIAC ACTOS-1 IN THE PRESENCE OF ATP.
L.A. STEIN AND V.A. HARWALKAR; LMC, SUNY AT STONY BROOK MEDICAL CENTER
Models for the activation of the myosin ATPase activity by actin describe transitions that occur between myosin-nucleotide complexes that are in rapid equilibrium binding with actin. In order to determine the individual binding constants involved in the activation process, we have investigated the presteady state kinetics of the dissociation of actomyosin by ATP. We find that an actin flow artifact dominates the shape of the time course of dissociation. However we also find that if we vary the order of addition of actin, S-1 and ATP, the observed light scattering transients are superimposable. The simplest interpretation of these data is that the binding constants for myosin-ATP and myosin-ADP-Pi to actin are equal. We also find that the actin flow artifact does not significantly affect the measurement of the tryptophan fluorescence enhancement associated with the initial Pi burst.

Tu-Pos442

ACTIN PROMOTED REASSEMBLY OF SMOOTH MUSCLE MYOSIN FILAMENTS.

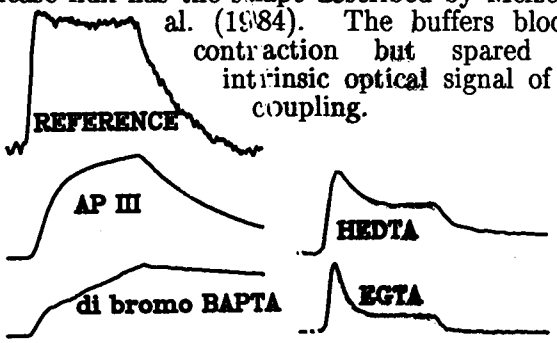
D. Applegate & J. Pardee; Mt. Sinai Med. Sch. & Cornell Med. Coll., N.Y., N.Y.

The binding of AMPPNP to nonphosphorylated myosin of chicken gizzard smooth muscle in 0.15M KCl, 10mM Imid. (pH 7.3), 1mM $MgCl_2$, 1mM EGTA, 1mM DTT promotes transition of myosin to the soluble 10S conformation (M^{10S}), due to the formation of a highly stable M^{10S}-$(AMPPNP)_2$ complex. When the M^{10S}-$(AMPPNP)_2$ complex is added to filamentous actin (final molar ratio of 10:1 actin to myosin), E.M. of negatively stained samples reveals: 1) progressive assembly of myosin filaments adjacent to actin filaments; and 2) organization of actin filaments around the newly assembled myosin filaments. The end products appear to be highly organized contractile units. The increase in the light scattering accompanying reassembly and realignment can be fitted by a single exponential. While varying either the myosin or actin concentrations alters the rate of the process, the important factor appears to be the actin:myosin molar ratio. The process is strikingly similar to the actin filament mediated assembly of Dictyostelium myosin (J. Pardee PNAS 86, 6161-5.). Supported by NIH grant DK1R2940154.

Tu-Pos443

EFFECTS OF INTRACELLULAR Ca BUFFERS ON Ca TRANSIENTS IN SKELETAL MUSCLE. E. Ríos, G. Brum, G. Pizarro, and M. Rodríguez (Introduced by B. Eisenberg). Rush University, Chicago, IL.

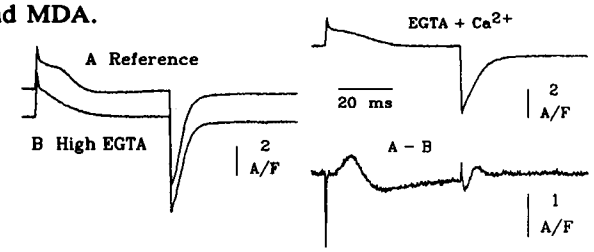
Ca transients were measured in cut muscle cells from signals of Antipyrylazo III. Ca buffers, diffused intracellularly, can be classified in two groups according to their effect on the Ca transient. Left: Dibromo BAPTA and the dye ApIII caused a "low-pass filtering", to a limiting shape equal to the time integral of the Ca release flux. Right: EGTA and HEDTA altered the Ca transient exaggerating its initial peak and making the decay faster, to a limiting shape that is a scaled version of the release flux itself. These two types of alteration can be explained if buffers at left equilibrate rapidly and the others have very slow reaction rates. A first consequence: the release flux has the shape described by Melzer et al. (1984). The buffers blocked contraction but spared the intrinsic optical signal of EC coupling.



Tu-Pos444

DEPLETION OF THE SR REDUCES THE DELAYED CHARGE MOVEMENT OF FROG SKELETAL MUSCLE. J. García, G. Pizarro*, E. Ríos* & E. Stefani. Dept. Molecular Physiology & Biophysics, Baylor College of Medicine, Houston, TX, 77030 & *Rush University, Dept. Physiology, Chicago, IL, 60612.

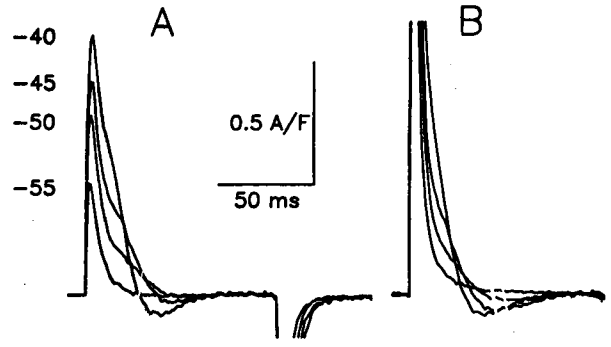
The vaseline gap voltage clamp technique was used in fibers with high $[EGTA]_i$. García et al. (1989, J. Gen. Physiol., 94) showed that repetitive pulses in high $[EGTA]_i$ impair Ca^{2+} release, probably by depleting the SR. Depolarizing pulses (-20 mV every 30 s) rapidly eliminated $Q\gamma$ in 15 mM $[EGTA]_i$. This effect was partially reversible after 5 min rest. When $[EGTA]_i$ was raised to 62.5 mM, $Q\gamma$ was suppressed at lower pulsing frequency (0.004 Hz). The supression was reversed with 200 nM free Ca^{2+} intracellularly. The intrinsic optical signal, thought to reflect Ca^{2+} release into the myoplasm (Ríos et al., this meeting), showed parallel changes to those found in $Q\gamma$. These results support the idea that $Q\gamma$ is caused by Ca^{2+} release from the SR. See also Pizarro et al., this meeting. Supported by NIH and MDA.



Tu-Pos445

AN INWARD PHASE IN INTRAMEMBRANE CHARGE MOVEMENT DURING A DEPOLARIZING PULSE. G. Pizarro, L. Csernoch and E. Ríos. Rush University, Chicago.

Intramembrane charge movement currents were measured in cut skeletal muscle fibers. At -50 to -30mV a hump component was visible ("$Q\gamma$"). In fibers containing EGTA a negative going phase followed the hump (A). This phase was also seen in the total current transient, (B), and was maximum (up to 0.1 A/F) when the hump was most noticeable. It was not blocked by any channel blockers other than the ones that blocked $Q\gamma$. It was voltage-shifted by interventions that shifted $Q\gamma$. It is a true inward charge movement during depolarization, in apparent conflict with electrodynamics. A model, with Ca binding to negative sites on the voltage sensor, explains the inward current as a feature of $Q\gamma$.



Tu-Pos446

EFFECTS OF 1-ALKANOLS ON E-C COUPLING. A Gonzalez, G. Pizarro, J. Ma, C. Caputo and E. Ríos (Intro. by R.S. Eisenberg). Rush University, Chicago, IL and IVIC, Caracas, Venezuela.

1-octanol and 1-heptanol block the reconstituted SR Ca channel (Ma and Coronado) while hexa and decanol have no effect. 1-alkanols had a similar pattern of effects when applied to cut frog skeletal fibers in a Vaseline gap. 1-octanol at 0.5 mM rapidly and reversibly reduced Ca release flux at all voltages by ≃ 50% (n=5). Hexanol had no effect or a potentiation (n=2). Decanol had no effect initially (n=2), but made cells leaky in ≃ 5 min. The effect of octanol on Ca release was accompanied by reduction of the hump component of charge movement ($Q\gamma$). $Q\beta$ was not altered. Hexanol had no effect on charge movement.

On small bundles of intact fibers 0.5 to 1 mM octanol suppressed twitches, action potentials and K contractures without changing resting potentials. The octanol-blocked fibers responded with contracture to 5 mM caffeine (n=2).

The pattern observed suggests an effect of octanol on the Ca release channel, that can be overcome by caffeine. The suppression of $Q\gamma$ is consistent with this component being a consequence of Ca release (Csernoch et al., Biophys. J. 55:88a). Supported by NIH and MDA.

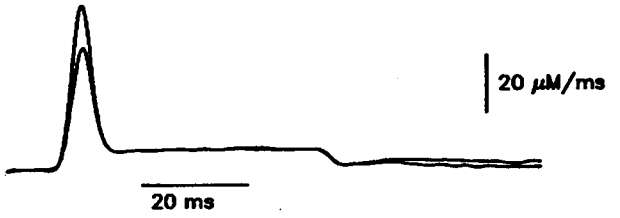
Tu-Pos447

EFFECTS OF ADRENALINE ON CALCIUM RELEASE IN SINGLE FIBERS OF FROG SKELETAL MUSCLE.

G. Brum, S. Gonzalez, G. Ferreira, M. Maggi and C. Santi (Intro. by E. Ríos). Dept. of Biophysics, School of Medicine, G. Flores 2125, Montevideo, Uruguay.

Single cut fibers of frog skeletal muscle, were voltage clamped in a double Vaseline gap chamber. Solutions blocked Na and K conductances. The internal solution contained antipyrylazo III. The Ca^{2+} transients were monitored optically during depolarizing pulses. From these transients the time course of calcium release was derived. Ca^{2+} transients and charge movement were recorded simultaneously.

5–50 μM adrenaline added to the external solution increased the amplitude of the Ca^{2+} transients in 11 fibers. This effect is the consequence of an increase in the early, inactivating phase of calcium release (Fig.). The steady component of release remained unaltered. Adrenaline did not modify the charge moved by depolarizing (charge 1) or hyperpolarizing (charge 2) pulses.



Tu-Pos448

DELAYED CHARGE MOVEMENT NEAR THE CONTRACTION THRESHOLD IN SKELETAL MUSCLE.

L. Kovacs, J. Magyar, L. Csernoch and G. Szucs (Int. by B.J. Simon) Dept. of Physiol., Univ. Med. School, Debrecen, Hungary, H-4012

Charge movements and calcium transients were recorded at and around the contraction threshold on single skeletal muscle fibers of the frog. Humps were clearly present on current records measured at the rheobase and their appearance was accompanied by a difference in ON and OFF charge (Q_{ON} being greater). Caffeine shifted both the hump and this inequality to the new rheobase, that is to more negative voltages. When applying 10 mV voltage steps on top of different prepulse levels Q_{ON} had a maximum at the contraction threshold while Q_{OFF} increased monotonically in the voltage range examined. Caffeine influenced only the ON charge shifting its maximum towards the resting potential. Difference records, obtained as caffeine-control, were bell shaped and resembled the hump. We believe that the delayed component reflects an interference of calcium release with charge movement process. Supported by the MDA.

Tu-Pos449

INTRACELLULAR CALCIUM AND MAGNESIUM MOVEMENTS FOLLOWING CALCIUM RELEASE IN FROG SKELETAL MUSCLE.

V. Jacquemond, M.G. Klein and M. F. Schneider, Dept. Biol. Chem., U. Maryland Med. School, Baltimore, MD 21201

Myoplasmic $\Delta[Ca^{2+}]$ and $\Delta[Mg^{2+}]$ were determined from antipyrylazo III absorbance signals at 700 and 590 nm in voltage clamped cut fibers (end pool solution predominately Cs glutamate). Calcium release from the sarcoplasmic reticulum (SR) was calculated from $\Delta[Ca^{2+}]$. The return of Ca^{2+} to the SR following a conditioning pulse (P1) was determined from the recovery of calcium release for test pulses 1 to 16 s after P1. P1 increased $\Delta[Mg^{2+}]$, presumably due to Ca^{2+} replacing Mg^{2+} on parvalbumin (parv) during elevated $[Ca^{2+}]$. After P1 $[Mg^{2+}]$ declined in parallel with the return of Ca^{2+} to the SR, as expected if Ca^{2+} moves from parv to the SR and Mg^{2+} returns to parv during recovery. In 4 fibers the mean initial SR Ca content was 1.1 mM (if dissolved in myoplasm) and the calcium unavailable for release at 2 and 4 s after P1 was 11.8 $\pm$ 1.9 and 9.1 $\pm$ 1.9 % of the initial content. For these recovery times the calcium unavailable for release was 2.6 $\pm$ 0.3 fold greater than $\Delta[Mg^{2+}]$. Since most of the calcium missing from the SR should displace magnesium from parv, the above discrepancy could be due to (i) magnesium binding to other myoplasmic sites, (ii) magnesium entry into the SR in exchange for released calcium, or (iii) calcium being in the SR but unavailable for release.

Tu-Pos450

INOSITOL TRISPHOSPHATE BINDS TO HEAVY SARCOPLASMIC RETICULUM MEMBRANES ISOLATED FROM FROG SKELETAL MUSCLE.

Cecilia Rojas and Cecilia Hidalgo. Depto de Fisiología y Biofísica, Fac. Medicina, U. de Chile and Centro de Estudios Científicos de Santiago, Casilla 16443, Santiago 9, Chile.

Inositol 1,4,5-trisphosphate (IP3), a putative chemical messenger in excitation-contraction coupling in skeletal muscle, activates the ryanodine-sensitive high conductance calcium channels present in heavy SR membranes. We have investigated IP3 binding to heavy SR vesicles, using 32P-IP3 at pCa 7.0 and in the absence of Mg to prevent IP3 phosphatase activity. We did not detect high affinity (nM Kd) binding in SR vesicles with a filtration assay to separate bound from free ligand. However, using a centrifugation assay, we detected low affinity binding sites, 3-5 pmoles per mg of protein, with a Kd of about 0.1uM. This Kd value is in the range of concentrations needed to activate the SR calcium channels incorporated in bilayers and calcium release in 'scratched' frog skeletal muscle fibers. These results indicate that heavy SR vesicles have receptors for IP3 that are different from the high affinity (nM Kd) receptors present in other cell systems. Supported by FONDECYT 972, MDA and NIH GM35981.

Tu-Pos451

EFFECTS OF TETRACAINE ON CALCIUM CHANNELS FROM SARCOPLASMIC RETICULUM (SR). R. Bull and J.J. Marengo (Int. by B.A. Suárez-Isla). Departamento de Fisiología y Biofísica, Facultad de Medicina, Universidad de Chile, Casilla 70005, Santiago 7, Chile.

We studied the effects of tetracaine on high conductance (90 pS) calcium channels from SR from frog skeletal muscle incorporated into POPE:PS:PC (5:3:2) bilayers, in 37 mM Ca or Ba HEPES trans, 225 mM HEPES-Tris cis, pH 7.4, pCa controlled in the range 7.0 to 5.0.

Tetracaine displayed a complex effect on channel kinetics. In channels activated by 1 mM ATP (pCa 6 or 5), tetracaine further increased fractional open time (Po) from 0.56 to 1 in a concentration dependent manner (0.25 to 4 mM). with no change in conductance. Mean duration of open events increased and that of closed events decreased. In channels activated by caffeine (5 or 10 mM) at pCa 7, tetracaine (0.05 to 1 mM) decreased Po from 0.8 to 0.3. These results suggest that a) there are at least two types of high conductance channels with different pharmacological profiles or b) there is a single population of channels with different sensitivities to tetracaine depending on the activation modes and the tetracaine concentration. Supported by FONDECYT 902 and 972, and by NIH GM 35981.

Tu-Pos452

LOCALIZATION OF PROTEIN KINASE C IN JUNCTIONAL TRANSVERSE TUBULE MEMBRANES.
G. Salviati, B. Cunningham, C. Jachec-Schmidt, J-J, Kang, A.S. Dahms and R. Sabbadini. Univ. of Padova and Molecular Biology Institute, San Diego State Univ. 92182

Junctional transverse tubular (JTT) membranes were prepared from rabbit and chicken skeletal muscle according the the procedures of Horgan and Kuypers (ABB 253:377, 1987) and compared to longitudinal SR (LSR), isolated junctional SR (JSR or R4 fraction of Saito et al. JBC99:875,1984) and TT prepared by several methods. Western immunoblots of the various membrane fractions were probed with a monoclonal antibody to the 79 kDa subunit of protein kinase C. PKC was most concentrated in isolated JTT fractions followed by very light staining in the sarcolemma preparations and in isolated triads. Immunoreactive PKC was not present in either junctional or non-junctional SR fractions. The effects of TPA and other modifiers of PKC activity upon caffeine-induced Ca release from both chemically skinned muscle fibers and isolated SR terminal cisternae from the rabbit were tested. At 0.2 uM TPA, potentiation of caffeine-induced Ca release was observed, while at 4 uM it significantly inhibited Ca release. Caffeine-induced Ca release from Ca-loaded JSR was only slightly inhibited by TPA in the 0.2-0.4 uM range but was significantly inhibited in the 2-4 uM range. The responses seen at high TPA could be physiological or a cmc effect. These results are related to our recent observation that low concentrations of phorbol esters and diacylglycerols (DAG) can significantly inhibit the TT Mg-ATPase, an effect which may be independent of PKC activation. The Mg-ATPase is also located in junctional transverse tubular membranes, suggesting that both the ATPase and PKC of the junction serve as physiological DAG receptors. This work was supported by the NSF, the California Metabolic Research Foundation and the MDA.

Tu-Pos453

RAPID, REVERSIBLE FATIGUE IN STIMULATED DIAPHRAGM: INFLUENCE OF THEOPHYLLINE. RC Kolbeck, TM Nosek, & WA Speir. Depts of Med & Physiol/ Endo, Med Coll Georgia, Augusta, GA 30912.

The time course of fatigue was measured in isolated, intact, perfused rat diaphragms stimulated for 10 min with 20Hz train-pulses at a frequency of 1.5Hz. At 50% of duty cycle, each 333 mSec train delivered 6 pulses. Two patterns of fatigue were noted. First, there was a rapid, reversible, loss of tension during each duty cycle: the 6th contraction was ~99% less than the 1st. Second, the contractions showed a slow, progressive fall in tension throughout the 10 min stimulus period. Theophylline did not alter the two patterns of fatigue; however, at 10^{-4}M, it increased the force of all contractions during each duty cycle for the entire period of stimulation. To our knowledge, the first pattern observed, that of rapid reversible fatigue in response to train-pulse stimulation, has not been previously reported. (Am Lung Assoc, Ga Affiliate)

Tu-Pos454

THE SPATIAL DISTRIBUTION OF INTRACELLULAR CA^{2+} AND THE POSSIBLE ROLE OF ORGANELLE BUFFERING IN CULTURED NEONATAL RAT VENTRICULAR MYOCYTES. Mei-Jie Jou and Shey-Shing Sheu, Dept. Pharmacology, Univ. Rochester, Rochester, NY 14642.

This study demonstrates developmental changes in the spatial distribution of intracellular Ca^{2+} ($[Ca^{2+}]_i$) determined via fluorescence digital imaging microscopy in fura-2 loaded ventricular myocytes cultured from 1-day-old newborn rats. In 2-day-old cultured myocytes, the Ca^{2+} images showed a high $[Ca^{2+}]$ that correlated with the nuclear location. In 5-day-old myocytes, a ring-like region of high Ca^{2+} appeared around the nuclear envelope. After two weeks, the contrast in $[Ca^{2+}]$ between the nucleus and cytosol decreased, however, the high $[Ca^{2+}]$ in the perinuclear region still remained. Furthermore, there was a lattice-like network of elevated $[Ca^{2+}]_i$ in the perinuclear region which extended to the cell periphery. Caffeine, 10 mM, induced a transient increase in $[Ca^{2+}]_i$, however did not diminish the high Ca^{2+} network nor the perinuclear Ca^{2+} ring suggesting that the high Ca^{2+} areas are not caffeine-sensitive sarcoplasmic reticulum. Rhodamine 123 images indicated that this network was spatially associated with the mitochondrial location. Treatment of cells with the protonophore, CCCP, reduced the contrast between the high $[Ca^{2+}]_i$ areas and the cytosol. Finally, the contrast of Ca^{2+} gradients remained during spontaneous contractions or subsequent to 50 mM KCl-induced increases in cytosolic calcium. These results suggest that the high $[Ca^{2+}]_i$ areas may act as a calcium buffer during elevations of cytosolic calcium.

Tu-Pos455

DIRECT CONTRIBUTION OF ELECTROGENIC Na-Ca EXCHANGE TOWARD DEVELOPED TENSION IN MAMMALIAN VENTRICLE. R. A. Bouchard and D. Bose. Department of Pharmacology & Therapeutics, University of Manitoba, Winnipeg, Canada.

Voltage-clamp studies have shown that membrane depolarization can increase cytosolic Ca directly by inhibiting the Ca efflux mode of electrogenic Na-Ca exchange and perhaps by facilitating Ca entry through a reverse mode of the exchanger. This work was undertaken to determine whether changes in the driving force for the exchange process similarly affects SR Ca loading and force of contraction following changes in stimulus interval which were designed to increase the time spent by the muscle in a depolarized state. Total SR Ca content was estimated with rapid cooling contractures (RCC) and the fractional release of this Ca during stimulation assayed by the magnitude of contraction. In normal $[Na]_o$, increasing the rate of stimulation from 0.5 through 1.5 Hz resulted in a positive force staircase in ten canine ventricular trabeculae. This was accompanied by a smaller increase in steady state RCC and postrest contraction, indicative of the change in SR Ca loading. Reduction of $[Na]_o$ from 140 to 105 mM by equimolar substitution with LiCl led to a 200% increase in both twitch and RCC magnitude, taking ~20 min to reach the steady state. Increasing the rate of stimulation resulted in a marked reduction in contraction amplitude, which was accompanied by a slight increase in the amplitude of postrest contractions, while little effect on steady state RCC was observed at the different frequencies. The results suggest that electrogenic Na-Ca exchange might be responsible for a significant component of beat-dependent changes in contraction amplitude which may occur independent of SR Ca loading and peak I_{si}. (Supported by the Canadian Heart Foundation).

Tu-Pos456

MYOPLASMIC CALCIUM TRANSIENTS IN INTACT FROG TWITCH FIBERS MONITORED WITH FURAPTRA (=MAG-FURA-2) AND PURPURATE-DIACETIC-ACID (=PDAA). M. Konishi, S. Hollingworth and S.M. Baylor. Dept. of Physiology, Univ. of Pennsylvania.

Our in vitro calibrations confirm that Mag-fura-2 (Molecular Probes Inc.; cf. Raju et al., Amer. J. Physiol. 256, C540, 1989) and PDAA (a gift of Dr. W. K. Chandler; cf. Hirota et al., J. Gen. Physiol. 92, 1989) react with Ca^{2+} with 1:1 stoichiometry (K_D's of 44 μM and 870 μM, respectively; 16°C). In stretched, dye-injected fibers, changes in indicator fluorescence and/or absorbance were measured at 16°C in response to action potential stimulation. With both indicators, $\Delta[Ca^{2+}]$, the change in myoplasmic free Ca^{2+} inferred from the optical signal, was found to have an essentially identical time course (time to peak, 6-7 ms; half-width, 8-9 ms), in close agreement with the brief waveform reported in cut muscle fibers (Hirota et al. 1989). The calibrated peak amplitude of $\Delta[Ca^{2+}]$, however, differed by about a factor of two for the indicators (4-5 μM, Mag-fura-2; 8-11 μM, PDAA). Several observations, including the relatively small fraction of PDAA estimated to be bound to myoplasmic constituents (ca. 35%), suggest that peak $\Delta[Ca^{2+}]$ in intact muscle fibers may be about 10 μM or slightly larger.

Tu-Pos457

DEPOLARIZATION-INDUCED Ca^2 RELEASE MEASURED WITH FURA-2 IN ISOLATED TRIADS FROM RABBIT SKELETAL MUSCLE. **A.M. Corbett, J.H. Bian, J.B. Wade and M.F. Schneider. Univ. of Maryland, Baltimore, Md 21201.**

Depolarization-induced Ca^2 release was studied in a triad/terminal cisternae mixture isolated from rabbit skeletal muscle. These vesicles were actively loaded with multiple Ca additions, in the absence of precipitating anions, in a solution containing 100 mM K propionate, 2 mM MgCl, 36 mM imidazole, 2 mM NaATP and an ATP regenerating system. Both loading and release solutions contained 10 uM impermeant Fura-2 and free Ca was calculated to be in the nanomolar range at the time of release. Ca release was initiated by diluting an aliquot of the loaded vesicles into depolarizing TEACl release solutions which maintained a constant KCl product. Fast release was defined as the percent of the total Ca loaded which released immediately after dilution (less than 10 sec). Fast release was unaffected by 5 mM caffeine and increasing Mg concentration from 0.1 mM to 0.2 mM. Thus, the Ca release measured in these studies may be different from Ca-induced Ca release. The fast release more than doubled when a greater dilution (1:20 versus 1:10) of the loaded vesicles into the release solution, which would produce a larger depolarization, was used. Dilution of loaded light sarcoplasmic reticulum vesicles into the release solution produced no fast release. The percentage of loaded Ca which released fast in a particular triad preparation was similar to the percentage of vesicles structurally coupled as visualized by electron microscopy.

Tu-Pos458

CALCIUM WAVES IN CARDIAC MYOCYTES VIEWED WITH CONFOCAL MICROSCOPY D.A. Williams, L.M. Delbridge, S.H. Cody, P.J. Harris & T.O. Morgan, Dept. of Physiol., Univ. of Melbourne, Victoria, Australia.

Laser-scanning confocal microscopy has been used with fluo-3, a fluorescent, visible wavelength Ca^{2+} probe, to visualise Ca^{2+}-dynamics during spontaneous contractility of isolated rat cardiac myocytes. Intracellular [fluo-3] of 50 to 80μM did not affect stimulated isotonic shortening in single cells. Some cells (10-35%) showed spontaneous contractility at $[Ca^{2+}]$ of 1.0-2.5mM. Underlying each contraction was a wave (50-150μm/sec) of elevated Ca^{2+} (1.0-1.5μM). Ca^{2+} waves initiated concomitantly at cell ends did not propagate past their point of intersection. Waves were abolished by caffeine (10mM) confirming the SR as the primary Ca^{2+} source. A series of waves often induced a global (2-6msec) Ca^{2+}-release ('flash'), presumably through a progressive Ca^{2+}-induced cell depolarisation. Ca^{2+} kinetics (timecourse and levels) during spontaneous waves and flashes were similar, indicating that Ca^{2+} influx may play a triggering rather than a direct activation role in the Excitation-Contraction process of rat cardiac muscle. *Supported by the National Heart Foundation of Australia.*

Tu-Pos459

OPTICAL PROBE ANALYSIS OF CHARGE MOVEMENT IN SKELETAL MUSCLE. J. A. Heiny and D. Jong. Dept. of Physiology & Biophysics, Univ. of Cincinnati, Cincinnati, OH.

The passive electrical behavior of the T-system was examined in the voltage-region where charge movement is elicited. Charge movement currents and T-system voltage-transients were recorded from single voltage-clamped muscle fibers stained with a potentiometric dye. They were compared with a radial cable model of the T-system which included a voltage-dependent capacitance with parameters matching those of the charge movement. The presence of a non-linear capacitance has two effects on the T-system voltage-transients. At every potential where charge movement appears, the T-system takes longer to reach a constant voltage than at the equivalent hyperpolarizing potential. Additionally, the time course of the T-system voltage transient is voltage-dependent and parallels the kinetics of charge movement, being slowest near the mechanical threshold and more rapid at positive potentials. These results are consistent with theoretical calculations of the expected electrical behavior of the T-system under these conditions. The T-system optical transients provide an independent verification of the tubular location of charge movement, and provide a method for analyzing the kinetic parameters of charge movement from its effects on the T-system voltage transient. (Supported by the NSF and AHA)

Tu-Pos461

Ca^{2+} RELEASE BY IPS_3 IN TRIADS OF RABBIT SKELETAL MUSCLE. C. Valdivia, H.H. Valdivia, B.V.L. Potter*, and R. Coronado. Department of Physiology, University of Wisconsin-Madison; and *Department of Chemistry, University of Leicester, U.K.

The effectiveness of IP_3 and that of the non-metabolizable analog, DL-myo-inositol 1,4,5-trisphosphorothioate (IPS_3) synthesized by Cooke et al, (J. Chem. Soc. Chem. Comm 1987, 1525) was examined in triads purified from rabbit skeletal muscle. In a Ca^{2+} electrode assay, 5 μM to 25 μM IP_3 or IPS_3, consistently released, 5% to 20% of the Ca^{2+} pool actively loaded into triads. In recordings of triads fused to planar bilayers, open probability of ryanodine receptor Ca^{2+} release channels were not significantly different in the presence or absence of 30 μM IPS_3. IPS_3 increased slightly the activity of release channels open at pCa~5 (P_o=0.16 $\pm$ 0.03 in control; P_o=0.2 $\pm$ 0.03 in 30 μM IPS_3) but failed to open release channels held closed by physiological resting levels of Ca^{2+} (pCa~7). This suggests that IP_3 mobilizes SR Ca^{2+} by a mechanism which is independent of the activity of Ca^{2+} release channels. Supported by NIH, AHA, and MDA.

Tu-Pos460

VOLTAGE-DEPENDENCE OF $INSP_3$-INDUCED CA^{2+} RELEASE IN NORMAL AND MALIGNANT HYPERTHERMIC PORCINE PEELED SKELETAL MUSCLE FIBERS. Sue K. Donaldson, Esther M. Gallant, and Daniel A. Huetteman. University of Minnesota, Minneapolis, MN 55455

The sensitivity of the sarcoplasmic reticulum (SR) to inositol trisphosphate ($InsP_3$) as a stimulus for Ca^{2+} release has been shown to be dependent upon voltage of the transverse tubules (TTs) in the peeled (sarcolemma mechanically removed) skeletal fiber preparation (PNAS, USA, 85:5749-5753, 1988). In this study we demonstrate the same voltage dependence in peeled porcine fibers. The magnitude of the IP_3-induced SR Ca^{2+} release is significantly larger as under conditions for depolarized as compared to polarized sealed TTs in the peeled fibers. There are no statistically significant differences between porcine and rabbit or between MH and normal porcine peeled fibers in IP_3-induced SR Ca^{2+} releases. Thus the voltage-dependence of SR-sensitivity to IP_3 is a characteristic of peeled skeletal fibers in general but is unlikely to be part of the defect responsible for MH abnormalities. Supported by grants from MDA and NIH (AR35132).

Tu-Pos462

DIRECT BINDING OF VERAPAMIL TO THE RYANODINE RECEPTOR Ca^{2+} RELEASE CHANNEL OF SARCOPLASMIC RETICULUM. H. H. Valdivia, C. Valdivia, and R. Coronado. Department of Physiology, University of Wisconsin, Madison WI 53706.

In isolated triads of rabbit skeletal muscle, verapamil decreased binding of [^{3}H]Ryanodine with an IC_{50} ~8 μM at an optimal pH 8.5 and pCa 4.3. Nitrendipine and d-cis-diltiazem, did not interfere with binding of [^{3}H]Ryanodine suggesting that the action of verapamil does not involve the dihydropyridine receptor. Single channel recordings showed that verapamil blocked Ca^{2+} release channels by decreasing open probability, duration of open events, and number of events per unit time. A direct interaction of verapamil with the purified ~400 kDa ryanodine receptor peptide was demonstrated. Verapamil and D600 decreased [^{3}H]Ryanodine binding to the purified receptor non-competitively, by reducing the B_{max} only. Verapamil blockade of Ca^{2+} release channels may account for some of the paralysing effects of phenylalkylamines observed during excitation-contraction coupling of skeletal muscle. Supported by NIH, MDA, and AHA.

Tu-Pos463

INTRACELLULAR SODIUM INFLUENCES THE VOLTAGE - CONTRACTION RELATIONSHIP IN FELINE VENTRICULOCYTES. H. Brad Nuss and Steven R. Houser, Dept. of Physiology, Temple Univ. Sch. of Med. Phila. PA

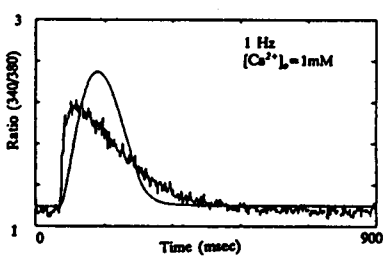
The voltage dependence of myocyte contraction was studied to examine the potential role of the Na/Ca exchanger in the regulation of contraction (C). To minimize or promote Na/Ca exchange mediated Ca entry following depolarization, pipettes (2-4 megohms) contained either 0 or 20 mM Na. Cells were voltage clamped from -40 mV to test potentials (TP) between -35 and +40 mV to induce I_{Ca} and C. In all cells, C increased with depolarization and reached a maximum at +10 mV. C at more positive TP remained at this maximal level in cells studied with 20 mM Na in pipettes but decreased to 63% of the maximal level in those with 0 mM Na. The voltage dependence of I_{Ca} was similar in all cells. These findings support the idea that Na/Ca exchange contributes to the normal Ca^{2+} transient at plateau potentials.

Tu-Pos465

CALCIUM MEASUREMENTS IN RAT CARDIAC TRABECULAE USING FURA-2

P.H. Backx, C. Lamont and H.E.D.J. ter Keurs, U. of Calgary, Calgary, Canada.

Calcium dependent fluorescence (CaDF) from FURA-2 AM loaded cardiac trabeculae decreases to 20-30% after skinning by saponin. CaDF, then, tracks [Ca] in the solution if A 23187 is present; it is inhibited by ruthenium red (10 μM) and disappears at $[Mn^{++}]$=10mM or in 1% Triton X100. This suggests significant loading of mitochondria with FURA-2. Mitochondrial CaDF, in intact muscles, varied with the time-averaged cytosolic $[Ca^{++}]$, thereby confounding the interpretation of diastolic CaDF. Hence, we loaded Fura-2 salt, by iontophoresis, into rat cardiac trabeculae using a microelectrode; a single impalement was needed. The Fura-2 spread uniformly throughout the muscles, during stimulation at 1Hz for 1 hour. Twitch force was, then, reduced by 50%, indicating significant calcium buffering. Diastolic $[Ca^{++}]$ was 100±15nM; $[Ca^{++}]$ during the twitch reached 2μM maximally. Typical recordings (cf the figure; twitch $[Ca^{++}]$ = 700nM) suggest, that adequate signal to noise ratio of CaDF during single twitches can be achieved with 10x less loading.



Tu-Pos464

ROLE OF CALCIUM CURRENT IN HYPOTHERMIC INOTROPY IN MYOCYTES ISOLATED FROM RABBIT VENTRICLES. G. Maurice Briggs and Donald M. Bers, Biomedical Sciences Div., University of Ca, Riverside, CA 92521

Cooling cardiac muscle from 37 to 24°C leads to a 5 fold increase in twitch force. Previous studies have indicated that cooling decreases myofilament Ca sensitivity (*J Gen Physiol.* 93:411-428) and that hypothermic inotropy persists after inhibition of the sarcoplasmic reticulum (*Circulation Res.* 61:761-771). In this study we investigated whether Ca entry via Ca current (I_{Ca}) could account for the increased inotropy utilizing solutions which help isolate I_{Ca}. Myocytes isolated from rabbit ventricle were superfused with (mM) TEA-Cl 140, CsCl 6, $MgCl_2$ 1, $CaCl_2$ 2, Glucose 10, HEPES 10. The pH was adjusted to 7.4 for each temperature (24, 30 & 37°C). Whole cell voltage clamp was accomplished with electrodes (0.5 - 1 MΩ) filled with (mM) CsCl 125, MgATP 5, HEPES 5, EGTA 10, pH 7.1. To assess the Ca dependent current, clamp protocols were repeated with Ca replaced by Mg, Co or Mn. This current was subsequently subtracted from the original current. Cooling reduced both peak I_{Ca} and the rate of I_{Ca} inactivation for test potentials (V_t=-40 to +50 mV, from V_h=-80, n=19). The integral of the I_{Ca} was unchanged or slightly reduced by cooling, for pulse durations from 180 to 400 msec (n=14). These results lead us to conclude that neither changes in the peak I_{Ca} nor the integrated Ca entry via I_{Ca} are likely to account for the observed hypothermic inotropy.

Tu-Pos466

PHOSPHOLIPASE C ACTIVITY OF ISOLATED TRANSVERSE TUBULE FROM RABBIT SKELETAL MUSCLE

G. Salviati and R. Betto
CNR Unit for Muscle Biology and Physiopathology, University of Padova. Padova, Italy.

We have previously shown that isolated TT membranes from rabbit muscle contain phospholipase C activity. In this work we carried out a further characterization of this enzyme. The PIP_2-PDE activity is membrane bound; 0.6M KCl washing does not extract appreciable amounts of activity and the pH-dependence of the soluble and the membrane bound activities is different. The TT membrane enzyme requires Ca^{2+}, being fully activated at 10^{-7}M Ca^{2+}, and is not inhibited by Mg^{2+}. The activity is stimulated by NaF (50% of activation is achieved at 1mM NaF) and by 250uM GTP-γ-S, suggesting that it is controlled by a G protein. These results indicate that TT membrane contains, in addition to the cAMP generating system, the signal transduction system generating IP_3 that may have a role in the EC coupling.

Work supported by institutional funds from CNR, MPI, and MDA.

Tu-Poe467

pH IN CUT FROG MUSCLE FIBERS. P. C. Pape.
Department of Cellular and Molecular Physiology, Yale School of Medicine, New Haven, CT 06510.

Single fibers were mounted in a double Vaseline-gap chamber and, at time = 0, 650 μM phenol red was added to the end pools. The apparent intracellular pH (pH_{app}) was estimated from measurements of dye absorbance at 570 and 480 nm. With an internal solution containing 5 mM glucose (Irving et al., 1987, J. Gen. Physiol., 80, 1-40), pH_{app} decreased from 6 9 ± 0.1 (mean ± SEM) at 10 min to an approximately steady state level of 6.5 ± 0.1 at 90 min (N=5). This latter value of pH, if real, indicates a large decrease in pH from the end pools to the center of the fiber, perhaps due to a high rate of acid production. A less acidic steady state level of 6.9 ± 0.2 pH units (N=9) was reached if glucose was not present. A possible explanation for this difference is that glygogen metabolism produces fewer protons than glucose metabolism. Measurements of pH_{app} were calibrated by controlling the internal pH by the pH of the external solution (pH_{ext}) in the presence of 10 μM nigericin (a H^+/K^+ exchanger), 120 mM $[K]_{ext}$ and zero membrane potential. Changes in pH_{ext} produced approximately equal changes in pH_{app}, indicating that phenol red reliably monitors differences in internal pH. The absolute level of pH_{app}, however, was 0.1-0.4 pH units more acidic than pH_{ext}. Thus, the pK of phenol red inside the fiber may be 0.1-0.4 greater than that in a calibration solution.

Tu-Pos468

AN α_2 DIMER OF KINESIN WITH ELEVATED MICROTUBULE-STIMULATED ATPase ACTIVITY. D.D. Hackney and D.D. Wagner, Depart. Biol. Sci., Carnegie Mellon Univ., Pittsburgh, PA.

Bovine brain kinesin contains a variable amount of a component which sediments in a sucrose gradient at an apparent $s_{20,w}$ value of 6.7S, in addition to the $\alpha_2\beta_2$ tetramer which sediments at 9.3S. This second peak at 6.7S is observed when the fractions are analyzed either for microtubule(MT)-stimulated ATPase activity, tightly bound [α-^{32}P]ADP, or the presence of the 120 kDa α subunit. The 6.7S component lacks β subunits and is likely to be an α_2 dimer. In the absence of MTs both the tetramer and the dimer bind one mole of [α-^{32}P]ADP per α subunit and release this bound ADP at a similar rate. The turnover rate of the dimer in the presence of MTs, however, is elevated 3-5 fold over the turnover rate of the tetramer (per α subunit). The level of the 6.7S component is variable from batch to batch and its presence may at least partially account for the wide range of reported specific activities for kinesin.

Supported by NIH grant AR25980.

Tu-Pos469

PYRUVATE KINASE BINDING TO MICROTUBULES IS DEPENDENT ON THE ABSENCE OF PEP. D.D. Hackney, Depart. Biol. Sci., Carnegie Mellon Univ., Pittsburgh, PA. (Intro. by W.R. McClure.)

Rabbit muscle pyruvate kinase (PK) binds strongly to brain microtubules (MTs) in the absence of PEP as determined by cosedimentation. The binding is strongly inhibited by PEP, but only weakly inhibited by ATP or ADP. Mixing MTs with pyruvate kinase results in cross linking of the MTs as indicated by a large increase in light scattering. The increased light scattering is completely reversed within 1 sec by addition of submillimolar levels of PEP, but is insensitive to ATP or ADP. The cross-linked material appears amorphous when viewed by dark field microscopy, but needle shaped bundles of MTs over 20 microns in length can be formed by slow removal of PEP using a low level of hexokinase in the presence of glucose and ADP.

Supported by NIH grant AR25980.

Tu-Pos470

Q-BAND EPR STUDIES ON THE INTERACTION OF MN WITH THE E-SITE AND N-SITE OF αβ-TUBULIN. J.J. Correia and A.H. Beth*, Dept. of Biochem., Univ. of Miss. Medical Center, Jackson, MS 39216 and *Dept. of Mol. Phys. & Biophys., Vanderbilt Univ. School of Medicine, Nashville, TN 37232.

Previous studies have shown that GTP occupied E- and N-sites of the tubulin heterodimer are high affinity divalent cation binding sites ($4.5*10^7$ M^{-1} for Mn [II]). Q-band EPR (Brucker ESP300) spectra were collected on specimens of Mn-treated tubulin with GTP at the N-site and either GDP or GTP at the E-site to investigate the coordination geometry of these high affinity environments. Experiments were performed on samples of tubulin that were treated with Mn for various periods of time and gel filtered to remove excess Mn or with stoichiometric concentrations of Mn and GTP. The samples were used directly or concentrated and studied as pellets. The N-site spectra reflect octahedral symmetry with slight zero-field splitting, while the E-site is broadened, apparently reflecting its rapid exchangeability with free Mn. In frozen samples the spectra for the E- and N-site become identical, reflecting the similarity of these GTP binding sites. (Supported by NIH grants GM41117 and HL34737.)

Tu-Pos471

DIFFERENTIAL MICROTUBULE BINDING AND TRANSLOCATION BY INNER DYNEIN ARM COMPONENTS. *Elizabeth F. Smith and W. S. Sale*. Dept. of Anatomy & Cell Biology, Emory University, Atlanta GA 30322.

A variety of evidence has demonstrated there are three inner dynein arm isotypes which differ in organization and composition (Piperno et al.,1989 JCB 109:156a). Using Chlamydomonas outer armless mutant pf28 we have begun to define the function of different inner arm types. Inner arms and their corresponding heavy chains were efficiently extracted in 0.6M NaCl. Light and electron microscopy revealed that, in the absence of ATP, the dialyzed extract induced crosslinking of microtubules in parallel bundles. Of the five or six heavy chains in the extract, two, 1α and 1β which cosediment as a 21S particle in sucrose gradients, bound to microtubules in an ATP sensitve manner. In the dialyzed extract the remaining heavy chains, which sediment as a mixture of 11S particles, bound poorly to the microtubules. Both the 21S and 11S fractions supported microtubule gliding in in vitro motility assays. However, in 1mM MgATP, the 21S fraction produced slower sliding velocities (0.76um/sec ± 0.4) compared to the 11S fraction (4.15um/sec ± 1.0). These results provide additional evidence for the functional heterogeneity among inner dynein arm isotypes. Supported by NIH HD/GM 20497 and HD 00553.

Tu-Pos472

KINETICS OF E1P PHOSPHOENZYME OF Na,K-ATPase IN 0.5 M NITRATE. K. Suzuki & R. L. Post, Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, TN 37232-0615

High concentrations of lyotropic anions (e.g. ClO_4^-, SCN^-, I^-, NO_3^-) stabilize the E1P conformation or reactive state (Biophys. J. 53:138a, 55:337a). The E1P conformation donates its phosphate group easily to ADP and is insensitive to K^+. Na,K-ATPase from kidney by Jørgensen's method was phosphorylated at 0 °C from [^{32}P]ATP in 0.5 M $NaNO_3$ with 0.1 to 10 mM Mg^{2+}. The phosphoenzyme was chased with an excess of unlabeled ATP or by chelation of free Mg^{2+} with EDTA or CDTA. To eliminate K^+-sensitive states of the phosphoenzyme, 6 mM KNO_3 was included in the chase. Also ADP was included at various concentrations. ADP induced a diphasic dephosphorylation with time. An immediate splitting burst was followed by a slower phase. The amplitude of the burst and the slope of the slow phase were proportional. The kinetics is consistent with the Pickart-Jencks model for the Ca-ATPase of sarcoplasmic reticulum (J.Biol.Chem. 257:5319). Varying the concentration of free Mg^{2+} suggested that ATP· E about 2.5-fold more rapidly than from ATP· Mg· E. Replacement of Na^+ with organic cations suggested that ATP· E· Na ⟷ ADP· E1~P + Na^+. Thus, in this variety of E1P, Na appears not to be occluded. In contrast, the literature favors occlusion of Na in E1P stabilized by binding of oligomycin. Supported by grant HL-01974 from Heart, Lung and Blood Institute, NIH.

Tu-Pos473

EVIDENCE FOR BINDING OF ATP TO THE E2-CONFORMATION OF THE PLASMA MEMBRANE H^+-ATPase FROM NEUROSPORA. Georg Nagel[1], Irena Klodos[2], Jian-Chao Xu[1], and Clifford Slayman[1]. Cellular & Molecular Physiology[1], Yale University, New Haven, CT 06510; & Institute of Biophysics[2] DK 8000 Aarhus C.

Klodos & Forbush (J. Gen. Physiol. 92:46a 1988) discovered that the fluorescent dye RH-160 responds rapidly to conformational changes of the Na^+,K^+-ATPase. We tested this dye on the closely related plasma membrane H^+-ATPase of the fungus Neurospora and found similar responses (Biophys.J. 55:338a 1989).

The largest shift in the excitation spectrum of RH-160 was obtained upon addition of ortho-vanadate. We used the initial rate of vanadate-stimulated fluorescence shift (dF/dt) as a measure of the amount of enzyme in the E_2-state. dF/dt increased with increasing pH to a maximum at pH 8.4 (at zero ATP). The result suggests that H^+ favors the E_1-state, as does Na^+ in the case of the Na^+,K^+-ATPase.

ATP increased dF/dt with a K_m of ca.1 mM, but then inhibited with a somewhat larger K_I. Mg-ADP also inhibited the vanadate reaction (K_I= ca 0.3 mM). The data could be simulated by a simple 3-state model. The biphasic dependence of dF/dt upon ATP was most easily explained by binding of ATP to both major conformations (E_1 and E_2), a result which also explains the previously observed sigmoidal activation of ATP hydrolysis by ATP (B.J.Bowman & C.W.Slayman, 1979, JBC 254:2928-2934).

[Supported by Grant GM-15858 from N.I.G.M.S. and M12-7961 & M12-9089 from the Danish Medical Research Council.]

Tu-Pos474

^{1}H NMR DETERMINED STRUCTURAL FEATURES OF THE DICYCLOHEXYLCARBODIIMIDE-BINDING SUBUNIT OF THE E. COLI F_1F_0 ATPASE. Mark E. Girvin and Robert H. Fillingame, Department of Physiological Chemistry, University of Wisconsin, Madison, WI 53706. (Intro. by M. Sundaralingam).

Subunit c, the DCCD-binding subunit of the H^+ ATP synthase of E. coli, is a 79 residue protein which is thought to be inserted in the membrane as a hairpin of two alpha helices. We are studying the structure of the purified subunit dissolved in organic solvents. Biochemical and NMR studies of wild type and mutant subunit c proteins show that the solubilized protein in chloroform-methanol-water retains properties seen in the native complex, e.g. specific DCCD binding to Asp_{61}, and an interaction between the two postulated transmembrane helices. Using 2D ^{1}H NMR we have assigned the spin systems of 64 of the 79 amino acid side chains to residue type, and have made 28 sequence specific residue assignments. Four regions of secondary structure (alpha helix) have been observed, three of which correspond to regions of the protein labeled by the hydrophobic photolabel 3-trifluoromethyl-m-(iodophenyl)diazirine in the native membrane.

Tu-Pos475

THE H,K-ATPASE α,β HETERODIMER

E. Rabon, G.Sachs and K. Hall. UCLA and VAMC Los Angeles, CA 90073

Glutaraldehyde treatment of active $C_{12}E_8$ solubilized H,K-ATPase crosslinks the catalytic monomer at M_r = 94 kDa into particles migrating between 148-188 kDa in SDS polyacrylamide gels. The subunit composition of this particle was determined from the comparative distribution of FITC fluorescence and wheat germ agglutinin reactivity in control and crosslinked preparations. FITC labelling was confined exclusively to the catalytic monomer in control samples and was initially distributed into 2 bands migrating at M_r = 147 and 173 kDa with crosslinking. These fluorescent bands coincided with protein migration detected by coomassie blue stain. A glycoprotein(s), detected by wheat germ agglutinin reactivity, was present in a diffuse area between 65 and 86 kDa in the untreated ATPase preparation and showed UDP-gal incorporation into 2 diffuse bands at M_r 65 and 80 kDa. With crosslinking, the distribution of the WGA reactive protein coincided with crosslinked particles labelled by FITC. The simultaneous appearance of both the catalytic monomer and the WGA reactive glycoprotein in the crosslinked particles indicated that these proteins remain associated in the $C_{12}E_8$ solution. This suggests that the fundamental structural particle of the H,K-ATPase is an α,β heterodimer.

Tu-Pos476

MELITTIN BINDING DOMAIN ON THE $(H^+{+}K^+)$ATPASE: EVIDENCE FOR A POLYPEPTIDE BINDING DOMAIN ON ION PUMPS. John Cuppoletti, Dept. Physiology and Biophysics, Univ. Cincinnati, College Medicine, Cincinnati, OH 45267-0576

The catalytic and transport functions of the gastric $(H^+{+}K^+)$ATPase are potently inhibited by the bee venom toxin melittin. A radioactive photoaffinity label, [^{125}I]azidosalicylyl melittin, labels the 95kDa $(H^+{+}K^+)$ATPase suggesting that the $(H^+{+}K^+)$ATPase contains a polypeptide binding site (ABB, In Press). Controlled tryptic hydrolysis of the labelled $(H^+{+}K^+)$ATPase was carried out to localize the melittin (polypeptide) binding domain. In the presence of KCl (E_2 form) trypsinolysis leads to accumulation of 56kDa and 42kDa fragments. The 56kDa fragment was not labelled, whereas the 42kDa fragment was labelled. The 42kDa fragment (which contains the aspartyl residue responsible for phosphoenzyme formation and the cation discriminating domains), also retains sufficient structure to bind melittin even after tryptic cleavage of the $(H^+{+}K^+)$ATPase. Another peptide of similar structure but dissimilar sequence was also inhibitory. A variety of other ion pumps also contain a similar polypeptide binding domain. This site may represent a polypeptide binding site for regulation of the activity and/or distribution of ion pumps.

Supported by DK38808.

Tu-Pos477

STUDIES ON F_1 WITH BzAF, A BIFUNCTIONAL FLUORESCENT PHOTOAFFINITY PROBE FOR ENZYME NUCLEOTIDE BINDING SITES. Pranab K. Pal and Peter S. Coleman, Lab. of Biochemistry, Dept. of Biology, New York University, NY, NY 10003

4-Benzoyl(Benzoyl)-1-Amino-Fluorescein (BzAF) was designed and synthesized as a structural analog of adenine nucleotides that would potentially possess both site-specific covalent photoliganding and environmentally sensitive fluorescent properties. With the complex F_1 (beef heart) ATPase the new probe may provide very useful mechanistic information, particularly regarding subunit conformational adjustments that have been assumed (but never accurately monitored) to occur upon multiple AdN ligand binding to the enzyme. Consonant with our previous studies using BzATP and BzADP, reaction of BzAF with F_1 exhibits pseudo-first order photoinhibition kinetics. Photoinactivation displayed rate-saturation, indicating that F_1 binds BzAF (K_I = 5.9 x 10^{-5} M) prior to irreversible covalent modification. Both Mg^{2+}-ADP and ATP provide protection against BzAF photoinactivation ($K_{protect}$ = 4.7 x 10^{-4} M for Mg^{2+}-ADP). In the absence of photolysis, BzAF inhibits ATPase reversibly and competitively vs. ATP (K_I = 5.2 x 10^{-5} M). Utilizing [^{3}H]BzAF (with the ^{3}H on the benzophenone moiety), we find a covalent binding stoichiometry of 1 mole probe bound/mole F_1 for complete inactivation, suggesting that the covalent labeling is extremely site specific. This permits us to expect that upon such unimolar covalent BzAF binding, the F_1 remains capable of further (at least one other) AdN site occupancy. Site-site cooperative conformational changes via the filling of additional AdN binding sites on F_1 may be monitored via BzAF fluorescence. (NIH GM 36619 to PSC).

Tu-Pos478

$(Na^+{+}K^+)$-ATPase: REGULATION OF PHOSPHORYLATION BY THE N-TERMINAL DOMAIN. Mehdi Ganjeizadeh, W.-H. Huang, and Amir Askari, Department of Pharmacology, Medical College of Ohio, Toledo, Ohio 43699-0008.

Chymotryptic cleavage of the α-subunit of the kidney enzyme yields an 83 kDa peptide, from Ala 267 to the C-terminus, that is phosphorylated by MgATP but not by MgPi. Phosphorylation of this peptide does not require Na^+, is not inhibited by K^+, and is stimulated by ouabain. The phosphopeptide is sensitive to ADP but not to K^+, and is more stable ($t_{0.5}$=8 min; 0°C; pH 7) than the native EP. The peptide also catalyzes a ouabain-stimulated ADP-ATP exchange in the absence of Na^+ and K^+. Further cleavage of this peptide by trypsin yields an 18 kDa peptide (Ala 267-Arg 438) whose phosphorylation-dephosphorylation properties are similar to those of the 83 kDa peptide. Thus, detachment of the N-terminal domain (a) yields a species that is neither E_1 nor E_2; (b) reverses the nature of ouabain effect on phosphorylation; and (c) removes Na^+ and K^+ regulation of phosphorylation-dephosphorylation (Supported by NIH grant HL-36573).

Tu-Pos479

$(Na^+{+}K^+)$-ATPase: CONTROL OF Rb^+ DEOCCLUSION AT 0°C. W.-H. Huang and A. Askari, Dept. of Pharmacol., Med. Coll. of Ohio, Toledo, OH 43699-0008.

Occluded K^+ (or Rb^+) is assumed to be an intermediate of the reaction cycle of the enzyme. While most studies on the reaction mechanism have been done at 0°C, nearly all studies on occluded Rb^+ have been done at 20°C. Our examination of the regulation of Rb^+ deocclusion at 0°C revealed the following: 1. In a medium of low ionic strength, occluded $^{86}Rb^+$ (about 2Rb^+/EP) was released slowly (k=0.004 min^{-1}) from the purified canine kidney enzyme. 2. Neither ATP nor free Rb^+ affected deocclusion rate; however, combination of the two accelerated the release (k=0.35 min^{-1}) of 50-80% of $^{86}Rb^+$ ($K_{0.5}$ of ATP, 30-50 μM; $K_{0.5}$ of Rb^+, 3-5 mM). 3. Pi+Mg^{2+} caused rapid release (in less than 10 s) of (a) 1/2 of $^{86}Rb^+$ in the absence of free Rb^+; and (b) 1/4 of $^{86}Rb^+$ in the presence of free Rb^+. These properties of occluded Rb^+, which are different from those previously observed at 20°C, indicate the existence of ligand-induced cooperativities (both positive and negative) among the multiple Rb^+ sites of the oligomeric enzyme (Supported by NIH Grant HL36573).

Tu-Pos480

INSULIN ACTIONS ON THE PROPERTY OF NA,K-PUMP. Yoshinori Marunaka Dept. of Physiol., Emory Univ. Sch. of Med., Atlanta, Georgia 30322.

Effects of $[Na]_i$ and $[K]_o$ on the insulin-stimulated and basal Na,K-pump have been reported (Marunaka, Y., J. Membrane Biology 91:165-172 (1986); Comp. Biochem. Physiol. 86A:133-136 (1987); J. Membrane Biology 101:19-31 (1988)). In the present study, I investigated the effect of insulin on the property of Na,K-pump. Insulin decreased the dependency of the ouabain-sensitive Na efflux on Na_i and increased the dependency of ouabain-sensitive potential on Na_i without any significant change in the dependency on K_o. Insulin had no significant effect on the membrane resistance at any $[Na]_i$ or $[K]_o$. These observations suggest that insulin increases the dependency of the Na/K coupling of the pump on $[Na]_i$ and had no significant effect on the dependency of the Na/K coupling on $[K]_o$. Insulin didn't affect the action of ouabain on Na efflux. This work was supported by a grant from the National Kidney Foundation to Y. Marunaka.

Tu-Pos481

Na,K-ATPase TOPOGRAPHY: EXTRACELLULAR CLEAVAGE SITES. K-Y. Xu and K.J. Sweadner (Intro. J.W. Peterson) Harvard Medical School and Mass. General Hospital, Boston, MA.

Extracellular tryptic cleavage sites were sought to help define the transmembrane folding of the alpha subunit of the Na,K-ATPase, a protein thought to pass the bilayer 6-8 times. Past work indicated that the extracellular surface was very resistant to cleavage, while three intracellular cleavage sites were known. Right-side-out vesicles (85% sealed to ATP) were prepared from rat renal medulla. The alpha subunit and its fragments were visualized by antibody stain of Western blots. Low trypsin levels (used in the past to see selective intracellular sites) cleaved only in the presence of saponin; extracellular sites were resistant. At higher trypsin concentrations, however, cleavage in intact vesicles could be seen. Cleavage was rapid (less than 5 min), but the fragments generated were stable to further cleavage (up to 30 min). 5-7 fragments (65-25K) stained with an antibody to the N-terminus, indicating at least 5 cleavage sites. Another fragment (70K) was stained by an antibody to the C-terminus. None of the fragments corresponded to those generated by cleavage of open membrane fragments. The rate of cleavage at the intracellular sites is known to differ when Na^+ or K^+ is present; in contrast, cleavage at the extracellular sites was the same in either Na^+ or K^+. Raising the ionic strength markedly increased cleavage, however, and a fragment of 25K became the major product stained for the N-terminus. It is hoped that sequencing of the N-termini of some of the fragments will define extracellular tryptic cleavage sites.

Tu-Pos482

THE SODIUM PUMP HAS VARIABLE CAPACITANCE

Luca Turin and Philippe Béhé(1)
Unité Neurobiologie Cellulaire [U261 INSERM], Institut Pasteur, 75724 Paris Cedex 15, France
(1) Physiologisches Institut, Universitat Homburg, Saar,FRG

Using two electrode voltage-clamp and lock-in detection [f=8Hz, ΔV=7 mV], we have measured the membrane capacitance of isolated Xenopus laevis blastomeres which possess a low and linear membrane conductance and a large [100-800 pA] steady-state sodium pump current. When the Na pump is blocked in K-free external solution, membrane capacitance rises. This rise in capacitance is proportional [0.24 pF/pA] to pump current, which varies from cell to cell. When the pump is inhibited with 10 µM ouabain, capacitance falls, also in proportion to pump current, but only by 0.17 pF/pA. ΔC is voltage-independent in the 0 to -150 mV range. Calculation of the *capacitance change per pump*, assuming a 3:2 Na/K stoichiometry and a turnover of 100/sec, yields a value of 3.8 attofarads (aF) /pump. We note that this capacitance change is large enough to store the 0.08 aJ supplied on average by the hydrolysis of a molecule of ATP under a reasonable voltage [less than 150 mV]. Therefore, it is suggested that the sodium pump stores ATP hydrolysis energy in electrostatic form, and that capacitance changes reflect the magnitude of stored energy: maximum in K-free medium (pumps blocked in conformation E1), minimum in ouabain (E2) and intermediate when the pump is running and converting it into ion translocation.

Tu-Pos483

INDIRECTLY COUPLED ACTIVITIES OF TONOPLAST H^+-ATPASE

Shu-I Tu, David Brauer, and Deidre L. Patterson, USDA-ARS-NAA, Eastern Regional Research Center, 600 E. Mermaid Lane, Wyndmoor, PA 19118

Modification of corn root tonoplast vesicles by fluorescamine (FL), caused a differential inhibition to the coupled activities of the H^+-ATPase. Within the range of 0 to 5 µmol of Fl per mg of protein, the proton pumping rate was significantly reduced more than that of ATP hydrolysis. FL treatment resulted in 1) a decrease of the number of primary amine groups, and 2) an implantation of multiple H-bonding moiety in the membrane. To test the possible effects of the latter, FL derivatives of benzylamine (Bz-FL), butylamine (Bu-FL), and phenylalanine (Phe-FL) of both acyclic and cyclic forms were used. Only the presence of the high H-bonding potential acyclic modifiers, at concentrations of 10 µM, inhibited the proton pumping rate by 50% without a significant effect to ATP hydrolysis rate. The results suggested that the proton pumping could be indirectly linked to ATP hydrolysis.

Tu-Pos484

Na/K PUMP CURRENT IN GUINEA PIG VENTRICULAR MYOCYTES IS LITTLE AFFECTED BY ACTIVATION OF ß-ADRENOCEPTORS OR BY INTRACELLULAR APPLICATION OF cAMP. Anthony Bahinski and David C. Gadsby. *Laboratory of Cardiac Physiology, The Rockefeller University, New York, NY 10021.*

Na/K pump current was determined at 36°C as strophanthidin-sensitive whole-cell current in ventricular myocytes internally-dialyzed via wide-tipped pipettes. Experimental solutions and holding potentials were selected to minimize K- and Na-channel currents and Na/Ca exchange current while strongly activating the Na/K pump (5.4 mM external K; 20-50 mM internal Na and 10 mM MgATP). The cAMP-dependent protein kinase pathway was activated with 0.1-1 µM isoproterenol or by switching to pipette solution containing 0.1-1 mM cAMP, and kinase activation was confirmed by observed increases in Cl^- current (steeply voltage-dependent outward current at positive potentials) and/or in Ca^{2+}-channel current (up to 5-fold). Although such current changes tended to interfere with Na/K pump current determination, in most cases Na/K pump current appeared to be unaltered, and in no case was it enhanced more than 20% at 0 mV. Similarly weak effects of 0.1 µM isoproterenol were observed even when the pump was less than maximally activated, e.g., using 10 mM internal Na, or 1.5 mM external K, and when interfering changes of Cl^- and Ca^{2+} currents were prevented. *(Supported by NIH HL-36783 and the Irma T. Hirschl Trust).*

Tu-Pos485

STABLE EXPRESSION OF THE MUTANT Na, K-ATPase. K.Takeyasu, A.Mizushima, D.B.Luckie, & K.L.Boyd. Department of Physiology, University of Virginia, Charlottesville, VA 22908. The Na,K-ATPase is a plasma membrane ion pump consisting of two heterologous subunits (~100 Kd catalytic α-subunit and ~45 Kd glycoprotein β-subunit) which are assembled co-translationally, and travel through the ER and Golgi apparatus as a complex. Recent analysis on the amino acid sequences deduced from cDNAs suggests that the 100 kd polypeptides may form a very similar higher order structure with ~8-10 membrane spanning domains to those of the other membrane ion-pumps such as the H,K-ATPase and the SR Ca-ATPase. We have constructed a series of mutant cDNAs encoding chicken Na,K-ATPase α-subunit. These mutants include partially deleted α-subunits, glycosylated α-subunits, and Na,K-/SR Ca-chimeric ATPase. The chimeric cDNAs were designed so that the overall topological structure of the encoding proteins as ion-pumps would be preserved. Mouse L cells were transfected with these mutant cDNAs, and the stable expression and subcellular distribution of the mutant proteins were studied with chicken specific monoclonal antibodies. These mutant Na,K-ATPases will be useful materials with which to identify specific domains responsible for (i) assembly with the β-subunit, (ii) ion-selectivity, (iii)topological orientation, and (iv) targeting to distinct subcellular membranes.

Tu-Pos486

MUTANTS IN SUBUNIT a OF THE E. COLI F_1F_0-ATPASE. Charles R. Paule and Robert H. Fillingame. Department of Physiological Chemistry, University of Wisconsin, Madison, Wisconsin 53706.

Mutants in subunit a of the E. coli F_1F_0-ATPase were isolated and characterized after mutagenesis of a plasmid carrying the subunit a (uncB) gene as described (Paule, C.R. and Fillingame, R.H. (1989) Arch Biochem. Biophys. 274, 270-284). Mutations that impair function without totally abolishing it lead to slow growth on succinate. These mutations, which include Asp-119→His, Ser-152→Phe, and Gly-197→Arg, do disrupt ATP-driven H^+ translocation by the F_1F_0 complex. Other partially defective mutants are being analyzed. Eighteen mutants that show complete loss of function have been generated by random mutagenesis. Fourteen of these were termination mutations, and the other four were missense mutations. The missense mutations include Arg-210→Gln, His-245→Tyr and Gly-213→Asp, the latter being found twice. A more extensive analysis of randomly generated mutants should provide more information on regions of the protein that are crucial to function.

Tu-Pos487

MODIFICATION OF Na, K-ATPase BY PALYTOXIN. S.Y. Kim, C.H. Wu, T. Narahashi, and L. Beress. Dept. of Pharmacol., Northwestern University Medical School, Chicago, IL

Palytoxin (PTX) is a marine toxin that has been shown to bind to the Na,K-ATPase and to depolarize excitable cells by forming nonselective cation channels within the cell membrane. We have found that PTX (100 nM) depolarizes the crayfish giant axon by 20 mV. This depolarization was antagonized by ouabain (10 µM). ATP (6mM) further depolarized the axon when it was internally applied after the PTX-induced depolarization had reached a steady level. These observations suggest that PTX binds to both the phosphorylated and unphosphorylated states of the sodium pump, and that phosphorylation promotes the conversion of the pump into an ion channel. The nature of the ion channel induced by PTX is being studied by patch clamp techniques on frog red blood cell membranes. PTX-induced single channels carrying inward Na currents were observed in cell-attached and excised patches. These channels exhibited prolonged openings and a conductance of about 10 pS. These results indicate that the depolarizing action of PTX may be due to the conversion of the sodium pump into an ion channel, and that the conversion occurs in the phosphorylated state of the pump.

Tu-Pos488

DISSOCIATION OF THE OAT TONOPLAST H^+-ATPASE WITH Mg-ATP AND ANIONS.

J. Ward and H. Sze, Department of Botany, University of Maryland, College Park, MD 20742.

The subunit composition of the peripheral sector of the oat root (*Avena sativa* L. var. Lang) tonoplast H^+-pumping ATPase was investigated. Incubation of tonoplast vesicles with Mg-ATP and anions resulted in dissociation and inactivation of the ATPase complex. Polypeptides of 72, 60, 44, and 36 kDa which reacted with polyclonal antibodies against the holoenzyme of tonoplast ATPase were solubilized by treatments which inactivated the ATPase. In the presence of Mg-ATP, the effectiveness of anions at 100 mM in inactivation of ATPase activity was $SCN^- > I^- > NO_3^- > Br^- > CH_3COO^- > SO_4^{2-} = Cl^-$. The binding of Mg-ATP at a site with a K_d of 34 uM lowered the concentration of anions required for maximum inhibition and dissociation which occurred more rapidly at 4C than at 20C. Proton-pumping driven by Mg-ATP was abolished by pretreatment with KI and Mg-ATP while PPi driven H^+-pumping was unaffected. The 44 and 36 kDa polypeptides appear to be additional peripheral subunits of the ATPase.

Tu-Pos489

ANTIBODIES TO THE γ SUBUNIT OF THE Na,K-ATPase.

Bliss Forbush III, Daniel Biemesderfer, Robert W. Mercer, and John H. Collins; with the assistance of John Barberia. Dept. of Cell. and Mol. Physiol. Yale Univ. School of Med., New Haven, CT, 06510, Dept. Cell Biol. and Physiol. Wash. Univ. School of Medicine, St. Louis, MO, 63110, and Dept. Biol. Chem. Univ. Md. School of Med., Baltimore, MD, 21201.

We have raised two rabbit antibodies to the putative γ subunit of Na,K-ATPase: one (Ab-gam) by immunization with protein eluted from the low molecular weight region of SDS gels of purified dog kidney Na,K-ATPase, and one (Ab-G17) by immunization with a 17 amino acid synthetic peptide (G17) prepared according to the sequence obtained by Collins and Leszyk (Biochem. 26, 8665, 1987) for sheep γ; for some purposes the antibodies were purified (Ab/p) by immunoabsorbtion to the γ subunit on nitrocellulose. By Elisa assay, both antibodies react with G17 (Ab-G17 at 1:30000 titer and Ab-gam at 1:1000), but only Ab-gam reacts detectably with dog kidney membranes. Both antibodies (Ab-gam 15% efficiency, Ab-G17 5% efficiency) immunoprecipitate solubilized [^{3}H]NAB-ouabain labeled γ subunit, and the precipitation is partially (Ab-gam) or completely (Ab-G17) blocked by co-incubation with G17. By immunofluorescence microscopy Ab/p-gam is found to label the basolateral membrane of the tubular epithelium in rat kidney. In the tricine SDS gel system of Schagger and von Jagow (Anal Biochem, 1987), [^{3}H]NAB-ouabain labeled γ subunit runs with a MW of ~7.6 K, the label probably contributing substantially to the apparent size. In Western blot analysis of dog kidney membranes, Ab-gam and Ab/p-G17 react *only* with a doublet of ~7.6K and ~6.7K and not with higher molecular weight species or with other small peptides. These results are strong additional evidence that the γ peptide(s) is not a proteolytic fragment of other subunits, and that the identified and sequenced peptide is specifically associated with the Na,K-ATPase. (Supported by NIH GM-31782 and NIH GM-39746).

Tu-Pos490

MOLECULAR CLONING OF THE γ-SUBUNIT OF THE NA,K-ATPASE.

Robert W. Mercer, David P. Bliss, Jr., John H. Collins, and Bliss Forbush III; Dept. Cell Biol. and Physiol. Washington Univ. School of Medicine, St. Louis, MO 63110, Dept. Biol. Chem. Univ. Md. School of Med., Baltimore, MD, 21201 and Dept. of Cell. and Mol. Physiol., Yale Univ. Sch. Med., New Haven, CT 06510.

The γ-subunit of the Na,K-ATPase is a small membrane bound protein which copurifies with the α and β subunits of the enzyme and may form part of the cadiac glycoside binding site. We have isolated the cDNAs coding for the sheep and rat γ-subunits. mRNA from sheep kidney was isolated and reverse transcribed using a 35-base oligonucleotide with 17 dT residues and an adaptor sequence containing three endonuclease recognition sites. An oligonucleotide based on the purified γ protein amino acid sequence (Collins and Leszyk, Biochem. 26:8665,87) was annealed to the cDNA strand and used as a primer to generate the complementary second strand. Repetitive polymerase chain reaction cycles using the γ-subunit primer and the 3' adaptor sequence resulted in the amplification of the double-stranded γ-subunit cDNA. From this reaction a cDNA clone corresponding to the sheep γ-subunit was isolated and used to identify a full length rat kidney cDNA clone. The nucleotide and deduced amino acid sequences predict a polypeptide consisting of 58 amino acids with a molecular weight of 6497 D. The protein has no significant homology with other proteins and no sites for N-linked glycosylation. Hydropathy analysis suggests a single transmembrane domain with highly charged intra- and extracellular domains. The γ-subunit gene encodes two RNA species of ~1.5 and 0.8 kb. The smaller transcript is most abundant in kidney, with low levels in spleen, lung and heart, whereas the larger transcript is most abundant in the spleen and lung. Interestingly, in contrast to the α and β mRNA, there is little expression of γ-subunit mRNA in the brain. (Supported by NIH GM-39746 and NIH 31782).

Tu-Pos491

VOLTAGE-DEPENDENCE OF THE Na/K-PUMP CURRENT OF RANA OOCYTES.

Michael M. Wu† and Mortimer M. Civan. Depts. of Bioengineering† and Physiology & Medicine, Univ. of Pennsylvania, Phila., PA 19104.

Current (I_p) through the Na/K-pump in frog oocytes depends on membrane potential (V_m), but the nature of the dependence has been unclear. We have shown previously that the non-pump conductances of the oocyte can generate errors in the derived I_p. I_p was measured only after successive applications and wash-outs of str yielded reproducible difference currents. The dose response to str in 4 oocytes gave a mean K_i of 0.4 μM, with 2-5 μM representing 83-93% pump block. With K^+ and Ca^{++} channel blockers in the bath, Na^+ pre-loaded oocytes of Rana pipiens were voltage clamped [-170,50 mV] with and without 2-5 μM strophanthidin (str). The average I_p of 12 oocytes depended directly on V_m up to a plateau at 0 mV with current reversal at -165 mV. Decreases in $[Na_o]$ decreased the voltage sensitivity of I_p, with no dependence at all at $[Na_o]=0$. As our results with frog oocytes are in very good agreement with work done in cardiac myocytes and squid axon, we conclude that the I-V characteristics of the Na/K-pump are conserved across the 3 tissues.

Tu-Pos492

Na,K-ATPase FUNCTION IN ALTERNATING CURRENTS. M.Blank & L.Soo (Intro. by H.S.Chase),Dept.of Physiology and Cellular Biophysics,Columbia University, NY,NY 10032 The ouabain sensitive accumulation of cations by erythrocytes in alternating currents (AC) is frequency dependent (Serpersu & Tsong,J. Biol. Chem. 259:7155, 1984). The electric field may act directly upon the Na,K-ATPase, but we have shown that AC across membranes causes frequency dependent ion concentration changes that could activate the enzyme (Blank, J.Electrochem. Soc. 134:343,1112, 1987). We measured the rate of ATP splitting by Na,K-ATPase from rabbit kidneys in AC currents over an amplitude range of 1mV to 1V and a frequency range of 10 Hz to 100kHz. We found that AC decreased ATP splitting by the normal enzyme, and increased the activity only when the control activity was low. Enzyme activity was lowered by natural decay during storage under refrigeration, by lowering the temperature, and by introducing partially effective concentrations of ouabain. Our experiments suggest that ion movement in AC plays an important role in the observed enhancement and inhibition of the Na,K-ATPase. (We thank the ONR for their support.)

Tu-Pos493

IN VIVO RESTING [NA^+, K^+]-ATPASE ACTIVITY IN THE ELECTRIC ORGAN OF *NARCINE BRASILIENSIS* MEASURED BY SATURATION TRANSFER. H. Blum[a], J. A. Balschi[b] and R. G. Johnson, Jr.[a], [a]HHMI, Univ of Penna Med Center, Phila, PA 19104 and [b]Univ of Ala Med Center, Cardiovasc NMR Lab, Birmingham, AL 35294.

^{31}P-NMR spectroscopy has been applied to the creatine phosphokinase (CPK) reaction to measure the unidirectional flux from ATP to PCr in the resting electric organ and skeletal muscle of the electric fish *Narcine brasiliensis*. The pseudo first-order rate constant k_f and relaxation time T_1 are compared with other organ systems below.

These results show that the electric organ, although well supplied with enzymatically highly active CPK and PCr, has no flux through this reaction, implying very low ADP levels. This is consistent with virtually no turnover of ATP in the resting *versus* the stimulated organ.

Sample	Temp (C)	pH	T_1 (sec)	k_f (sec-1)	ref
Narcine Muscle	24	7.5	3.73	0.08 +/- .03	
Narcine Organ	24	7.5	4.0	0.00 +/- .03	
Human Arm		7.0	3.74	0.239	1
Human Leg		7.0	3.4	0.398	2
Rat Thigh		7.0	2.7	0.52	3
Rabbit Leg		7.0		0.32	4
Rabbit Enzyme	21	7.0		0.16 (1400 u/ml)	5
	21	8.0		0.08 "	5
Rabbit Enzyme	37	7.2	2.0	0.35 (10^4 u/ml)	6

[1] D. Rees, et al. *Magn Reson Med* 9: 39-52, 1989.
[2] S. Englander, et al. Unpublished data, 1989.
[3] J. A. Bittl, et al. *Biochem* 26: 6083-6090, 1987.
[4] P. S. Hsieh et al. *Magn Reson Med* 7: 56-64, 1988.
[5] H. Eldar et al. *Magn Reson Med* 11: 121-126, 1989.
[6] H. L. Kantor et al. *Biochim Biophys Acta* 789: 128-135, 1984.

Tu-Pos494

PRIMARY STRUCTURAL LOCATION OF FLUORESCEIN-MALEIMIDE LABELING SITES IN SR CA-ATPASE. J.E. Bishop, A. Wawrzynow, J.L. Theibert, D.J. Bigelow, and J.H. Collins. Dept. Biological Chemistry, Univ. Maryland School of Medicine, Baltimore, MD 21201.

Previous studies of the Ca-ATPase have shown selectivity of chemical modification of the ~16 sulfhydryls on the protein's surface: (1) Cys-344 and Cys-364 by N-ethyl-maleimide (Mal-Et); and (2) Cys-674 and (in one case) Cys-670 by iodoacetate (IA), IA-fluorescein (IA-Fl) and IA-diethylaminonaphthalene sulfonate (IA-Dans). We are investigating factors influencing reagent selectivity: the reactive moiety of the reagent versus the attached chromophore. To this end, we are identifying the site(s) labeled by Mal-Fl.

One preparation of labeled Ca-ATPase was completely digested with trypsin; labeled peptides were purified by HPLC and sequenced. Preliminary results indicate a lack of selectivity, with several Cys residues (12, 364, 498, 636, 614, 670) labeled. Another preparation, however, when subjected to limited trypsinolysis and gel electrophoresis, showed most of the label to be in the "A1" fragment (residues 199-505). The reasons for this discrepancy are now being investigated.

Supported by F.C. Bressler and NIH (P01-HL-27867) grants.

Tu-Pos495

CA-ATPASE OF SARCOPLASMIC RETICULUM: IDENTIFICATION OF THIOL GROUPS LABELED BY STRUCTURAL PROBES. A. Wawrzynow, D.J. Bigelow, J.E. Bishop, and J.H. Collins. Dept. Biological Chemistry, Univ. Maryland School of Medicine, Baltimore, MD 21201.

We are using 4-dimethylaminophenylazophenyl- -4'-maleimide (DABMI), 4-dimethylaminophenyl-azophenyl-4'-iodoacetamide (DABIA), and 2-(4'-maleimidylanilino)naphthalene-6-sulfonate (MIANS) as SH-specific structural probes of the Ca-ATPase of rabbit skeletal muscle sarcoplasmic reticulum. Intramolecular fluorescence resonance energy transfer is being used for distance measurements between these and other site-specific probes; it is therefore necessary to know their locations in the primary structure of the Ca-ATPase. The labeled Ca-ATPase was completely proteolyzed with trypsin, and the unlabeled membrane-bound peptides were removed by centrifugation. Soluble labeled peptides were purified by HPLC and sequenced in order to identify the labeled thiol groups within the primary structure of the Ca-ATPase. The details of these findings will be presented.

Supported by NIH grant P01-HL-27867.

Tu-Pos496

KINETIC ANALYSIS OF THE INTERACTION OF Mg WITH Na/K-ATPase. Yolanda G.-M. Covarrubias and Paul De Weer, Washington University School of Medicine, St. Louis, Missouri.

Several reactions catalyzed by the sodium pump, including ATP hydrolysis in the presence of Na or Na+K, and ADP/ATP exchange, are accelerated by μM free Mg and inhibited by mM free Mg. A single Mg binding site suffices to account for this dual effect. Steady-state analysis of hydrolysis kinetics alone cannot resolve the question whether MgATP complex binds to the enzyme rather than ATP and Mg separately: either model fits the data equally well, as judged by the least-squares criterion. On the other hand, steady-state kinetic analysis of ADP/ATP exchange reactions favors the separate-binding model and suggests that the low-affinity inhibitory effect of Mg is exerted at the Mg release step. We present a minimal model that accounts for the observed rates of hydrolysis and isotope exchange in the absence of K. Simultaneous least-squares fitting of all the data to the model resolves 5 parameters at fixed nucleotide concentrations, and correspondingly more when the latter are varied. Resolution of all 9 rate constants will require presteady-state analysis.

(Supported by the American Heart Association).

Tu-Pos497

Glycosylation state of the Na,K-ATPase α-subunit. C. H. Pedemonte[1], K. Hall[2], G. Sachs[2] and J. H. Kaplan[1]. [1]Dept. of Physiology, Univ. of Pennsylvania,Phila., PA 19104 and [2]UCLA.

The β-subunit of the Na-pump is usually described as the glycosylated subunit. We have investigated the glycosylated status of the α-subunit. Reaction of the purified dog kidney Na,K-ATPase withUDP-[^{14}C]-galactose catalyzed by bovine milk galactosyl transferase results in radiolabel incorporation into both the α- and β-subunits of the enzyme. The label in β- is 3 to 5 times that in the α-subunit. Treatment of the labelled α-subunit with sodium borohydride in basic medium give no evidence for β-elimination of sugars suggesting that glycosylation of the α-subunit did not occur through O-linkages. This treatment produces partial degradation of the protein but it does not affect the N-linked glycosides. Na,K-ATPase separated by PAGE and transferred to a nitrocullulose membrane shows positive staining in the α-subunit when probed with concanavaline A (Con A)-biotin and extravidin-peroxidase.The Na,K-ATPase was treated with proteases (proteinase K or trypsin), the fragments separated by PAGE and electroblotted to an Immobilon membrane. The amino terminal sequence was determined of some of the fragments which were glycosylated as shown by positive reaction with Con A. Based on the NH_2-terminal sequence and the apparent molecular weight, the following segments were shown to be glycosylated: 171-270, 406-586, 557-707, 266-end, 593-end, 31-411, in the primary structure of the Na,K-ATPase α-subunit. If the α-subunit is N-glycosylated, these data unambiguosly indicate that asparagine residues 211, 479 and 634 are glycosylated. These three of the possible five consensus N-glycosylation sequences in the α-subunit primary structure appear to be located on the cytoplasmic side of the membrane. This suggests either there are considerable errors in the current folding models based on hydropathy plots or the Na-pump is post-translationally processed in a novel way. Supported by NIH Grant GM-39500.

Tu-Pos498

PROPERTIES OF THE NA,K-ATPASE INACTIVATED AT AN ESSENTIAL AND UNIQUE NA-SENSITIVE SITE **Graham C.R. Ellis-Davies and Jack. H. Kaplan**
Dept. of Physiology, University of Pennsylvania, Philadelphia, PA 19104-6085.

Treatment of purified Na,K-ATPase from canine renal medulla with N-(2-nitro-4-isothiocyano-phenyl)-imidazole (NIPI) results in the rapid, irreversible inactivation of the enzyme (*Biophys. J.* **55**,424a,[1989]). The rate of covalent modification is dramatically increased by the presence of Na and fully protected by ATP. The synthesis of [^{3}H]-NIPI has enabled us to demonstrate that both in the presence and absence of Na only one molecule of NIPI reacts covalently with the α-subunit of the enzyme and that no binding occurs in the presence of ATP. Furthermore, ligand (K, P_i, ADP) binding properties have been altered in the NIPI-modified enzyme. Principally, NIPI-treated enzyme has lost its high affinity ATP binding site. This result alone is sufficient to account for the loss of enzyme activity. Phosphorylation from P_i, on the other hand, is uninhibited and is consistently slightly higher than native enzyme. Also, NIPI modified enzyme only partially occludes Rb (used as a cogener for K). Trypsin treatment of the modified enzyme shows a new cleavage site which is common to the Na and K forms of the enzyme, whilst the presence of the fragments characteristic of native enzyme in these forms is diminished. These results imply that, upon Na binding to the enzyme, an essential lysine residue becomes more exposed, which may be in or near the ATP binding domain. Supported by NIH GM 39500.

Tu-Pos499

4-(DIAZOMETHYL)-7-(DIETHYLAMINO)-COUMARIN INACTIVATION OF THE Na,K-ATPase IS K-PROTECTABLE. **José M. Argüello and Jack H. Kaplan.** Dept. of Physiology, Univ. of Pennsylvania, Philadelphia, PA, 19104-6085.

4-(diazomethyl)-7-(diethylamino)-coumarin (DEAC) is a stable diazomethane analog which can be used as a fluorescent reagent for carboxyl residues. Isolated dog kidney Na,K-ATPase was inactivated by treatment with DEAC. The ATPase and p-NPPase activities were affected to the same extent. The ATPase inactivation was partially prevented by K^+, but not by ATP, ATP-Mg^{+2}, Na^+, Mg^{+2} or inorganic phosphate (Pi). The inactivation velocity was dramatically increased when the enzyme was treated in the presence of 3 mM $MgCl_2$, 3 mM Pi (DEAC-enzyme). In this condition the inactivation was partially prevented by Na^+ and almost completely prevented by K^+. The DEAC-treated enzyme shows normal levels of high affinity ATP binding and phosphorylation by Pi. However, the presence of ouabain did not increase the phosphorylation level of DEAC-enzyme. Trypsin treatment indicated that DEAC-enzyme could form E1 conformation in the presence of Na^+, but the E2 form tryptic pattern could not be obtained with K^+. DEAC treated enzyme showed only minimal Rb^+ binding and occlusion. These results suggest that the modified carboxyl residue(s) are closely related to the K^+ binding site. Preliminary determinations indicate that only one or two amino acids per α subunit are modified by DEAC. Supported by NIH grant GM 39500

Tu-Pos500

THE TEMPERATURE INDEPENDENCE OF ATP COMPARTMENTATION IN HUMAN AND GROUND SQUIRREL RED CELLS.

M. Marjanovic, P. Ghosh, J.S. Willis and M.J. Dawson, Dept. of Physiol. and Biophys., Univ. of Il, Urbana, Il, 61801

Red cells from hibernating species such as the ground squirrel maintain more active transport of Na^+ and K^+ at low temperature than do red cells from humans. We have been using ^{31}P NMR spectroscopy together with chemical analysis to determine whether differences in [ATP], its hydrolysis products, or ions with which it interacts could account for this species difference. We have previously shown species differences in the effect of temperature on free intracellular [Mg^{2+}] (Biophys J 51:77a, 1987).

We report here that the freely mobile [ATP], as determined by ^{31}P NMR, corresponds to approximately 80% of that measured by the luciferin/luciferase method in lysed red cells of both species at T = 2 to 30°C. The T_1 relaxation time for ATP in fresh human red cells at 20°C (55% hematocrit) was 1.04s; and fully relaxed spectra were obtained at this temperature. The relative ß-ATP peak intensity and total ATP in lysed cells was independent of temperature in both species.

Tu-Pos501

ATP KINETICS OF NA-K PUMP IN INTACT ERYTHOCYTES SENSITIVE AND RESISTANT TO LOW TEMPERATURE.

Marina Marjanovic and John S. Willis
(Intro. by Paul C. Lauterbur)
Department of Physiology and Biophysics, University of Illinois, Urbana, IL 61801

Activity of the Na-K pump is less reduced at 5°C in ground squirrel red cells than in human or guinea pig. There are no relevant differences in Na or K kinetics of the pump in the red cells of these species. We have now estimated the ATP kinetics of the pump in intact erythrocytes by progressively depleting ATP in the presence of 2-deoxy-D-glucose and then measuring ouabain-sensitive K influx. Linear transforms of the Michaelis-Menten curve for human and guinea pig red cells at 37°C indicate two components: a low affinity activation with Km of about 500 μM and a high affinity activation with a Km of about 40. Cooling lowers the affinity of both components so that below 20°C only the high affinity component is in range. In ground squirrel cells at 37°C there is only a single, low affinity component with a Km of 300. Cooling in these cells *raises* the affinity. Thus loss of sensitivity for ATP in guinea pig and human cells accords with their lower pump activity relative to ground squirrel cells at low temperature. (Support, NIH GM11494.)

Tu-Pos502

IDENTIFICATION OF A REACTIVE LYS RESIDUE IN THE PUTATIVE ATP BINDING SITE OF LAMB KIDNEY (Na^+,K^+)-ATPase.

Hellmuth R. Hinz and Terence L. Kirley, Department of Pharmacology and Cell Biophysics, College of Medicine, University of Cincinnati, Cincinnati, OH 45267-0575.

A single lysine residue of lamb kidney (Na^+,K^+)-ATPase is protected from labelling with pyridoxal-5'-phosphate (PLP) in the presence of ATP. After trypsin digestion of the purified α subunit, the fluorescently labeled peptide was isolated using high performance liquid chromatography (HPLC) equipped with an absorbance and fluorescence detector linked in series. The purified peptide was analyzed by amino acid combustion analysis and sequenced. The peptide had the sequence Ile^{470}-Val-Glu-Ile-Pro-Phe-Asn-Ser-Thr-Asn-Lys^{480}-Tyr-Gln-Leu-Ser-Ile-His-Lys^{487}. Lys^{480} was identified as the labelled amino acid. The beta subunit is not differentially labelled by PLP in presence or absence of ATP. The homology of the binding/modification site is discussed with respect to other ATPases. Supported by Training Grant HL07382-13 and Program Project Grant PO1-HL22619 (Core 2).

Tu-Pos503

TWO ACTIVE SPECIES OF ERYTHROCYTE Ca^{2+}-ATPase.

D. Kosk-Kosicka, T. Bzdega, University of Maryland, Baltimore, MD 21201.

The study was performed on the purified human erythrocyte Ca^{2+}-ATPase to test whether or not calmodulin promotes enzyme oligomerization. Two models were considered to explain the observed activation of this enzyme by calmodulin binding to monomeric enzyme and by enzyme oligomerization. The first model assumed two active enzyme species, oligomers and monomers with bound calmodulin, whereas the second assumed that only oligomers are fully active and calmodulin facilitates enzyme oligomerization.

We show that addition of calmodulin does not decrease enzyme concentration at which half-maximal oligomerization occurs (determined by FRET between separately labeled Ca^{2+}-ATPase molecules) and that compound 48/80, a calmodulin inhibitor that totally inhibits Ca^{2+}-ATPase activity of monomeric enzyme, has no effect on either Ca^{2+}-ATPase activity of oligomers or on the extent of oligomerization.

These, together with other findings, demonstrate that calmodulin is not involved in the oligomerization-induced activation pathway. Calmodulin neither promotes oligomerization nor stimulates the Ca^{2+}-ATPase activity of oligomers.

Supported by NIH GM37143 and AHA 880832 grants.

Tu-Pos504

SPECIFIC EFFECT OF K^+ ON ERYTHROCYTE Ca^{2+}-ATPase ACTIVITY. O. Juhasz, A. Wawrzynow, D. Kosk-Kosicka. University of Maryland, Dep. Biol. Chem., Baltimore.

The effect of K^+ and ionic strength on the Ca^{2+}-ATPase activity of the erythrocyte Ca^{2+}-ATPase in ghost membranes and in the detergent purified enzyme was studied. In the purified enzyme the calmodulin-independent Ca^{2+}-ATPase activity, representing oligomers, was increased 2-3 fold when KCl concentration was raised from 10 to 200 mM. The effect of the respective ionic strength was significantly lower. At the same time the calmodulin-dependent Ca^{2+}-ATPase activity of monomers was slightly decreased in this KCl concentration range, and the effect of KCl was very similar to the effect of ionic strength.

Preliminary data suggest that the specific effect of K^+ on the oligomeric enzyme is Ca^{2+} dependent: at higher K^+ the enzyme reveals higher affinity for Ca^{2+}.

Additionally, we have found the same effects of K^+ on the enzyme studied in erythrocyte ghost membranes in the absence and presence of calmodulin, respectively. Supported by NIH GM37143 and AHA 880832.

Tu-Pos505

STRUCTURAL STUDY OF H,K-ATPase BY ELECTRON MICROSCOPY AND IMAGE PROCESSING. M. Mohraz, S. Sathe, and P.R. Smith. New York Univ. Sch. Med. New York, NY.

H,K-ATPase is an integral protein of the plasma membrane of gastric parietal cells. It constitutes the pump responsible for the secretion of acid into stomach. It has one polypeptide chain (M_r 110,000) that shows striking sequence homology to the catalytic subunit of other transport ATPases. The enzyme is isolated in protein-rich membrane vesicles from hog stomach. Crystallization is induced in suspensions of the enzyme by phospholipases in the presence of various ions. Electron micrographs of negatively stained specimens show that ordered arrays of different morphologies (long tubes or rods, basic sheets, multi-stacked sheets, and vesicles) form concurrently in a given suspension. The crystalline fragments are extremely large (some more than 2 μm in diameter) and very well-ordered. The unit-cell parameters are: a=b=14.6 nm, $\gamma=90^\circ$, and the lattice has $p12_12_1$ symmetry. Computer-filtration of projection images shows four sets of dimers (eight molecules) in the unit-cell. Viewed normal to the membrane, the H,K-ATPase molecule has an elongated, rod-shape structure.

Supported by NIH Grant GM-35399 to MM.

Tu-Pos506

EVIDENCE FOR ROTATIONAL MOTION OF SENSORY RHODOPSIN-I IN HALOBACTERIAL CELL MEMBRANES.
Steven Sundberg* and Roberto Bogomolni*†,
* Cardiovascular Research Institute, UCSF, San Francisco, CA, and †Dept. of Chemistry, UCSC, Santa Cruz, CA.

Sensory rhodopsin-I (sR-I) is a photoactive retinal-protein believed to function as a photosensory receptor in Halobacterium halobium. We have observed transient dichroism on the micro- to millisecond timescale in measurements of the main sR-I absorption depletion band following excitation of the sample by a polarized laser pulse. In membrane pellets containing sR-I as the only detectable retinal pigment, the flash-induced anisotropy decayed with a halftime of 30-200 μs to nonzero limiting values > 0.02, indicating restricted molecular motion over this timespan. We tentatively attribute this to rotational diffusion of the protein molecule within the plane of the membrane. This assignment is based on the shape (Cherry, et al., Nature 263:389-393 (1976)) and kinetics of the anisotropy decay curve, which are faster than expected for membrane vesicle tumbling (Heyn, et al., J. Mol. Biol. 117:607-620 (1977)). As yet, we have not entirely ruled out possible contributions by motion of the chromophore relative to the protein.

Tu-Pos507

SPECTROPHOTOMETRIC & FLASH PHOTOLYSIS STUDIES OF SENSORY RHODOPSIN-I RECONSTITUTED WITH 3,4-DEHYDRORETINAL.
Steven Sundberg* and Roberto Bogomolni*†,
*Cardiovascular Research Institute, UCSF, San Francisco, CA, and †Dept. of Chemistry, UCSC, Santa Cruz, CA.

Spectroscopic studies of sensory rhodopsin-I (sR-I) were carried out using membrane preparations from a retinal-deficient strain of Halobacterium halobium which contains the sR-I apoprotein. Reconstitution with 3,4-dehydroretinal produced a pigment absorbing at ∿ 620 nm, which underwent cyclic photoconversion to a long-lived species absorbing at ∿ 400 nm. The risetime of this intermediate (∿ 300 μs) was similar to that of the native pigment's S-intermediate, while its thermal decay was slower (∿ 1.5 s halftime at room temperature compared to 0.8 s for the native pigment). Photosteady-state difference spectra reveal fine structure in both the native and analogue S-intermediate spectra. Like that of the native pigment, the analogue S-intermediate can be photoconverted back to the ground-state, again with altered kinetics. The results are of relevance to ongoing studies of chromophore/protein interactions in retinal pigments, and to studies of photosensory transduction in this microorganism.

Tu-Pos508

EVIDENCE FOR A NEW PHOTOLYSIS INTERMEDIATE IN VISUAL PIGMENTS

D.S. Kliger, J.W. Lewis, S.J. Hug, C.M. Einterz, T. Thorgeirsson, and S.C. Bjorling; Chemistry Department Univ. of California, Santa Cruz

Nanosecond time-resolved absorption studies of rhodopsin, isorhodopsin, and a variety of artificial visual pigments reveal that the reaction in which bathorhodopsin (BATHO) is transformed into lumirhodopsin (LUMI) is more complicated than previously thought. Prior to LUMI formation a new, blue-shifted intermediate (BSI) forms a pseudo-equilibrium with BATHO. BSI then is transformed into LUMI. Early events in visual transduction are thus:

... BATHO <---> BSI ---> LUMI ...

Information is presented on the spectral, kinetic, and thermodynamic properties of the new BSI intermediate as well as information of chromophore orientations associated with each of the intermediates.

Tu-Pos509

COLOR CONTROL OF THE GECKO 521-VISUAL PIGMENT
F. Crescitelli and B. Karvaly, Dept. Biology, UCLA, Los Angeles, CA 90024 and Dept. Physics ECU, Greenville, NC 27858

The 521-pigment in the retina of the Tokay gecko (Gekko gekko) readily responds to particular physical and chemical changes in its environment. When solubilized in chloride deficient state the addition of Class I anions (Cl^-, Br^-) induces a bathochromic shift of the absorption spectrum. Class II anions (NO_3^-, IO_3^-, N_3^-, OCN^-, SCN^-, $SeCN^-$, $N(CN)_2^-$) cause an hypsochromic shift. Class III anions (F^-, I^-, NO_2^-, CN^-, AsO_3^-, $SO_4^=$, $S_2O_3^=$) have no spectral effect on the 521-pigment. Cations appear to have no influence on the pigment absorption and Class I anions prevent or reverse the hypsochromic shift caused by Class II anions. It is suggested that the spectral displacements reflect specific changes in the opsin conformation, which alter the dipolar environment of the retinal chromophore. The protein conformation seems to promote excited-state processes most in the native 521-pigment state and least in the presence of Class II anions. This in turn suggests that the photosensitivity of the 521-pigment (Crescitelli & Karvaly, Proc. Roy. Soc. Lond. 220B, 69, (1983)) is controlled by the excited- rather than by the ground-state properties of the pigment.

Tu-Pos510

BACTERIORHODOPSIN ASP 85 MUTANTS: pH DEPENDENCE OF PROTON RELEASE/UPTAKE
T. Marinetti+, S. Subramaniam*, T. Mogi*, T. Marti* and H.G. Khorana*
+ Rockefeller University, NY NY
* Depts. of Chemistry and Biology, M.I.T., Cambridge MA

H^+ release and uptake from site-specific bacteriorhodopsin mutants were detected by flash-induced conductivity changes. For Asp 85→Glu at pH 6 (blue), H^+ transients are seen (30-60 ms); the quantum yield is 50% of wild type. The blue form is inactive in steady state pumping, so the conductivity changes may be due to H^+ release/uptake from the same side of the molecule. Above pH 7 (purple) the uptake step is slowed ∿10 fold; at pH 7.8 the transient is clearly biphasic, with a 10 ms component. These signals were due only to H^+ movements. In contrast, the double mutant D85E/D212E (inactive in pumping), shows conductivity transients due to nonproton ions. The results show that "inactive" mutants undergo some structural changes since ions are transiently released and bound following light excitation.
+NIH GM32955 *NIH and ONR

Tu-Pos511

THE DISTANCE OF THE RETINAL'S RING FROM THE MEMBRANE SURFACE OF BACTERIORHODOPSIN AND RHODOPSIN DETERMINED WITH SECO-RING/SPACER ARM/END GROUP LABEL-BEARING RETINALS.
Valeria Balogh-Nair, Liang Chen and Wei-Xing Li, Department of Chemistry, City College of the City University of New York, New York, N.Y. 10031.

Retinal analogs with seco rings attached to spacer arms of 3-8 carbon length bearing methyl, carboxyl and diazoacetoxy end group labels have been synthesized efficiently via Pd-catalyzed Heck olefination of the vinyl triflate prepared from 2-methyl-2-siloxy-3-pentanone, followed by attachment of the spacer arms bearing the labels. The all-trans and 11-cis isomers were combined with bacterio- and bovine opsins to yield remarkably stable bR and Rh analogs. Both bR and Rh analogs had 30-45 nm blue shifted absorption maxima, and the bR analogs light-adapted readily (8 nm red shift). In the case of 7-carbon spacer/carboxyl label, bR analog formation of the intermediates of regeneration could be observed at room temperature. Systematic investigation with progressively longer spacer armed analogs showed that 7-carbon spacers form bR and Rh analogs while 8-carbon spacers do not. This studies indicate that the retinal's cyclohexyl ring is no more than 12Å from the membrane surface. Labeling patterns to determine the location of protein amino acid residues surrounding the label, yielding precise information on the tertiary structure is in progress.

Tu-Pos512

QUANTUM MECHANICAL CALCULATION OF VIBRATIONAL SPECTRA
Earl McMaster* and Aaron Lewis+* *Department of Applied Physics, Cornell University, Ithaca, New York 14853 +Department of Applied Physics, Hebrew University, Jerusalem, Israel

We have developed a computational method to facilitate the normal mode analysis of vibrational spectra. The method involves two key steps. In step one, a quantum mechanically calculated potential energy surface is empirically corrected to produce improved values of the calculated Wilson **F** and **G** matrices. This is done by adding a power series to second order designed to correct for the failings of the quantum mechanical calculation (e.g. basis set truncation, neglect of electron correlation, and some anharmonic influences). In step two, the calculated Wilson **F** and **G** matrices from step one are used to calculate an improved set of vibrational frequencies and their corresponding normal modes. The *calculated* normal modes and the *experimental* vibrational frequencies are used to further correct the calculated force constants. Using this method with both *ab initio* (RHF/4-31G) and semiempirical (AM1) calculations, we have seen dramatic improvement in the calculated force field as evidenced by good prediction of isotopic shifts. The goal is to use the method to help elucidate the structural changes of retinal and lysine in the Bacteriorhodopsin photocycle via analysis of our FTIR spectra.

Tu-Pos513

Quantum Efficiencies of Bacteriorhodopsin Photochemical Reactions at 110K.

Aihua Xie, Department of Physics, University of Illinois at Urbana-Champaign, Urbana, IL 61801.

For a bacteriorhodopsin (bR) sample prepared in 70% glycerol/water (v/v) buffered to pH 7 with 10 μM potassium phosphate, the K intermediate generated by photo-excitation of bR is stable in the dark at 110K. We measured the absorption spectra of pure bR, and the K+bR mixtures photo-saturated by illumination at 420, 460, 500, 520, 530, 560, 570, 580, 590 and 600nm at 110K. The amount of K formation, f_K, under saturation is determined by $f_K^{-1}=1+(\phi_2/\phi_1)(A_K/A_{bR})$, where ϕ_1 is the quantum efficiency for bR to K photoconversion, ϕ_2 is the quantum efficiency of K to bR, and A_K/A_{bR} is the ratio of absorptions of K to bR at the excitation wavelength. By assuming that the ratio ϕ_1/ϕ_2 is the same at 570 and 580nm, the spectrum of K and the value of ϕ_1/ϕ_2 at 570 and 580nm were obtained. The values of ϕ_1/ϕ_2 at the other excitation wavelengths could then be evaluated. It was found that ϕ_1/ϕ_2 does depend on the excitation wavelength: small in the red, and large in the blue, where the scattering effect was shown to be negligible. The resulting values of ϕ_1/ϕ_2 are 0.62, 0.54 and 0.52, and the amounts of K formed under saturation are 46%, 50% and 42% at 420, 530 and 580nm respectively, while the peak wavelengths for K and bR are at 608 and 578nm. Even though the results shown here disagree with those of several other groups, it should be emphasized that this method has advantages over other methods for its minimal assumptions and straight-forward procedure.
This work was supported in part by NIH grant GM32455, and ONR grants N00014-86-K-0270 and N00014-86-J-1300.

Tu-Pos514

BACTERIORHODOPSIN'S PHOTOCYCLE KINETICS ARE ACCURATELY MODELED WITH BACKREACTIONS AND PROTONATION IN THE N → O STEP.

J. B. Ames, S. P. A. Fodor, and R. A. Mathies, Chem. Dept., Univ. of Calif., Berkeley, CA 94720

The pH-dependent kinetics of bacteriorhodopsin's photocycle have been analyzed using time-resolved resonance Raman spectroscopy. The absolute concentrations of each photocycle intermediate are measured as a function of time from pH 5 to 8.6. These data are optimally modeled by the scheme: BR → L ↔ M ↔ N → O → BR. Backreactions between L, M and N are sufficient to accurately model the biphasic decay of L and M at all pH's. Branched reactions or multiple photocycles are not required. Three rate constants are found to be pH-dependent. k_{LM} and k_{ML} both increase sigmoidally with pH, confirming earlier proposals that Schiff base deprotonation is catalyzed by a titratable tyrosine residue. k_{NO} depends linearly on $[H^+]$ above pH 8 in 3 M KCl, but it becomes pH-independent below pH 8. This suggests that proton uptake occurs along with cis → trans isomerization during the N to O transition. The rate determining step shifts from proton uptake above pH 8 to cis → trans isomerization below pH 8. A model for the proton pumping mechanism is presented which incorporates these kinetic results into a two state T-C Model (Fodor et al., *Biochem.*, 1988, 27, 7097)

Tu-Pos515

NMR STUDIES OF $-CD_3$ GROUP DYNAMICS IN BACTERIORHODOPSIN AND RETINOIC ACID MODEL COMPOUNDS: EVIDENCE FOR A 6-s-TRANS RETINAL CHROMOPHORE IN THE PROTEIN.

V. Copié*, A. McDermott*, K. Beshah*, M. Spijker#, J. Lugtenburg#, J. Herzfeld$, and R. Griffin*. *MIT, Cambridge, MA 02139. $Brandeis University. #University of Leiden

Solid-state ^{2}H-NMR has been used to examine the dynamics of $-CD_3$ groups in 6-s-cis and 6-s-trans retinoic acid, and in the membrane protein bacteriorhodopsin (bR), reconstituted with 18-CD_3 retinal. For the 6-s-cis retinoic acid, intermediate exchange lineshapes indicating three-site hopping rates on the order of 10^4-10^5 s^{-1} are observed from -160°C to -185°C. In contrast, the 18-CD_3 in the 6-s-trans retinoic acid exhibits Pake patterns due to fast exchange, from -140°C to -185°C. For bR the quadrupolar echo lineshapes observed are similar to those of the 18-CD_3 6-s-trans retinoic acid, and exhibit motionally narrowed Pake patterns at temperatures down to -175°C, indicating a 6-s-trans form in the protein. These results illustrate the manner in which molecular dynamics studies can be used to probe the structure of proteins.

Tu-Pos516

SOLID-STATE NMR DETERMINATION OF A CARBON-CARBON DISTANCE IN BACTERIORHODOPSIN BY ROTATIONAL RESONANCE

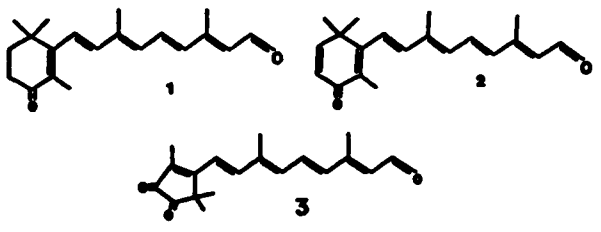
A. McDermott, F. Creuzet, M. Levitt, R. Griffin - MIT; R. Gebhardt, J. Lugtenburg - University of Leiden; J. Herzfeld - Brandeis University

Rotational resonance (R2), a recently proposed technique for measuring homonuclear distances in solids, was successfully used to demonstrate that the retinal in bR is in a 6-s-trans configuration. With R2 the magnetization transfer within an isolated spin pair is driven by rotation of the sample at a speed equal to the difference in chemical shifts (or a submultiple of that difference); as a result, it is spectrally selective for the spin pair of interest. We measured the rate of rotationally driven magnetization exchange between the C8 and the C18 resonances of the retinal in bR and in 6-s-cis and 6-s-trans model compounds. The rate of magnetization exchange for the spin pair in the retinal in bR was found to be in good agreement with the internuclear distance for a 6-s-trans configuration (4.2 Å) and inconsistent with that for a 6-s-cis configuration (3.1 Å).

Tu-Pos517

OXIDIZED RETINAL BASED ANALOGUE PIGMENTS OF BACTERIORHODOPSIN

CJ Beischel, V Mani, TC Williams, RK Crouch (Intro. by DR Menick). Departments of Pharmacology and Opthalmology, Medical University of South Carolina. A series of oxidized retinal analogues has been synthesized and fully characterized. 1 and 2 have similar absorption maxima near 378nm whereas 3 has a greatly red shifted maximum at 432nm. Two dimensional NMR spectroscopy was used to confirm the structures of 1-3. Analysis of the NOE correlations of these retinals in solution indicates that 1 and 2 possess the twisted 6-s-*cis* conformation, but that 3 is 6-s-*trans*. The three analogues form similar bacteriorhodopsin analogue pigments which absorb maximally near 520nm, are stable to hydroxylamine and all-trans retinal, and exhibit an unusual blue shift on exposure to white light. Interestingly, the pigment formation from 3 is quicker than that from 1 and 2. The M-intermediate spectra of pigments formed from 1 and 2 in both the non-irradiated and irradiated forms are conventional, whereas the M-intermediate spectra of both forms of the pigment based on 3 are highly unusual. (Supported by R01-EY04939)



Tu-Pos518

QUANTUM YIELD OF THE PHOTOCHEMICAL CYCLE OF BACTERIORHODOPSIN

R. Govindjee, S. Balashov and T.G. Ebrey
Dept. of Physiology and Biophysics,
University of Illinois, Urbana, IL 61801

Values in the literature for the quantum efficiency of the photochemical cycle of bacteriorhodopsin (BR) range from 0.25 to 0.79, and, the sum of the quantum yields of the forward and back photoreactions (BR $\langle\frac{\phi_f}{\phi_b}\rangle$ K), $\phi_1+\phi_2$, has been proposed to be 1. In the present work low intensity laser flashes (532nm) and kinetic spectroscopy were used to determine the quantum efficiency of BR photoconversion by measuring transient bleaching of BR in the submillisecond time scale. Bovine rhodopsin in 2% ammonyx LO was used as a photon counter. The quantum yield of the photobleaching of rhodopsin was taken as 0.67. We find that the quantum efficiency of the BR photoconversion at 20°C is 0.63 ±.03. In a saturating laser flash the maximum amount of BR cycling was found to be ≥45% (measured as a ratio of ΔOD/OD at 610nm). From this value and from the spectrum of K published in the literature, the ratio of the efficiencies of the forward and back light reactions, ϕ_1/ϕ_2, was estimated to be ≈0.65 and ϕ_2≈1. The sum of $\phi_1+\phi_2$≈1.6.

Tu-Pos520

BIOSYNTHETIC INCORPORATION OF FLUORINATED TYROSINE INTO PURPLE MEMBRANE.

E.S. Hazard , R. Govindjee*,T. Ebrey* and R.K. Crouch. *Departments of. Pharmacology and Ophthamology. Medical University of South Carolina, Charleston,SC and *Department of Biophysics ,University of Illinois,Urbana,IL*

The m-fluoro-derivative of tyrosine has been biosynthetically incorporated into bacteriorhodopsin (BR) of Halobacterium halobium. Incorporation varies from 25 to 50 percent of the tyrosines substituted as determined from analysis of PTH derivatives of amino acids after complete hydrolysis *in vacuo* with HCl. Yields of BR decreased with increasing incorporation of m-fluoro-tyrosine. The CD spectra of fluorinated BR in water are not distinct from unsubstituted BR. The substituted BR shows normal light-dark adaptation in the visible with maxima at 568 nm for light-adapted and 562nm for dark-adapted forms. The M/BR difference spectrum shows a maximum at 410 nm at 1 ms after a laser flash with an O state appearing around 4 ms post-flash. The rate of M-state decay appears to be slowed in the derivatized BR . At pH 7.2 , this reduction scales with the level of fluorinated tyrosine incorporation. For 50 percent incorporated BR , the rate of M state decay is roughly half that of the native BR. These results imply that incorporation of fluorinated derivatives provides a useful means for studying the role of tyrosine in the function of BR. Funded by grants NIH EY03949, NSF DMB8815824 and DOE 88-ER13948.

Tu-Pos519

HIGH RESOLUTION SOLID STATE NMR OF 13C- AND 15N-ARG LABELED BACTERIORHODOPSIN

M.Engelhard, B.Hess, G.Metz, and F.Siebert (Intro.by E.Heinz)

Bacteriorhodopsin (bR), the light driven proton pump from Halobacterium halobium was biosynthetically modified with 13C- and 15N-labeled Arg. The isotope labels were introduced at the terminal N, the guanidinium C, and the δ-N of Arg. Isotope enrichment was approximately 30%. The Solid State NMR spectra revealed two classes of Arg. The 15N and 13C chemical shifts of three Arg are close to the normal positions of the guanidinium group. The other class (4 Arg) had chemical shifts which are similar to the one of peptide bonds. This unusual shift of four Arg residues might be caused by the interaction of the guanidinium group with the membrane surface. Using a pulse sequence which was introduced by Harbison et al. (JACS 110, 7221, 1988) it could be shown that all Arg residues do not freely exchange their nitrogen hydrogens with bulk water.

Tu-Pos521

PATHWAYS OF THE RISE AND DECAY OF THE M PHOTOINTERMEDIATE(S) OF BACTERIORHODOPSIN.

György Váró and Janos K. Lanyi. *Department of Physiology and Biophysics, University of California, Irvine, CA 92717*

Studies of the photocycle of bacteriorhodopsin (BR) at alkaline pH with a gated multichannel analyzer indicate that the biphasic rise and decay kinetics of M are unrelated to a photoreaction of the N intermediate, proposed earlier by others (Kouyama et al., *Biochemistry 27*, 5855-5863, 1988). Under conditions where N did not accumulate in appreciable amounts, conventional kinetic schemes sufficed. Conditions were then created where N accumulated in a photosteady state. The apparent increase in the of the slow M decay component by background illumination could be quantitatively accounted for by the mixing of the relaxation of the background-light induced photosteady-state with the inherent kinetics of the photocycle. Absorption of light by N appeared to generate only one intermediate, with a maximum near 610 nm.

Tu-Pos522

FTIR EVIDENCE FOR THE INTERACTION OF PRO-186 WITH THE RETINYLIDENE CHROMOPHORE OF BACTERIORHODOPSIN

K. J. Rothschild, Y.W. He
T. Mogi†, T. Marti†, and H. G. Khorana†
Dept. of Physics and Cellular Biophysics Program
Boston University, Boston, MA 02215
†Depts. of Chem. and Biol., MIT, Cambridge, MA 02139

The role of the three membrane embedded proline residues, Pro-50, Pro-91 and Pro-186, in the structure and function of bacteriorhodopsin has been studied by FTIR. All three prolines were replaced by alanine and glycine; in addition, Pro-186 was changed to valine. Difference spectra were recorded for the bR ⇒ K and bR ⇒ M photoreactions of each of these mutants and compared to wild type bacteriorhodopsin. Only substitutions of Pro-186 caused significant perturbations in the frequency of the C=C and C-C stretching modes of the retinylidene chromophore. In addition, these substitutions abolished signals previously assigned to the adjacent Tyr-185 for the bR ⇒ K photoreaction. Pro-186 ⇒ Val caused the largest alterations, producing a second species similar to bR_{548} and nearly blocking chromophore isomerisation at 78 K but not at 250 K. These results are consistent with a structural model of the retinal binding site in which Pro-186 and Tyr-185 are located in direct proximity to the chromophore. [NSF-PCM-8110992, NIH-GM28289-06 and ONR-N00014-82-K-0668 to HGK; NSF-DMB-8509587 and ONR-N00014-88-K-0464 to KJR.]

Tu-Pos523

UV/VIS TRANSIENT SPECTROSCOPY OF BACTERIORHODOPSIN: TWO FORMS OF THE MUTANT Y185F

M. Duñach, S. Berkowitz, T. Marti†, T. Mogi†,
H. G. Khorana† and K. J. Rothschild
Dept. of Physics, Boston University, Boston, MA 02215
†Depts. of Chem. and Biol., MIT, Cambridge, MA 02139

The photocycle kinetics of the bacteriorhodopsin mutant Tyr-185→Phe has been investigated by UV-Vis transient spectroscopy. Flash induced spectral changes were measured from 100 ns–500 ms using a gated optical multichannel analyser. At pH 5, the dominant photoactive species has a λ_{max} near 610 nm. This red-shifted species does not form any M-like intermediate and exhibits a photocycle similar to deionised blue membrane. At pH 8, the dominant photoactive form exhibits a λ_{max} near 550 nm. This purple species exhibits a photocycle which includes the normal K, L, M and O intermediates. However, M formation occurs in 2.5 μs, approximately 3 times faster than wild type bR at pH 8. In the UV region, a broad band near 300-310 nm is absent in the mutant relative to wild type. These results are consistent with the occurrence of protonation changes of Tyr-185 during the photocycle. In addition, Tyr-185 appears to help stabilise bR in its native conformation. It is hypothesised that replacement of Tyr-185 by Phe stabilises a protein conformation present in both blue membrane and the M intermediate.

NSF (PCM-8110992), NIH (GM28289-06), and ONR (N00014-82-K-0668) to HGK, and from the NSF (DMB-8509587) and ONR (N00014-88-K-0464) to KJR.

Tu-Pos524

RAMAN SPECTROSCOPIC EVIDENCE FOR A STABLE M-LIKE FORM OF THE BACTERIORHODOPSIN MUTANT ASP-85⇒ASN

P. Rath, M. Duñach, T. Marti†, T. Mogi†
H. G. Khorana† and K. J. Rothschild
Dept. of Physics and Cellular Biophysics Program,
Boston University, Boston, MA 02215
† Dept. of Chemistry and Biology, MIT
Cambridge, MA 02139

The Asp-85⇒Asn mutant (D85N) of bacteriorhodopsin (bR) is a blue colored pigment absorbing near 600 nm (in DMPC-CHAPS micelles at pH 6) with no known proton translocation capability. Recent studies using FTIR difference spectroscopy (Braiman et al., *Biochemistry* 27, 8516 (1988)) indicate that in native bR, Asp-85 is ionised and serves as the acceptor for the Schiff base proton during M intermediate formation. At pH 6, the photocycle of D85N resembles that of the blue membrane formed by deionisation or acid treament of purple membrane. The resonance Raman vibrational spectrum of D85N also shows marked similarities with blue membrane. D85N exhibits upon increasing pH a reversible transition to a species with a λ_{max} near 412 nm. The resonance Raman spectrum of this alkaline form of the mutant is similar to that of the M-intermediate of bR photocycle.
(NSF-DMB-8509857, NIH-EY05499-5 (KJR), NIH-GM28289-06, NSF-PCM-8110992 (HGK))

Tu-Pos525

THE DEFECTIVE PROTON PUMP, POINT-MUTATED BACTERIORHODOPSIN ASP96->ASN IS FULLY REACTIVATED BY AZIDE

Jörg Tittor, D. Oesterhelt, H.J. Butt* and E. Bamberg*
MPI for Biochemie, D-8033 Martinsried *MPI for Biophysik, D-6000 Frankfurt, FRG

Replacement of Asp96 by Asn leads to a drastically reduced M-decay (=reprotonation of the Schiff base) during the photocycle of BR. Addition of azide accelerates the M-decay to the wildtype value and restores proton pump activity of the protein. Under this condition the recovery of the initial state is not longer correlated with the decay of M. This opens the possibility to characterize the intermediates following after M. The activation energy of the M-decay in the mutant is higher in the presence of azide than in the absence despite the fact, that the decay time is drastically increased. These facts indicate that Asp96 serves as an internal proton donor, whereas the mutation leads to an increased activation energy, which is restored to the wiltyp value after addition of azide.

Tu-Pos526

STRUCTURE DETERMINATION OF BACTERIORHODOPSIN BY EPR SPECTROSCOPY. T.Marti, C. Altenbach+, W.L.Hubbell+, and H.G.Khorana, Depts. of Chem. and Biol., MIT,Cambridge, MA 02139; and Jules Stein Eye Inst.,Univ. of CA, Los Angeles, CA 90024. 18 single X→Cys mutants of bR (residues 125-142) were generated by site-directed mutagenesis and reacted with a specific spin label. To obtain structural information, the collision frequency of oxygen or chromium oxalate with the spin label was measured with power saturation EPR spectroscopy. Collision with chromium oxalate indicates location of the spin label in a water exposed region. Collision with oxygen depends on the location of the spin label with respect to the protein surface. It is highest for a spin label facing the hydrocarbon chains inside the membrane and lower for a spin label facing the protein interior or water. The combined results show that residues 129-131 form a very short water exposed loop while residues 131-138 show an oxygen accessibility that varies with a periodicity of 3.6 residues, indicating a transmembrane α-helix. A slight change in phase of the periodicity at position 140-142 can be explained by a helix tilt, in agreement with the known low resolution structure.

Tu-Pos527

PHOTOSENSORY BEHAVIOR OF *HALOBACTERIUM* AND *CHLAMYDOMONAS* WITH ISOMERIZATION-LOCKED RETINAL ANALOGS BING YAN[1], TETSUO TAKAHASHI[2], MOIRA LAWSON[2], RANDY JOHNSON[1], FADILA DERGUINI[1], KOJI NAKANISHI[1], JOHN L. SPUDICH[2] 1.Columbia University, New York, NY 10027. 2.Albert Einstein College of Medicine, Bronx, NY 10461.

An analog of all-*trans* retinal in which all-*trans*/13-*cis* isomerization is blocked by a carbon bridge from C12 to C14 was incorporated into the apoproteins of sensory rhodopsins I and II (SR-I and SR-II) in retinal-deficient *Halobacterium halobium* membranes. Blocking isomerization prevents the formation of the long-lived intermediates of the SR-I and SR-II photocycles (S_{373} and $SR\text{-}II_{360}$, respectively). Introduction of the locked analog into SR-I or SR-II apoprotein *in vivo* did not restore phototactic responses through any of the three known photosensory systems (SR-I attractant, SR-I repellent, or SR-II repellent), as assessed by sensitive (2% detection) computerized cell tracking and motion analysis. The results demonstrate that unlike the phototaxis receptor of *Chlamydomonas reinhardtii*, which has been reported to mediate physiological responses without isomerization of its retinal chromophore (Foster *et al.*,1989, Biochemistry, 28: 819), all-*trans*/13-*cis* isomerization is essential for SR-I and SR-II phototaxis signaling. We have applied computerized motion analysis also to the flash-induced swimming responses of *C. reinhardtii*. Addition of all-*trans* retinal to a phototaxis-deficient mutant (lts-1, strain CC2359) generates photophobic responses; effects of incorporating isomerization-locked analogs will be presented.

Tu-Pos528

TIME-RESOLVED FTIR DIFFERENCE SPECTROSCOPY OF BACTERIORHODOPSIN

M. S. Braiman, K. J. Wilson*, O. Bousché †, and K. J. Rothschild†. From the *Department of Biochemistry, University of Virginia School of Medicine, Charlottesville, VA 22908 and the †Physics Department, Boston University, Boston, MA 02215.*

Earlier time-resolved FTIR difference spectra of bacteriorhodopsin (bR) photoreactions were obtained using a "rapid-sweep" technique [Braiman et al., 1987, *Proc. Nat. Acad. Sci. USA* **84**, 5221], which resulted in 8- cm^{-1} spectral resolution for a time resolution of 5 ms. We have now developed a stroboscopic time-resolved FTIR difference technique that produces higher-resolution data. Portions of interferograms taken during different mirror scans were re-combined to form a complete interferogram. For the bR→M spectrum, 512 points (corresponding to 5.1 ms, or 0.158 mm of mirror travel) were taken from each scan. Twenty scans, with the photolysis flash timed to occur at a different mirror position in each, sufficed to build up a complete one-sided interferogram at 2- cm^{-1} resolution. Baseline drifts were corrected to first order by subtracting a flash-off reference scan, taken prior to each flash-on scan, before compiling points from different scans to form a complete difference interferogram. Averaging 2500 such difference interferograms gave a bR→M absorbance difference spectrum with $< 5\times10^{-4}$ *A* noise. This spectrum confirms the presence of difference bands in the 1270–1280 cm^{-1} region that were previously attributed to transient tyrosine protonation changes. *M.S.B. is a Lucille P. Markey Scholar and this work was supported by a grant from the Lucille P. Markey Charitable Trust.*

Tu-Pos529

STRATEGIES FOR SEMISYNTHESIS OF BACTERIORHODOPSIN. R. Renthal, T. Guth and D. Gonzalez, U. of Texas, San Antonio, TX

We are developing methods for semisynthesis of bacteriorhodopsin substituted with stable isotopes at each of 4 Asp residues involved in proton pump activity. 1) **Asp 212.** Chromophore regeneration of BR from proteolytic fragments V1 (residues 1-166) plus V2 (167-248) is unaffected by removal of the carboxyl terminal tail (232-248). The shortened fragment V2' (167-231) contains Asp 212 in a position ideal for semisynthesis by combining synthetic 210-231 and a CNBr peptide, CB164-209. 2) **Asp 85, 96 and 115.** Proteolytic fragment C1 (72-248) self-associates to dimers (K_d = 27 uM), whereas V1, V2 and C2 (1-71) do not. C1 also forms a pigment with the overlapping fragment V1. This suggests the possibility of preparing a 3-fragment complex of CB72-118 plus C1 plus C2. The resulting pigment has diminished absorbance at 550 nm. The chromophore inhibition by CB72-118 is independent of the C1:C2 ratio, implying a direct interaction with the C1-C2 complex. Insertion of the half helix 107-118 may cause the inhibition, since neither V2 nor CB119-145 affects chromophore regeneration.

Tu-Pos530

A RAMAN SPECTRASCOPIC STUDY OF OCTOPUS BATHORHODOPSIN. H. Deng, D. Mannor, G. Weng, P. Rath, R. Vandersteen., J. Lugtenberg, Y. Koutalos, T. Ebrey, M. Tsuda, and R. Callender. Physics Department(H. D., D. M., G. W., P. R.,and R. C.), City College of City University of New York , New York New York 10031. Chemistry Department (R. V., and J. L), Leiden University , 2300 RA Leiden, The Netherlands. Department of Physiology and Biophysics (Y. K., and T. E.), University of Illinois at Urbana-Champaign, Urbana-Champaign, Illinois 61801. Department of Physics (M. T.), Sapporo Medical College, Sapporo, Japan.

We have obtained resonance Raman spectra of octopus bathorhodopsin regenerated with retinal deuterium-labeled at 7, 8, 10, 11, 12, 14, and 15th positions to study its hydrogen out of plane vibrational modes. Although the hydrogens along the retinal are widely perturbed by the opsin just like that in bovine bathorhodopsin, the bands positions and intensity patterns are quite different. The spectra will be presented and the significance of the difference between bovine and octopus bathorhodopsin will be discussed.

Tu-Pos531

PROTONATION STATE OF ASP (-GLU) 85 REGULATES PURPLE TO BLUE TRANSITION IN BACTERIORHODOPSIN MUTANTS ARG82→ALA AND ASP85→GLU S.Subramaniam, T.Marti and H.G.Khorana, Mass. Inst. of Technology, Cambridge, MA 02139. Site-directed mutagenesis experiments with bacteriorhodopsin show that between pH 5.5 and 7, the Asp85→Glu and Arg82→Ala mutants exist in a pH-dependent equilibrium between purple (λmax ∿550/540nm) and blue (λmax ∿600/590nm) forms of the pigment. We show here that the steady state proton pumping activities for the above mutants reside exclusively in their respective purple species. The Asp85→Asn mutant displays a blue chromophore (λmax ∿588nm), is inactive in proton translocation over the pH range 5 to 7.5, and shows no transition to the purple form. In contrast, the Asp212→Asn mutant is purple (λmax ∿555nm) at neutral pH and shows no transition to a blue chromophore with decreasing pH. The experiments show that the relative strengths of interaction between the protonated Schiff base, Asp85, Asp212 and Arg82 regulate the color and function of bacteriorhodopsin. (Supported by grants from the NIH and ONR to HGK and the Damon Runyon-Walter Winchell Cancer Fund to SS).

Tu-Pos532

ROLE OF ASP-85, ASP-212, ARG-82 AND ASP-96 IN THE RELEASE AND UPTAKE OF PROTONS BY BACTERIORHODOPSIN. H.Otto, T.Marti, M. Holz, T.Mogi, M.Lindau, L.Stern, S. Subramaniam, M.P.Heyn and H.G.Khorana, Freie Universitat Berlin and Mass. Inst. of Technology, Cambridge, MA 02139. Photocycle, photovoltage and pH-indicator experments with the mutants Asp-96→Asn and Asp-96→Ala show that Asp-96 serves as an internal donor in the reprotonation of the Schiff base (SB). The SB is first reprotonated from Asp-96 which is subsequently reprotonated from the cytoplasm. This conclusion is supported by measurements on wild-type which show that the charge displacement associated with the decay of M splits up in two components at alkaline pH. In the mutants Asp-85→Asn, Asp-85→Ala, Asp-212→Asn, Arg-82→Ala and Arg-82→Gln the early events in the photocycle, charge translocation and proton release are strongly perturbed. In the blue mutants Asp-85→Asn and Asp-85→Ala the pK of the SB deprotonation is lowered to 7 and 8.2 respectively suggesting a role for Asp-85 as part of a complex counterion.

Tu-Pos533

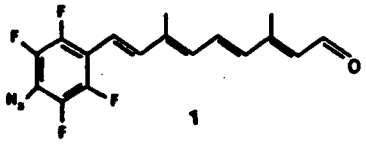
SOLID STATE NMR INVESTIGATIONS OF THE MECHANISM OF PROTON PUMPING IN BACTERIORHODOPSIN: TYROSINATE IS NOT FOUND IN BR_{568} OR THE M INTERMEDIATES

L.K. Thompson, A.E. McDermott, M.R. Farrar & R.G. Griffin, MIT; C. Winkel & J. Lugtenburg, Univ. of Leiden; R.S. Brown & J. Herzfeld, Brandeis Univ.

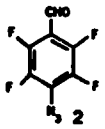
The mechanism of light-driven proton pumping by bacteriorhodopsin (bR) is thought to include proton transfer among ionizable amino acids forming a "proton wire" across the membrane. In particular, FTIR and UV spectroscopies have yielded evidence for the presence of a tyrosinate (Tyr^-) residue in bR_{568} and the M intermediates of the photocycle. A recent solid state NMR study of [4'-^{13}C]Tyr-labelled bR and tyrosine model compounds demonstrated that a single Tyr^- would give a well-resolved, observable resonance in bR, shifted 9 ppm downfield from the Tyr resonance. Such a tyrosinate was not observed in the dark-adapted protein, until the protein began to denature above pH 12. We have extended these studies to determine whether a tyrosinate is formed in the light-adapted protein (bR_{568}) or the M photointermediates. The ^{13}C-14 retinal resonance, which is sensitive to the retinal conformation, is used to monitor thermal trapping of the bR_{568} and M states. Two forms of M, which may correspond to M_{fast} and M_{slow}, are trapped at low temperature at pH 10, in the presence and absence of 500 mM guanidine hydrochloride. Although we cannot rule out small chemical shift changes corresponding to unusual Tyr interactions or hydrogen bonding, we can rule out the large chemical shift changes corresponding to deprotonation. We conclude that Tyr^- is not formed in bR_{568} or either form of M.

Tu-Pos534

A NOVEL PHOTOAFFINITY LABEL FOR BACTERIORHODOPSIN CJ Beischel, V Mani, EA Cohen, DR Knapp, RK Crouch. Departments of Pharmacology and Opthalmology, Medical University of South Carolina. Tetrafluoro-4-azidoretinal 1 has been synthesized and fully characterized as a potential photoaffinity label for bacteriorhodopsin. When incubated with bacterio-opsin this retinal analogue rapidly forms a stable analogue pigment with an absorption maximum at 524nm. Model photolysis studies have been performed with the readily synthesized tetrafluoro-4-azidobenzaldehyde 2 to determine the appropriate conditions for the activation of the photo-activatible azido moiety. Irradiation of 2 in hydrocarbon solvents indicates the formation of C-H insertion based addition products. Time course studies of the disappearance of the azido functionality (by FTIR) and the appearance of the addition product (by GC-MS) indicate that the appropriate conditions are unlikely to harm the pigment. Similar model studies are underway with a flash lamp apparatus with the objective of activating the analogue pigment at certain stages in the photocycle. (Supported by R01-EY04939 and EY08239)



1



2

Tu-Pos536

SOLID-STATE ^{13}C NMR OF [ε-^{13}C]LYS-BACTERIORHODOPSIN: STUDIES OF THE SCHIFF BASE CONFORMATION IN BR_{555} AND BR_{568} AND M INTERMEDIATES

M.R. Farrar, K.V. Lakshmi, R.S. Brown, & J. Herzfeld, Brandeis Univ.; S.O. Smith, R.G. Griffin, MIT; J. Raap, J. Lugtenburg, Univ. of Leiden

Previous solid-state ^{13}C NMR studies of bR have used the [14-^{13}C]retinal resonances to deduce the C=N configuration of the retinal-lysine Schiff base (SB) linkage. Here we extend these studies using [ε-^{13}C]Lys-bR. Proximity to the SB linkage causes the chemical shift of Lys-216 to be downfield from the remaining 6 lysines in bR. The chemical shift in bR_{555}, 48.1 ppm, is ~5 ppm upfield from that in bR_{568}, 53.3 ppm, due to steric interaction with the proton on C-14 of the retinal chromophore when the CN bond is in the syn configuration. Deprotonation of the SB upon formation of the M intermediate results in a downfield shift of the resonance to 59.9 ppm. This form of M, thermally trapped at pH 10 in 500 mM guanidine HCl, is thought to be CN anti. Results on the M intermediate trapped at pH10 in 100 mM NaCl, which is expected to be CN syn, will also be presented.

Tu-Pos535

Membrane Flapping During the Photocycle of Bacteriorhodopsin

József Czégé and Lou Reinisch

Laser Biophysics Center, USUHS, Bethesda, MD

Changes in the ultraviolet light scattering from a suspension of purple membrane fragments were detected during the photocycle of bacteriorhodopsin with a cross-correlation method. The scattered light intensity from the sample was measured on a logarithmic time scale of 1 μs to 0.1 s at pH 4.6 after the photocycle was initiated with a polarized 532 nm laser flash. A simple model of curved sheets with positive and negative changes in the curvature is used to describe the observed light scattering changes. The initial change, a decrease in the curvature of the asymmetrically charged membrane fragments, occurs faster than 10 μs. This is followed by a forced increase in the membrane curvature. Finally, the membrane relaxes to its initial, slightly curved state exponentially in time and slightly slower than the photocycle. The changes in the curvature are more than likely driven the by the motion of the proton during the photocycle. (This work supported in part by the Office of Naval Research, Grant N00014-WR-24020 and SDIO.)