T-Pos197 SIZE AND CHARGE HETEROGENEITY OF SLOW-TYPE ISOFORMS IN CHICKEN STRIATED MUSCLE. H. Takano-Ohmuro[1,2], S.M. Goldfine[1] and D.A. Fischman[1]. [1]Department of Cell Biol. and Anat., Cornell Univ. Med. Col., NY, NY 10021 and [2]Medical Engineering Sect., Tokyo Met. Inst. of Med. Sci., Bunkyo-ku, Tokyo 113.

Four C-protein variants have been identified in adult chicken striated muscles based on differential antigenicity, ion exchange chromatography and SDS-PAGE Mr (1,2). We now report that C-protein isoforms can be distinguished by IEF and NEPHGE 2-D gels and this technique used for examining C-protein expression in vivo and in cell-free translates. Crude myosin was prepared from adult pectoralis (PM), ALD, PLD and ventricular muscles, and from PM of 13d and 19d embryos. Slow, fast and cardiac-type isoforms were identified with McAbs ALD-66, MF-1, and C-315 (1,3). The fast-type isoforms in adult and 19dE PM were indistinguishable by 2-D gels. The same was true for the cardiac-type isoforms expressed in adult and 13 dE PM. In contrast, at least 4 slow-type C-proteins could be identified; these differed in both charge and Mr. Since identical variants were synthesized in reticulocyte lysates containing mRNA from these muscles, the isoforms are unlikely to result from post-translational modifications. We suggest the presence of at least 6 C-protein transcripts and isoforms in chicken striated muscles.

1. Reinach et al. J. Cell Biol. (1982) 95:78-84; 2. Obinata et al. Devel. Biol. (1984) 101:116-124; 3. Kawashima et al. J. Biochem. (1986) 99:1037-1047. Supported by NIH grant (AM 32147) and by a grant from the Keck Foundation.

T-Pos198 MYOSIN ISOFORMS IN SINGLE MUSCLE FIBRES FROM MOUSE SOLEUS. S. Wylie and D.J. Parry, Dept. Physiology, Univ. Ottawa, Ottawa Canada K1H 8M5.

Single muscle fibres were dissected out from mouse EDL and soleus which had been either freeze-dried or chemically skinned. Two-dimensional gels of freeze-dried soleus fibres revealed at least three (and possibly four) distinct combinations of light chains within a single fibre. Of 35 fibres examined, 13 (37%) contained only LC_{1s} and LC_{2s}, 20% only LC_{1f} and LC_{2f} while 25 fibres showed a combination of fast and slow light chains. Of these hybrid fibres most (35% of the total pool) contained four light chains while 9% lacked LC_{2s}. The fibres, which exhibited the fast light chains alone were the only ones to contain a spot on the 2-D gel which appears to be parvalbumin. All fibres from EDL appeared at least qualitatively identical insofar as only two light chains were seen (LC_{1f}, LC_{2f}) and all contained parvalbumin. Mouse EDL is known to contain few, if any, type I fibres. In a group of fibres, which had been chemically skinned, one dimensional gel electrophoresis was performed using the method of Carraro & Catani (Biochem. Biophys. Res. Commun. 116: 793- 801), i.e. 6% polyacrylamide gels containing 30% glycerol. This allowed resolution of the myosin heavy chains. In this initial series of 26 fibres from soleus, 58% contained type I MHC, 31% type IIA MHC and 11% contained both, although the relative proportions of the two varied among these fibres. Immunohistochemistry using monoclonal antibodies directed against the various MHC (courtesy of S. Schiaffino, Padova, Italy) has revealed between 5 and 10% of hybrid fibres in soleus. We are presently attempting to determine both the MHC and MLC pattern in the same fibre, in the hope that the various combinations may shed some light on the apparent inconsistency between the proportion of histochemically "slow" fibres and physiologically slow motor units (e.g. Lewis et al., J. Physiol. 325: 393-401). (Supported by Medical Research Council of Canada)

T-Pos199 MYOSIN ISOZYME TRANSITIONS DURING AVIAN DEVELOPMENT. **J. I. Rushbrook, C. Weiss, T.T. Yao and J.-M. Lin. Dept. Biochemistry, SUNY-Health Science Center at Brooklyn, New York 11203.**

Myosin isoform changes during development in the avian pectoralis major muscle (PM) were monitored by reverse-phase HPLC of S-1 tryptic fragments, SDS-gel electrophoresis and amino acid sequencing. 50 kDa fragments characteristic of adult and post-hatch myosin were identified, the latter prodominating between 12 and 20-days post-hatch. A third 50 kDa fragment was prominent at 5-days post-hatch. This species was present in greater amounts in the more slowly developing New Hampshire and Rhode Island Red strains than in the White Leghorn line, suggesting it may have an embryonic or perinatal isoform origin.

20 kDa species characteristic of adult and post-hatch isoforms each contained three components separable by HPLC and SDS-gel electrophoresis. Preliminary sequencing data indicates that cleavage to form the adult 20 kDa fragment occurs at a lysine triplet, residues 638-640 in the heavy chain sequence (1) and that the three 20 kDa fragments produced differ in the lysine cleaved.

(1) Strehler, E. E., Strehler-Page, M.-A., Perriard, J.-C., Periasamy, M. and Nadal-Ginard, B. (1986) J. Mol. Biol. 190, 291-317.

T-Pos200 HIGH RESOLUTION CHROMATOGRAPHIC ANALYSIS OF MYOSIN ISOFORMS IN AVIAN MUSCLES. **J. I. Rushbrook, C. Weiss and T.T. Yao. Dept. Biochemistry, SUNY-Health Science Center at Brooklyn, New York 11203.**

Pectoralis major (PM), posterior latissimus dorsi (PLD) and adductor superficialis (AS) muscles contain the following fiber types: PM-99% type IIB; PLD-87% type IIB, 13% type IIA; AS-10% type IIB, 87% type IIA, 3% type 1 (1). The myosin isoform content of these muscles was analyzed by high resolution anion-exchange chromatography of S-1 LC1 and S-1 LC3 species. PM myosin contained single major LC1 and LC3 isoforms (IIB). The finding of two major peaks contrasts with rabbit where four such species were present (2). The AS preparation, in addition to small amounts of IIB isoforms, contained 2 major peaks and 2 minor ones. The major peaks constituted 70% total protein and therefore contained type IIA myosin. The isoform contents of PM and AS muscles thus approximate predictions from histological studies, apart from the additional minor peaks in the AS.

The PLD chromatogram contained six major species. Two (40%) corresponded to the PM isoforms (type IIB), two (40%) to the AS major species (type IIA). The remaining peaks (20%) co-migrated with the unknown peaks in the AS profile. These peaks did not correspond to either slow tonic or PM post-hatch isoforms. Their origin as new isoforms or species differing in post-translational modifications is under investigation. (1) Barnard et al. (1982) J. Physiol. 331, 333-354. (2) Burke et al. (1986) J. Biol. Chem. 261, 253-256.

T-Pos201 ISOLATION OF NATIVE MYOSIN FROM MAMMALIAN MUSCLES USING HIGH PERFORMANCE ION-EXCHANGE CHROMATOGRAPHY. MJ Lema, MG Pluskal*, PD Allen and FJ Julian, Dept. of Anesthesia Research, Brigham & Women's Hospital, Boston, MA; *Dept. of Biochem. Research, Millipore Corp., Bedford, MA.

Ion-exchange high performance liquid chromatography (HPLC) was performed using extracts from small samples (20-30 mg) of various muscles to evaluate its capability of isolating native myosin. Rabbit soleus, semitendinous, left ventricle and uterus muscles, and biopsed human ventricular muscles were homogenized and myosin extracted as previously described (Lema MJ, et al. Circ. Res. 59(2):115,1986). All experiments were conducted at 4°C using a DEAE-5PW column (7.5 mm x 7.5 cm), two M6000A pumps, a U6K injector, a 660 solvent programmer, a 440 multi-wavelength detector set at 280 nm, and a 730 data module recorder (Waters). Column equilibration was achieved with 50 mM $Na_4P_2O_7$, 1 mM EDTA, pH 8.8, at a flow rate of 1 ml/min for 30 min. Extracts were injected into a sample loop and unretained material was eluted for 10 minutes. Myosin was then eluted for 20 minutes using a linear gradient of 0-0.6 M KCl in 20 mM Tris HCl pH 7.6 and the peaks collected. One dimensional high resolution 16% acrylamide gel electrophoresis with 1.6% lithium dodecyl sulfate (Kolbe HVJ, et al. J. Biol. Chem 259:9115, 1984) using the collected fractions confirmed the presence of myosin essentially free from contamination. Ca^{2+} and K^+/EDTA ATPase activities of human cardiac myosin were unchanged between the extract values (0.87 and 1.05 μmol Pi/mg protein/min) and the isolated HPLC fraction values (0.9 and 1.06). We conclude that highly purified myosin can be isolated from small samples using ion-exchange HPLC while maintaining full biological activity. Supported by the Parker B. Francis Investigatorship (MJL), NIH grants HL 27231 (PDA) and HL 35032 (FJJ).

T-Pos202 PLATINUM SHADOWING OF ISOLATED FISH MUSCLE THICK FILAMENTS. Robert W. Kensler. Department of Anatomy, The Medical College of Pennsylvania, Philadelphia, PA 19129.

We have previously demonstrated that skeletal muscle thick filaments from the goldfish (*Carassius auratus*) can be isolated with the helical arrangement of the myosin crossbridges largely preserved as indicated by optical diffraction analysis of electron micrographs of the isolated and negatively-stained filaments (Kensler. 1986. Biophys. J. 49:263a - abstract). Optical diffraction patterns show a series of layer lines indexing near the expected positions for a 43 nm helical (or near helical) repeat consistent with previous X-ray diffraction data (Harford and Squire. 1982. J. Muscle Res. & Cell Motil. 3:481- 482). We have now examined the appearance of the isolated fish filaments after platinum shadowing. Images of the shadowed filaments show a series of subunits arranged along a series of right-handed near-helical strands that occur every 43 nm axially along the filament arms. The optical diffraction patterns of the shadowed fish filaments show a departure from the pattern expected for helical symmetry similar to that we previously demonstrated for shadowed frog thick filaments (Kensler and Stewart. 1986. Biophys. J. 49:343-351) and which appears consistent with a regularly occurring perturbation in the helical arrangement of the myosin crossbridges. Optical filtrations of images of the shadowed fish filaments show 4-5 subunits per half-turn of the strands, consistent with a three-stranded arrangement of the crossbridges, as we have shown for frog thick filaments (Kensler and Stewart. 1983. J. Cell Biol. 96:1797-1802). Supported by UPHS grant AR30442.

T-Pos203 OPTICAL ANALYSIS OF ANTIBODY BINDING TO MYOSIN S-1 IN MUSCLE FIBERS. M. Jones, R. J. Baskin, and Y. Yeh, Depts. of Zoology and Applied Science, University of California, Davis, California, 95616.

Monoclonal antibodies were generated against frog skeletal muscle myosin by fusing NS/O myeloma cells with spleen cells from mice immunized with partially purified (according to SDS-gel electrophoresis) frog skeletal muscle myosin. The products of this fusion were screened by a solid phase immunoassay and the desired antibody secreting hybridomas were cloned by limiting dilution. Anti-S-1 activity was detected using immunoblots of SDS-gel electrophoretic patterns of chymotrypsin-digested myosin synthetic filaments in the presence of EDTA. Anti-S-1 antibody was added to a chemically skinned frog skeletal muscle fiber bathed in a solution containing 100 mM KCl, 1 mM $MgCl_2$, 30 mM EGTA, 10 mM imidazole and 4 mM ATP, pH = 7.0. Diffraction patterns were produced with a helium-neon laser and analyzed with optical ellipsometry. Control fibers were incubated with a monoclonal antibody directed against a microbial protein. Preliminary results are consistent with an increase in optical density of the A-band due to antibody binding, resulting in an increase in the phase angle and diffraction order intensity. Measurements of r, the depolarization ratio, gave results that are consistent with our earlier conclusions relating the value of r to average cross-bridge orientation. Work supported by NIH Grant #AM-26817.

T-Pos204 A MYOSIN SITE POSSIBLY ESSENTIAL TO ENERGY TRANSDUCTION IN MUSCLE CONTRACTION. TakayukiMiyanishi**†, Keisuki Horiuti**, Makoto Endo**, Genji Matsuda* (*Department of Biochemistry, Nagasaki University, School of Medicine, **Department of Pharmacology, Faculty of Medicine, University of Tokyo, †Cardiovascular Research Institute, University of California, San Francisco)

An antibody against the 25 K fragment of myosin heavy chain from chicken skeletal muscle was applied to a physiological preparation of rabbit skinned fibers. The antibody specifically and selectively abolished the capability of the fiber to develop active tension. A biochemical study revealed that the antigenic site was approximately the 77th to 80th amino acid residues from the N-terminus of the 25 K fragment, and was outside of the proposed ATP binding region on the heavy chain. The region identified in this study probably plays an important role in energy transduction in muscle contraction. This finding is consistent with our previous finding that a modification of a reactive lysine (Lys-83) results in a complete loss of key reaction intermediate responsible for muscle contraction. The antigenic site on the myosin head can be visualized by electron microscopic study. This work was done in collaboration with Drs. Chikashi Toyoshima and Takeyuki Wakabayashi (Univ. of Tokyo, Faculty of Science).

T-Pos205 HYDROSTATIC PRESSURE STUDIES OF SYNTHETIC THICK FILAMENTS PREPARED BY DILUTION Santa J. Tumminia, Jane F. Koretz, and Joseph V. Landau, Biophysics and Biochemistry Group and Biology Department, RPI, Troy, NY 12180-3590

Synthetic thick filaments were prepared from solutions of column-purified rabbit skeletal muscle myosin by rapid dilution at constant velocity to 0.10 M KCl, 10 mM imidazole, pH 7.0. The time course over which dilution took place was varied at intervals between "0" seconds and 120 seconds, and the resultant population placed in dialysis bags and exposed to increased hydrostatic pressure at 2000 psi intervals up to 14,000 psi. After pressure release, the treated populations were examined in the electron microscope. Filaments made as fast as possible (t="0") were .478 ± 116 microns in length. With increasing hydrostatic pressure, average filament length shortened slightly (.396 ± .097 microns) and the length distribution narrowed; it was impossible to determine, however, whether "skinny" filaments were generated because of poor filament organization initially. Filaments made over 60 second dilution, in contrast, were .980 ± .425 microns long, shortening steadily with increasing hydrostatic pressure to .419 ± .116 microns, the final length of the t="0" filaments and pressure-treated synthetic thick filaments made by dialysis. "Skinny" filaments were visible after pressure treatment of the t=60 filaments, again like the dialysis case. Filaments made at intermediate time courses showed intermediate behavior under pressure. Thus, filaments prepared by either dilution or dialysis show the same type of pressure sensitivity despite differences in apparent organization.

T-Pos206 STRUCTURE OF PARACRYSTALS OF MYOSIN ROD.
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In order to study the packing of myosin tails in the shaft of the vertebrate striated muscle thick filament, assemblies of the proteolytic fragment, myosin rod, equivalent to the whole tail, have been investigated using electron microscopy and image analysis. A large, single layered, ribbon-like species of myosin rod paracrystal, formed at 100 mM K^+, pH 8.0 and in the absence of divalent cations, was found to be suitable for structural study. The rod molecules, comprising the aggregates, appeared to be arranged in longitudinal filaments, and were sensitive to pH and addition of divalent cations in a similar manner to myosin in thick filaments. Optical diffraction analysis of images of negatively stained paracrystals indicate an approximately rectangular unit cell measuring 43.0 nm axially and 12.4 nm laterally. Often patterns are dominated by axial information and the presence of pertubations in the crystallinity of the structures is also strongly suggested. Fourier based image analysis indicates the presence of two antiparallel filaments in a unit cell and it is likely that the filaments consist of parallel rod molecules, staggered by 43.0 nm, and may be related to subfilaments in the thick filament. To obtain a more detailed and accurate structure, suspensions of unstained, unfixed paracrystals have been imaged, in the frozen-hydrated state, using cryo-electron microscopy. Transforms of such images, exhibiting a high signal:noise ratio, indicate a similar structure. Tilted views of frozen-hydrated paracrystals are being obtained so that 3-dimensional maps may be built.

T-Pos207 EVIDENCE FOR A DIFFERENCE IN THE ROLE OF THE LMM AND S2 PORTIONS OF THE MYOSIN MOLECULE IN LENGTH DETERMINATION. P. Chowrashi and F. Pepe. Dept. of Anatomy, Univ. of Penn. Phila. PA 19104.

The assembly properties of the LMM and rod fragments of myosin were compared to those of the intact myosin molecule, under the same conditions. From these studies some insight was obtained about the involvement of the LMM, S2 and S1 portions of the molecule in filament assembly. The conditions used were those which have been shown to give sharp length distributions around 1.5 um, or broad length distributions around the same length with intact myosin. Conditions which produce a sharp length distribution for myosin assembly produce LMM paracrystals with a sharp 43 nm axial repeat and those which produce a broad length distribution produce LMM aggregates which are non-periodic. Conditions which initally give a broad myosin filament length distribution and which with time lead to a redistribution to a sharp distribution around 1.5 um, concomitantly lead to the assembly of non-periodic LMM paracrystals which with time reorganize into paracrystals with a 43 nm axial repeat. The presence of the S2 portion of the molecule on the rod fragment leads to different assembly properties. Only a 14.3 nm axial repeat is obtained under all the conditions studied. We conclude the following: a) The assembly properties of the LMM portion of the molecule can be correlated with those of the intact molecule. Therefore, the LMM portion of the molecule is probably significantly involved in precise length determination for the myosin filament. b) The presence of the S2 portion of the molecule eliminates this correlation in the assembly with that of intact myosin. c) The S1 portion may, therefore, be involved in diminishing the effect of the S2 on the assembly of the intact molecule.

T-Pos208 STUDIES ON THE CONFORMATION OF MYOSIN S1 CONTAINING TRAPPED MgPPi or MgADP.
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The conformations of S1, containing MgPPi or MgADP trapped by the covalent bridging of the SH1 and SH2 thiols, was examined by employing the trifunctional, photoactivatable reagent bismaleimidyl-benzophenone. Modifications of S1 by this reagent in the presence of these ligands indicate that the two ATPase-related thiols SH1 and SH2 are bridged by this reagent and are constrained to lie between 1.3 and 1.8nm from one another. Photolysis of the modified S1 results in crosslinking between the 21kDa segment and the other segments of the heavy chain and this is found to be dependent on whether MgPPi or MgADP is trapped in the protein. For S1 with trapped MgPPi, the photo-induced crosslinking proceeds with low efficiency and crosslinking to both the 50kDa and the 27kDa segment is observed. When S1 with trapped MgADP is photolysed, the crosslinking occurs with high efficiency and is almost exclusively restricted to the 50kDa segment. The distance between these thiols and the linked residues in the 27kDa and the 50kDa segments ranges from 0.96 to about 1.04nm. These data suggest that trapping of MgPPi does not markedly immobilize the S1 structure and the 27kDa and 50kDa segments come within the crosslinking span of the benzophenone triplet only occasionally. On the other hand, the trapped MgADP causes significant immobilization of S1, at least with respect to the bridged thiols and a segment of the 50kDa segment. These results are consistent with the reports of several laboratories that the 50kDa segment is intimately involved in the ATPase function. Moreover, the data suggest that the ability of the SH1 and SH2 thiols, on modification to perturb the ATPase properties of myosin may be due to their perturbation of the 50kDa segment. Supported by USPHS grant NS 15319.

T-Pos209 THE STATE OF AGGREGATION OF MYOSIN S1 IN SOLUTION.
Walter F. Stafford & Sarkis S. Margossian, Department of Muscle Research, Boston Biomedical Research Institute, Boston, MA 02114 and Department of Orthopedic Research, Montefiore Medical Center, Bronx NY 10467

The question of myosin S1 dimerization in solution was investigated using high speed equilibrium ultracentrifugation. Experiments performed in 0.115 M KCl, 2 mM DTT, 0.3 mM EGTA and 50 mM imidizole (pH 7.0) revealed a uniform distribution of a single thermodynamic species with an M_r of 110 kDa for rabbit S1 prepared by α-chymotryptic digestion of myosin. When similar experiments were performed in the presence of nucleotides, it was reported that they induced formation of S1 dimers which could be visualized in the electron microscope and that the dimers had a higher MgATPase activity than the monomers (Bachouchi, N., et al., (1986) J. Mol. Biol. 191, 247). In view of the importance of these observations, these studies were repeated using 20 mM KCl, 2 mM DTT, 0.3 mM EGTA, 2 mM ADP and 5 mM $MgCl_2$, which was reported to favor pure dimer formation. No dimers of rabbit S1 could be detected. Including 0.1 mM $CaCl_2$ in the buffer did not generate dimerization either. In addition, an S1 from a different source, namely from rat cardiac myosin, behaved in a manner similar to skeletal S1. These results confirm those of our original report (Margossian, et al. (1981) Biochemistry 20, 2151) in that no dimerization of S1 occurs in buffers commonly used in kinetic or actin binding studies. (Supported by NIH grant HL 26569 (SSM)).

T-Pos210 TWO SEGMENTS SEPARATED BY ~45 kDa IN THE HEAVY CHAIN ARE CLOSE TO THE SH-1 OF MYOSIN S1. Kazuo Sutoh[+] & Renné Chen Lu[*,+] Dept. of Biophys. & Biochem., Univ. of Tokyo, Tokyo 113, Japan & [*]Dept. of Muscle Res., Boston Biomed. Res. Inst. Boston, MA 02114

The thiol specific photoactivatable reagent benzophenone iodoacetamide (BPIA) can be selectively incorporated into the SH-1 of myosin S1 and upon photolysis an intramolecular crosslink is formed between SH-1 and the N-terminal 25 kDa region of S1. If a Mg^{2+}-nucleotide is present during photolysis, crosslinks can be formed either with the 25 kDa or the central 50 kDa region (Lu et al., PNAS 83 6392, 1986). Heavy chains (HC) with these two types of intramolecular crosslinks and uncrosslinked HC have different mobility on SDS-PAGE and therefore can be purified electrophoretically. Each type of HC was cleaved with S. aureus protease, chymotrypsin or lysyl endopeptidase. The cleavage points were estimated based on the molecular weights of peptides containing the N-terminus which was identified with the use of antibody. Locations of the crosslinks were determined by comparing the peptide maps of crosslinked and uncrosslinked HC. The results indicate that the segement located about 12-16 kDa from the N-terminus of HC can be crosslinked to SH-1 via BPIA independently of the presence of a nucleotide, whereas the segment located 57-60 kDa from the N-terminus can be crosslinked to SH-1 only in the presence of a Mg^{2+}-nucleotide. Using the avidin-biotin system it has been shown that SH-1 is located 13 nm from the head/rod junction (Sutoh et al.,JMB 178 323, 1984). Since BPIA spans less than 1 nm, our results show that two regions, separated by 400 amino acid residues and located in the 25 kDa and 50 kda domains of S1, respectively, are also part of the head structure about 12-14 nm from the head/rod junction. Supported by grants from NIH (AM-28401 & HL 5949) and MDA.

T-Pos211 NUCLEOTIDE & ACTIN EFFECTS ON THE CONFORMATION OF MYOSIN S1 AS SHOWN BY INTRAMOLECULAR CROSSLINKING. R.C. Lu, L. Nyitray, A. Wong, & J. Gergely, Dept. Muscle Res., Boston Biomed. Res. Inst.; Depts. Neurol. & Biol. Chem., Harvard Med. Sch.; Dept. Neurol., MGH, Boston, MA 02114

The thiol specific photoactivatable reagent benzophenone iodoacetamide (BPIA) can be selectively incorporated into the SH-1 of myosin S1 in the absence of nucleotide. An intramolecular crosslink forms upon photolysis with the N-terminal 25 kDa region. If a Mg^{2+}-nucleotide is present during photolysis, crosslinking takes place either with the 25 kDa or with the central 50 kDa region. In the presence of Mg^{2+}-ATP SH-2 is also modified; the crosslinking via SH-2 with the 50 kDa region is nucleotide independent (Lu et al., PNAS 83 6392, 1986). Further studies show that crosslinking of S1 does not prevent the nucleotide induced conformational change resulting by tryptic cleavage in 21 kDa and 47 kDa peptides derived from the 25 kDa and 50 kDa regions, respectively, previously described for uncrosslinked S1 (Muhlrad & Hozumi, PNAS 79 958, 1982; Mocz et al., Eur. J. Biochem. 145 221, 1984). S1 modified with BPIA retains its ability to bind actin both before and after photolysis. Crosslinking of SH-1 with the 25 kDa region is not affected by the presence of actin, whether non-covalently bound or crosslinked with a carbodiimide, but the Mg^{2+}-nucleotide induced crosslinking with the 50 kDa region is inhibited. The nucleotide independent crosslinking between the 20 kDa and 50 kDa regions via SH-2 is also inhibited by actin. Our results suggest that actin induced conformational changes affect the relation of the 20 kDa and 50 kDa regions as well as some of the nucleotide induced structural changes in S1. Grants from NIH (AM-28401 & HL 5949) and MDA.

T-Pos212 SELECTIVE FLUORESCENT LABELING OF THE N-TERMINAL DOMAIN OF ACTIN AND THE 50K REGION OF THE S-1 HEAVY CHAIN. R.Bertrand,E.Audemard,P.Chaussepied,D.Mornet and R.Kassab. Centre de recherches de Biochimie Macromoleculaire, CNRS, Montpellier, FRANCE.

The water soluble 1-ethyl-3(-3-dimethylaminopropyl)carbodiimide (E.D.C.) and the hydrophobic N-ethoxycarbonyl-2-2' ethoxy-1-2 dihydroquinoline (E.E.D.Q.) were used as environnement-sensitive carboxyl groups activators for the specific incorporation within skeletal F-actin or Myosin-Subfragment-1 (S1), of the fluorescent nucleophilic label, 5-(2-aminoethylamino)1-naphthalene sulfonic acid (E-DANS).

F-actin was selectively labeled in the presence of EDC with the incorporation of 0.75 mole E-DANS /mole actin and the modification did not alter its polymerization properties.The major site of labeling was localized by limited proteolytic digestion with Staphylococcus protease and thrombin in the N-terminal segment including residues 1-28 (0.5 mole /mole peptide as calculated from amino acid analysis and spectroscopic measurements).

Subfragment-1 was modified using either EDC or EEDQ. Limited proteolysis by trypsin or Staphylococcus protease showed the fluorescent label to be incorporated mainly in the 50K fragment or in the 48K and 22K C-terminal heavy chain fragments respectively.However when starting with the preformed trypsin-split (27K-50K-20K)-S1 and S.protease-split (28K-48K-22K)-S1 the label was incorporated with EEDQ selectively into either the 50K or 48K central fragments and with EDC into the 22K part. The biological properties of the labeled S1 and actin are under study. (Supported by Grants from CNRS and MDA).

T-Pos213 THE PRESENCE OF ARGINYL RESIDUES AT THE ACTO-MYOSIN INTERFACE. R.Bertrand,D.Mornet and R.Kassab Centre de Recherches de Biochimie Macromoleculaire, CNRS, Montpellier,FRANCE.

The involvement of reactives arginyl side chains at the Actin-myosin interface was investigated by two approaches using A)-limited proteolysis of S1 by the arginine specific protease from mouse submaxillaris glands (ARG-C) B)-chemical crosslinking of the acto-S1 complex by phenyldiglyoxal (P.D.G.) which spans 6-8Å.

The ARG-C cleaves the 95K S1 heavy chain into only two fragments, 75K (N-terminal) and 21K (C-terminal), whereas the light chains were unaffected. The cleavage rate was decreased by Mg-ATP or Mg-ADP and it was completely abolished by F-actin. The actin-activated Mg-ATPase was concomittantly lost during the heavy chain cission and the clip site now under study is thought to be ARG 652. The two peptides are crosslinkable to actin by EDC. The PDG treatment of S1 alone led to a new species with Mr 115K due presumably to an intramolecular crosslinking, with a concomitant loss of S1 APTases. The amount of this product was markedly decreased by Mg-PPi but only slightly by Mg-ADP or Mg-ATP. The formation of Acto-S1 resulted in the production of a 160K-180K doublet together with a 200K entity corresponding to crosslinked Actin-S1 heavy chain. They were formed with fluorescent actin but not at all with S1 labeled by 1-5-IAEDANS at its SH1 thiol.This suggests that the crosslinked S1 arginyl residue is in a close spatial relationship with the SH1 thiol or the label on this thiol induces a change in the arginyl reactivity.(Supported by Grants from CNRS and MDA).

T-Pos214 MYOSIN LIGHT CHAIN EXCHANGE DIFFERS IN NATIVE AND SYNTHETIC FILAMENTS. S.M. Goldfine and D.A. Fischman. Department of Cell Biology and Anatomy, Cornell University Medical College, New York, NY 10021.

Using the method of Bouche and Fischman (Biophys. J. 49:223a, 1986) we have compared the myosin light chain (MLC) exchange properties of native and synthetic thick filaments. Poly(A^+)RNA from the pectoralis muscle of 19-d-old chick embryos was translated in a reticulocyte lysate system containing ^{35}S-methionine. After terminating protein synthesis, either adult myofibrils, native thick filaments, synthetic thick filaments (prepared from column-purified myosin) or filaments made from the chymotryptic rod fragments of myosin were added to equal aliquots of the lysate at 4°C and incubated for 1 hr. Samples were collected by centrifugation, washed twice and displayed by 1- and 2-D gel electrophoresis and fluorography. Myosin heavy chain (MHC) associated equivalently with all 4 samples but this was not the case for the MLCs. Myofibrils and native thick filaments predominantly captured MLC1 and 3, whereas synthetic myosin filaments captured MLC2. MLCs were not captured by the rod filaments. No detectable association of newly synthesized peptides was observed with synthetic filaments after zero length crosslinking with 1-ethyl-3 (3-dimethylaminopropyl)carbodiimide (EDC). These results suggest that MLC exchange can occur independently of the MHC and be affected by either the structure and/or composition of the thick filament. Supported by NIH grant AM32147 and by an American Heart Assoc. Participating Laboratory Fellowship.

T-Pos215 INCORPORATION OF A FLUORINE NMR PROBE IN MYOSIN S-1 ON THE ALKALI LIGHT CHAINS.

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^{19}F NMR studies have been initiated to investigate the existence of structural changes occurring at the Cys177 residue of the alkali light chains of rabbit skeletal myosin subfragment-1 (S-1). A fluorine probe N-(4(trifluoromethyl)phenyl) iodoacetamide was covalently attached to the Cys177 residue of isolated alkali light chains. The labeled light chains were subsequently incorporated into the myosin S-1 to yield labeled species with unaltered ATPase activity. It is anticipated that this species will allow studies of the topology of thermotropic transitions previously observed at other sites. The structural changes at the labeled site were studied as a function of the chemical shift of the fluorine probe over a temperature range of 0 to 25°C. Preliminary ^{19}F NMR results indicate the presence of two different species. One of the species has a linewidth consistent with a protein the size of S-1; thus indicating that the labeled light chain is firmly bound to S-1. It was observed that the chemical shift of this species varied over a temperature range of 2 to 25°C reminiscent of results of Shriver and Sykes (Biochem. 21 3022 (1982)). The other species had a narrower linewidth indicating a mobile, loosely bound light chain with greater mobility than expected for S-1. There was no temperature dependence observed for this line. The relative populations of the two species varied with each preparation. Further characterization of the two species is under way.
(Supported by a grant from the Muscular Dystrophy Association).

T-Pos216 THE EXTRACTION OF LC2 FROM VERTEBRATE FIBERS AND REPLACEMENT.

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Although the action of myosin light chain 2 (LC2) in invertebrate muscle is well established (Lehman & Szent-Gyorgyi: 66, 1, 1975), its role in vertebrate muscle remains uncertain. Here we demonstrate the extraction of a major fraction of LC2 in vertebrate fibers, which would suggest this as a possible approach in the future studies of LC2 function. Skinned single psoas fiber segments of hamsters and rabbits were used. The extraction procedure was similar to that used for TnC extraction, but prolonged. Fibers were transferred from the standard relaxing solution (190mM ionic strength) via a rigor solution (5 min, 5°C) to the extracting solution (Gulati & Babu, this volume) for up to 3 hours at 30°C. The extracted fibers were then run through SDS-PAGE and silver-stained. With 30 min. extraction when 80% or more of the TnC was lost, LC2 content (normalized to LC1) was within 10% of the original level in rabbit fibers, and within 25% in hamster fibers. In 2-3 hours, the residual LC2 was 49% and 16% in rabbit and hamster, respectively. LC3 content was not changed by prolonged extraction. When these fibers were incubated in solutions containing 1-2 mg/ml purified LC's (LC1+LC2+LC3), the lost LC2 was restored to the original level. Purified LC2 was also effective. (NSF-8303045, NIH-AM 33736 and HL 18824).

T-Pos217 SEQUENCE ANALYSIS OF CLAM MYOSIN CHAIN SEQUENCES. John H. Collins*, Winifred W. Barouch*, Kim E. Breese* and Andrew G. Szent-Györgyi#. *Dept. Biology, Clarkson Univ., Potsdam, NY 13676, and #Dept. Biology, Brandeis Univ., Waltham, MA 02254.

We are analyzing the amino acid sequences of the essential light chains (ELC) and regulatory light chains (RLC) of myosin from two species of clam: Mercenaria mercenaria and Macrocallista Nimbosa. Peptides derived from these proteins by chemical and enzymatic cleavage are being separated by reverse-phase HPLC and analyzed with an automated, gas-phase sequencer. Cleavage of the Mercenaria RLC at its 3 Arg yielded three blocked peptides which could not be sequenced directly, due to an N-terminal blocking group and 2Arg-Gln bonds. The fourth peptide was partially cleaved at a Met, apparently due to an unusual alkylation with iodoacetic acid, yielding a sulfonium ion which provided a site for tryptic hydrolysis. Clam ELC sequences seem to contain a single Ca-binding site in domain III which may be important in regulating molluscan muscle contraction (Collins et al., Biochemistry, in press). A comparison of the sequences of ELCs from Mercenaria and the scallop Aquipecten irradians shows an overall identity of only about 50%, but the sequences within the putative Ca-binding region are completely conserved. In contrast, the domain I Ca/Mg sites of clam and scallop RLCs are only about 40% identical, in agreement with other evidence that these sites are not involved in regulation. Supported by grants from the NIH (AM35120, AM15963) and the NSF (8510411).

T-Pos218 **IDENTIFICATION OF MYOSIN HEAVY CHAIN SITES DIGESTED BY PROTEOLYTIC ENZYMES.**
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We investigated the ability of a variety of proteases to cleave the myosin head in the vicinity of two trypsin sensitive sites and determined the specific location for several of these sites. It has been suggested that these trypsin sensitive sites occur within flexible connector regions between three spatially, and perhaps functionally, distinct domains of about 23, 50, and 20,000 daltons. Our results show that the specific proteases cleave the myosin head primarily at the 23/50 junctions and 50/20 junctions. In fact, the 50K and 20K fragments produced by clostripain and plasmin begin at the same positions as those produced by trypsin: Met-214 (50K), and Lys-641 (20K). The less specific proteases (papain, elastase, thermolysin, proteinase K, and subtilisins Carlsberg and BPN) appear to cleave at a number of sites within the 50K and 20K fragments, as well as at the 23/50 and 50/20 junctions. A fluorescent papain 70K fragment begins at Gly-202 or Pro-211 of the sequence, and a fluorescent 48K fragment begins at Gly-409. A fluorescent 50K subtilisin fragment starts at positions Leu-341, Gly-342, and Phe-343. A number of these enzymes produce 22K alkali-1 light chain derivatives which comigrate, on SDS gels, with the 22K carboxyl-terminal fragments produced by some of the less specific enzymes. We also determined the amino terminal sequence of a fluorescent 24K tryptic fragment which appears to be a precursor of the tryptic 20K carboxyl-terminal fragment. Our results indicate that a significant portion (> 30%) of this fragment begins at Lys 641, as does the tryptic 20K fragment. This indicates that the additional amino acid residues must arise from the carboxyl-terminal portion of the chymotryptic S1 heavy chain and not the 50/20K junction. (Supported by DOE and NIH).

T-Pos219 MAPPING MYOSIN LIGHT CHAINS BY IMMUNOELECTRON MICROSCOPY.
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The location and orientation of the two classes of light chains in myosin have been studied by immunoelectron microscopy. Cysteine residues located near the C-termini in both light chains were reacted with 5-(iodoacetamido) fluorescein (IAF), and the labeled light chains were exchanged into chicken skeletal muscle myosin. Anti-fluorescyl IgG antibodies, which bind specifically to the fluorescent probes, were used to visualize the position of the sulfhydryls in rotary-shadowed images of the myosin/antibody complex. A single cysteine residue at position 136 of the alkali 2 light chain (A2) was localized at about 90Å from the head/rod junction, in agreement with the value reported by Yamamoto et al. (J. Mol. Biol. 183, 287, 1985). Unexpectedly, the cysteine residues at position 125 and 154 in the sequence of the DTNB light chain (or LC2) were mapped close to the head/rod junction by this immunoelectron microscopic approach. The two cysteines appear to be close to each other in the tertiary structure of LC2, as evidenced by their ability to form an intramolecular disulfide bond upon the addition of DTNB. The N-terminal regions of the light chains also appear to be widely separated from each other: Antibodies specific for the amino-terminus of A2 (and A1) were located at about 80Å from the head/rod junction, whereas a monoclonal antibody specific for the amino-terminal third of the LC2 mapped close to the head/rod junction (Waller and Lowey, J. Biol. Chem. 260, 14368, 1985; Winkelmann and Lowey, J. Mol. Biol. 188, 595, 1986). These data suggest that the two classes of light chains do not lie adjacent to each other along their entire length, but instead have portions of their structure located in distinct regions of the myosin head.

T-Pos220 CELL-FREE INCORPORATION OF NONMUSCLE, BETA-ACTIN INTO MYOFIBRILS.
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Bouche and Fischman (Biophys. J. 49:233a, 1986) have established a cell-free system for analyzing the post-translation incorporation of newly synthesized contractile proteins into adult myofibrils or myofilaments. In that system, poly(A^+)RNA from muscle is translated in a reticulocyte lysate containing a ^{35}S-methionine and radiolabelled proteins "captured" by added myofibrils or myofilaments are detected by 1- or 2-D gel fluorography. In this report, we have determined whether a non-muscle actin will associate with myofibrils. A beta-actin cDNA (provided by B. Paterson) was cloned into an SP6 vector. Transcripts from this vector were capped, translated and the synthesized peptides analyzed by SDS gel fluorography. Proteins of 43Kd (assumed to be full length actin) and lower molecular weight peptides (assumed to be incomplete actin translation products) were obtained. Translation products were incubated with myofibrils for 1 hr on ice in the presence of BSA and Triton X-100. Myofibrils and associated ^{35}S labelled proteins were recovered by centrifugation, washed twice and displayed by SDS-PAGE and fluorography. The results clearly demonstrate that the 43Kd translation product from the beta-actin SP6 transcript is incorporated into myofibrils, consistant with prior microinjection studies (N. McKenna, J.B. Meigs and Y.-L Wang. J. Cell Biol. 100:292-296, 1985). (Supported by NIH grant AM 32147 to DAF and a MDA postdoctoral fellowship to IP).

T-Pos221 DMSDS CROSSLINKING OF MOLLUSCAN AND HYBRID MYOSINS. Peter D. Chantler & Susanne M. Bower. Department of Anatomy, Medical College of Pennsylvania, Philadelphia, PA. 19129.

4-4'-dimaleimidylstilbene-2-2'-disulfonic acid (DMSDS) is a bifunctional crosslinking reagent having the capability of reacting with cysteine residues at either end; only when both links are formed does the molecule become fluorescent. Mercenaria myosin and scallop pure hybrid myosin possessing Mercenaria regulatory light-chains (R-LC) were reacted with various concentrations of DMSDS and the products analyzed by fluorescence spectroscopy, SDS and urea-gel electrophoresis and by immunoblotting techniques. Crosslinks between heavy-chains were formed at an equimolar ratio of DMSDS to myosin. Maximum fluorescence and the production of LC dimers was achieved with a 2-5 molar ratio of DMSDS to myosin. A 5-10 molar ratio of DMSDS to myosin revealed oligomers of the essential light-chain (E-LC), presumably formed through multiple crosslinks occurring in myosin aggregates. Mercenaria R-LC possesses a single sulfhydryl (Cys-50). In the case of Mercenaria myosin, LC dimers were shown to be composed solely of R-LCs. In the case of the hybrid myosin, the SDS-gel LC dimer band was immunopositive for both Mercenaria R-LC and scallop E-LC. R-LC and E-LC homodimers were shown to be present here for subfragment-1 preparations, obtained by papain digestion of the hybrid myosin, did not form LC dimers even though Cys-50 was still present on the cleaved R-LC of S-1. Results were invariant, irrespective of the presence or absence of ATP +/- calcium. These results indicate that translationally equivalent positions (Cys-50) on both R-LCs situated on each head of a single myosin molecule, can come within 15-24Å of each other, the span of reacted DMSDS. This is true provided the reagent specificity is as expected - currently under investigation. The experiments complement and extend ongoing Førster transfer measurements (Chantler & Tao. J.Mol.Biol. In Press.). Supported by Grant #DMB-8602246 (N.S.F.); PDC is an Established Investigator of the A.H.A.

T-Pos222 DIFFUSIVITY OF PARVALBUMIN AND OTHER PROTEINS IN FRESHLY SKINNED FROG SKELETAL MUSCLE FIBERS. D. Maughan and E. Wegner, Dept. of Physiology & Biophysics, University of Vermont, Burlington, VT 05405 USA.

Kushmerick & Podolsky (Science 166, 1297-1298, 1969) have investigated the cytoplasmic diffusivities of trace amounts of radioisotopes of selected electrolytes (and sugars) injected into frog skinned fibers bathed in oil at 20° C. The apparent (longitudinal) diffusion coefficient (D) of Ca^{2+} was 50 times less than that in aqueous solution. This is in marked contrast to the D's of the other molecules tested which were only 2 times less than their values in aqueous solution. If injected Ca^{2+} binds to the major diffusible Ca^{2+}-binding protein parvalbumin, one would expect that D for Ca^{2+} and parvalbumin to be comparable. To investigate this, we measured the apparent (radial) diffusion coefficient (D') of parvalbumin and other proteins (e.g., G-3-P dehydrogenase) in skinned fibers bathed in relaxing solution at 20° C. These proteins were tentatively identified on the basis of their electrophoretic mobility (SDS-PAGE). D' of parvalbumin and other proteins were about a tenth of their value in aqueous solution. Tortuosity and viscosity factors probably contribute to most, if not all, of the apparent reduced diffusivity of the proteins, but binding to cytomatrix proteins or sarcoplasmic reticular structures (with binding free energy <1.3 kcal/mole) may also contribute. Parvalbumin D' was similar to Ca^{2+} D, thus supporting the notion that the low apparent diffusion coefficient of Ca^{2+} is actually that of a Ca^{2+}-parvalbumin complex. Supported by NIH RO1 DK33833.

T-Pos223 PHOSPHORYLATION OF PARAMYOSIN ALTERS ITS SOLUBILITY CHARACTERISTICS, William H. Johnson, Muscle Biology Group, University of Arizona, Tucson, AZ, 85721. (Intro. by D.H. Hartshorne.)

The unique solubility behaviour of the invertebrate muscle protein paramyosin has been extensively studied. Of special interest is the sharp zone of precipitation near pH 7.0; increasing the ionic strength shifts the zone to higher pH in a predictable and reversible manner. Subsequent work revealed that the earlier work had been done on a preparation (b-PM) in which a polypeptide had been somehow removed during extraction from c-terminal end of the native protein (a-PM). The pH and ionic strength dependence of the solubility of a-PM differed markedly from that of the b-PM preparation. The pH zone of precipitation was broadened, and considerable hysteresis is observed during ascending and descending pH changes. Paracrystals of b-PM are single and needle shaped with a marked 14.5 nm banding in negatively stained EM preparations, whereas paracrystals of a-PM consist of side-by-side aggregates of smaller, b-PM like needles with a 14.5 nm bands in register across the bundle. We recently found that a-PM can be phosphorylated near the c-terminal and by an enzyme isolated from molluscan muscles. In work reported here, we found that the solubility of phosphorylated a-PM was similar to that of b-PM. Paracrystalline structure was also similar, whereas dephosphorylated a-PM showed bundle formation with 14.5 nm bands in register. Very long, thin bundles are seen in the light microscope in wet mounts. Thus it would appear that phosphorylation modulates side-by-side aggregation of smaller paracrystals of paramyosin.

T-Pos224 THE KINETIC MECHANISM OF MAGNESIUM BINDING TO COD PARVALBUMIN, Xue-Zhong Zhang and Howard D. White, Department of Biochemistry, Eastern Virginia Medical School, Norfolk Va. 23501

Magnesium binding to cod parvalbumin induces a two fold enhancement and a 348 to 328 nm blue shift in the fluorescence emission spectrum of the protein that is similar to that previously observed for calcium binding to cod parvalbumin (Breen et. al. 1985 Biochem., 24, 4992) and to whiting parvalbumin (White, Biochem. in press). Titrations of 8 uM apoprotein with Mg are sigmoidal with a half maximal change in the fluorescence spectra at 250 10 uM Mg and are best fit by equilibrium constants of 2300 100 uM for the first Mg bound and 27 3 uM for the second Mg. The kinetics of Mg dissociation from parvalbumin have been measured in a stopped-flow fluorometer by mixing Mg-PA with excess EDTA. The time course of the change in fluorescence are best fit by the sum of two exponential terms of $2.6 \pm 0.1\ s^{-1}$ and $23 \pm 3\ s^{-1}$. Rate constants of $1.2\ s^{-1}$ and $3\ s^{-1}$ are measured for Ca dissociation under identical conditions. The time course of the fluorescence changes observed upon mixing apo cod parvalbumin with Mg are complex. At [Mg] < 5 mM the kinetics are nearly single exponential and can be described by an apparent second order rate constant of $4 \pm 0.5 \times 10^3\ M^{-1}s^{-1}$, which is 1/300 the apparent second order rate constant of Ca binding. At higher Mg concentrations the kinetics are fit by a sum of two exponentials which have maximum rate constants of $45 \pm 5\ s^{-1}$ and $8 \pm 0.4\ s^{-1}$ at [Mg] > 50 mM. (Experimental conditions: 0.1 M KCl, 20 mM bistrispropane, 0.1 mM DTT, pH 7, 20° C.) The results described here indicate that at 'in vivo' [Mg], ~3 mM, a large fraction of the parvalbumin in resting muscle cells will have Mg bound and that the kinetics of Mg binding and dissociation is likely to be important in determining the time course of intracellular Ca changes associated with muscle activation and relaxation.

T-Pos225 TITIN: SUBUNIT MOLECULAR WEIGHT BY HYDRODYNAMIC CHARACTERIZATION IN GUANIDINE HYDROCHLORIDE. Gary P. Kurzban & Kuan Wang (Intr. by Edward Barr) Clayton Foundation Biochemical Institute & Department of Chemistry, The University of Texas, Austin, TX 78712

Titin is a major myofibrillar protein of the cytoskeletal matrix and is unusually large. The reported subunit chain weight for the titin doublet, T1 and T2, has ranged from 1.2 - 2.8 megadaltons. To clarify this issue, we have initiated hydrodynamic studies of denatured titin in 6 M guanidine HCl. We have developed a new procedure to purify nucleic acid-free titin: rabbit back myofibrils were denatured in hot 6 M guanidine HCl, alkylated with N-ethylmaleimide, concentrated by ultrafiltration, and applied to a gel filtration column of Sephacryl S-1000 equilibrated in guanidine HCl. Myofibrillar macromolecules partitioned on the S-1000 with Kav as follows: nucleic acids, 0 (void); T1, 0.28; T2, 0.32; nebulin, 0.53; myosin heavy chain, 0.71; and actin, 0.79. Fractions containing predominantly T1 were used for further hydrodynamic studies. The sedimentation coefficient of titin is significantly concentration-dependent; within the concentration range of 0.1 to 0.6 A_{280}, sedimentation followed the relationship: $1/s = 0.25 + 0.3\ A_{280}$. Thus, in guanidine HCl, $s_{o,20°c} = 4.0S$. The denatured titin is extremely prone to aggregation during ultrafiltration, forming slowing dissociating, heterogeneous aggregates of larger than 500S. For this reason, sedimentation equilibrium studies have employed titin from column fractions without further concentration. Our results to date indicate a subunit chain weight in the range of 0.8 - 1.6 megadaltons.

T-Pos226 HIGH RESOLUTION IMMUNOCYTOCHEMICAL LOCALIZATION OF CARBONIC ANHYDRASE III IN SKELETAL MUSCLE FIBER SPECIFIC TYPES. P. Frémont, C. Côté, P.A. Rogers and P.M. Charest (Intr.by M.-C. Thibault), Muscle Biology Research Group, Laval University, Québec, Canada.

Carbonic anhydrase (CA) III is the predominant isoform of this enzyme in skeletal muscle tissue and is primarily found in type I muscle fibers where it can account for up to 20% of the soluble protein. The objective of the present series of experiments was to determine the ultrastructural localization of CA III using immunoelectron microscopy. Rat hindlimb was fixed in situ with 3% gluteraldehyde and small pieces of the soleus (SOL), deep vastus lateralis (DVL) and superficial vastus lateralis (SVL) muscles which contain predominantly type I, IIa and IIb fibers respectively, were embedded in polyvinyl alcohol (PVA). Immunolabelling of ultrathin sections with an antibody specific for CA III and gold conjugated protein A revealed that: 1) the concentration of labelling was significantly higher in the type I fibers than in type IIa or IIb, 2) mitochondria and nuclei were not labelled, 3) there was no particular association of the label with either the Z- or M-band structures, and 4) labelling was uniform in the sarcomere space between the thick and thin filaments but relatively weaker in the intermyofibril space. Myofibrils were prepared from each muscle and washed five times in a low ionic strength buffer containing 1% Triton X-100. Immunoblotting of the washed myofibrils demonstrated that no significant amount of CA III is bound to the myofibrils. It is concluded that CA III is localized primarily in the cytoplasmic domain within the myofibrils rather than between myofibrils. The identification of the specific sarcomeric localization of CA III should be useful in understanding the physiological significance of this enzyme.

T-Pos227 THE CELLULAR CONTENT OF DESMIN IS MUSCLE FIBER TYPE SPECIFIC. M.-J. Mailloux, C. Côté and P.A. Rogers (Intr. by N. Marceau). Muscle Biology Research Group, Laval University, Québec, Canada.

Desmin is the major subunit protein of the intermediate filament (IF) system in cardiac and skeletal muscle. In order to gain an insight into the possible role of the IF system in muscle tissue, a qualitative and quantitative analysis of desmin from the cytoskeletal fraction was performed using the following rat skeletal muscles: the soleus (SOL) deep vastus lateralis (DVL) and superficial vastus lateralis (SVL). These particular muscles are composed primarily of type 1, 2A and 2B fibers respectively. Immunochemical analyses were carried out using immunoblotting, a polyclonal antibody prepared against chicken gizzard desmin and radioiodinated goat antirabbit IgG. The desmin of all types of skeletal muscles was found to exist as three distinct isoelectric variants by two dimensional acrylamide gel analysis. This was confirmed by immunoblotting with the desmin antibody. Quantitative analysis revealed that the relative cellular content of desmin for the SOL, DVL and SVL was 1.4, 1.3 and 1.0, respectively, while the corresponding values for purified myofibrils were 2.1, 1.2 and 1.0. These results demonstrate that the cellular content of desmin varies according to the muscle fiber type. Considering the various, ultrastructural, biochemical and physiological differences between type 1, 2A and 2B skeletal muscle fibers, we found the desmin content to best correlate with Z-line thickness. Since the functional significance of the difference in Z-line width in the three fiber types is still unclear, the results regarding desmin are difficult to interpret. It is evident however that future studies on the IF system of muscle should take into account the various fiber types.

T-Pos228 CALMODULIN IN SKINNED FAST-TWITCH MUSCLE OF HAMSTER: STUDY WITH RADIO-IMMUNO-ASSAY.

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TnC-extracted skinned fibers are found to retain Ca-sensitivity for force development in low ionic strength even though no force is made in physiological ionic strength (Babu: FEBS Letts 203, 20, 1986). Here we check if residual calmodulin could have substituted for TnC in low ionic strength in the extracted fiber. Calmodulin contents of intact, skinned and TnC-extracted preparations were determined with NEN radioimmunoassay kit after Chafouleas et al. (J Biol Chem 254, 10262, 1979). Intact samples were excised immediately after sacrifice of the animal; skinned and extracted samples were prepared as before (Babu). The samples were weighed, suspended in 0.1% SDS and boiled for 1-3 min. Intense ultrasonication was used to help dissolve the tissue, and Triton-X was added to 0.7% before measuring calmodulin. Calmodulin content was found as (mg/1000g): intact, 18.7±3.7 (S.D., with n=12); skinned, 14.3±3.2; extracted, 11.6±3.3. Assuming a factor of 1.3 for swelling on skinning, the calmodulin content is found to be identical in intact and skinned preparations, and there is 19% loss in the extracted preparation. Ca-dependent low ionic strength force of the extracted fibers was found to be unaffected by including purified calmodulin (1 mg/ml) or the inhibitors trifluoperazine (0.1mM) and calmidazolium (0.1mM). Thus the low ionic strength force appears to be unrelated to calmodulin. Further, the results show that the majority of calmodulin in muscle is firmly associated with the structural components of the fiber even in the presence of EGTA, and that the diffusible fraction is insignificant. (NSF-8303045, NIH-AM 33736 & HL 18824).

T-Pos229 STRUCTURAL EFFECTS OF LIMULUS MYOSIN LIGHT CHAIN PHOSPHORYLATION. Rhea J.C. Levine & John L. Woodhead. Department of Anatomy, The Medical College of Pennsylvania, Philadelphia, PA. 19129

Limulus skeletal muscle is dually regulated (Lehman & Szent-Gyorgyi, J.Gen.Physiol.66:1,'75): both Ca^{2+}binding to troponin and myosin light chain (MLC) phosphorylation activate contraction. Unlike other tissues activated by MLC phosphorylation, Limulus myosin has 2 phosphorylatable LCs (Sellers, J.Biol.Chem.256:9274,'81). We studied both incorporation of ^{32}P into Limulus myosin by scintillation counting and autoradiography of SDS gels and the effect of MLC phosphorylation on the structure of thick filaments islated from previously unstimulated Limulus telson muscles.

MLC phosphorylation by ATP was catalyzed by either intact chicken gizzard myosin LC kinase (MLCK) (+ calmodulin, + Ca^{2+}) or trypsin-clipped, Ca^{2+}-insensitive MLCK. Isolated myosin incorporated 2 mol. ^{32}P/mol. protein. Autoradiography of SDS gels showed radioactivity limited to the 2 phosphorylatable LCs. Isolated filaments exposed to intact MLCK without Ca^{2+} remain long (~4.3μm) and retain their helical crossbridge order. Filaments exposed to either intact MLCK (+ calmodulin, + Ca^{2+}) show: (1) loss of helical crossbridge order as these extend away from the surface (Craig, et al.Biophys.J. 47: 469a,'85); (2) sharp bends and breaks, 0.65μm from the tapered ends; and (3) decrease in length to 3μm. Re-rinsing with relaxing solution restores some degree of crossbridge order but not filament length. MLC phosphorylation may weaken intermolecular interactions at a specific site on Limulus filaments, leading to breakage under appropriate force. We are examining the effect of MLC phosphorylation on isolated myosin molecules to determine if structural changes occur in this condition. Supported by USPHS grants: AM 33302 & HL 15835 to The Pennsylvania Muscle Institute.

T-Pos230 Sr^{2+}-ACTIVATION PROPERTIES OF RAT SKINNED SOLEUS AND VENTRICULAR CELLS ARE DETERMINED BY THE TYPE OF TNC. P.E. Hoar+, N.J. Liu+, J.D. Potter# and W.G.L. Kerrick+#. Depts. of +Physiology & Biophysics and #Pharmacology, Univ. of Miami Sch. of Medicine, Miami, FL 33101.

Troponin C (TnC) was extracted from skinned rat soleus (slow-twitch) fibers and bundles of cardiac ventricular cells by a method similar to that previously used to extract TnC from skinned rabbit skeletal muscle cells (Kerrick et al. J. Biol. Chem. 260:15687-15693, 1985) and replaced with either bovine cardiac or rabbit fast-twitch skeletal TnC. When the extracted TnC was replaced by bovine cardiac TnC, the Sr^{2+}-activated tension relationships were not altered from control values. In contrast, replacement of the endogenous TnCs with exogenous rabbit fast-twitch TnC caused the relationships between Sr^{2+} concentration and tension to shift towards higher concentrations of Sr^{2+}. The Sr^{2+}-activated tension of rat fibers with fast-twitch TnC was identical to that of rabbit fast-twitch skinned fibers. Partial skeletal TnC substitution in skinned soleus or cardiac cells gradually shifted the relationship between tension and $[Sr^{2+}]$ to higher Sr^{2+} concentrations and caused the cells to be activated over a wider range of Sr^{2+} concentrations. Thus it appears that the activation of rat soleus or cardiac skinned cells by Sr^{2+} is determined by characteristics of the TnC. In contrast, the Sr^{2+} activation of skinned fast-twitch skeletal fibers containing either cardiac or skeletal TnC is identical, strongly suggesting that protein-protein interactions determined the Sr^{2+}-activation properties in these fibers (Kerrick et al., 1985). Support: NIH (BRSG SO7 RR-05363, HL22619-09, & AR37701-01), Muscular Dystrophy Assoc., American Heart Assoc. (National and Florida Affiliate).

T-Pos231 MUSCLE REGULATORY PROTEINS IN HAMSTER CARDIOMYOPATHY. Ashwani Malhotra, Ph.D.

The relaxing factor (troponin-tropomyosin) is known to inhibit the Mg^{2+}-ATPase activity of striated muscle actomyosin in the absence of Ca^{2+} ion but potentiates ATPase in its presence. We have conducted reassociation studies of skeletal actomyosin in the presence of relaxing factor isolated from the skeletal muscle of control and myopathic hamsters at 7 months of age. Mg^{2+} ATPase of actomyosin was determined in the absence (2mm EGTA) and presence of different concentrations of free Ca^{2+} ion. Results are shown as:

Skeletal Myosin (Sk.)	Relaxing Factor	Sp. Activity (μ-mole Pi.min.mg^{-1}) $+Ca^{2+}$	Sp. Activity (μ-mole Pi.min.mg^{-1}) $-Ca^{2+}$	Calcium Sensitivity
C	C	0.280	0.040	86%
C	M	0.200	0.140	30%
M	C	0.200	0.055	74%
M	M	0.200	0.125	37%

C = control; M = myopathic; mean of 3-4 studies.

Actin used was from Sk. rabbit muscle in all the experiments. Ca^{2+}-sensitivity was markedly decreased in the actomyosin reconstituted with relaxing factor from M Sk. muscle. Electrophoretic patterns of Tn-Tm complex from C and M muscles on gradient SDS gels showed differences in their mobilities. These data suggest that Ca^{2+} sensitivity of regulatory proteins from Sk muscles of M hamsters have diminished inhibitory action on Mg^{2+} actomyosin ATPase activity. This may be due to an alteration in the make up of the regulatory proteins. Supported by NIH grant HL-18824.

T-Pos232 **CALMIDAZOLIUM, A CALMODULIN ANTAGONIST STIMULATES CA-CALMODULIN-DEPENDENT AND CA-TNC-DEPENDENT REGULATION OF STRIATED MYOFILAMENTS.** **Saleh C. El-Saleh and R. John Solaro, University of Cincinnati, College of Medicine, Cincinnati, OH.**

Recently Silver et al. (Biochem. Pharmacology, 35:2545, 1986) reported that calmidazolium (CDZ), a calmodulin antagonist can produce a stimulatory leftward shift (.23 pCa units at pCa_{50}) in the pCa-ATPase activity relationship of cardiac myofibrils. As part of our studies on the effects of calmodulin antagonists including CDZ on TNC Ca-binding, we have confirmed the results of Silver et al. in cardiac myofibrils. However, in the case of skeletal myofibrils the magnitude of the shift induced by CDZ was 40% of that for cardiac myofibrils. Similar stimulatory effects on cardiac myofilaments sub-maximal force production were also observed. These stimulatory effects by CDZ were associated with increase in the amount of Ca bound to TNC in myofibrils. Direct fluorescent measurements using cardiac TNC-IAANS and skeletal TNC-DANZ showed that CDZ reversibly enhances the TnC Ca-binding properties in proportional degrees to the degree of stimulation in both skeletal and cardiac myofibrils. CDZ also stimulated the Ca-dependent ATPase of skeletal myofibrils with calmodulin substituted for TNC. Furthermore, the degree of effect of CDZ on the Ca-sensitivity of ATPase activity was greater in skeletal myofibrils with cardiac TNC substituted for skeletal TNC. Therefore, the stimulatory or inhibitory actions (and their degrees) of CDZ appear to be dependent on specific interactions of the Ca-binding proteins with their neighbors in a particular regulatory scheme.

T-Pos233 THE EFFECT OF TROPONIN C REMOVAL ON THE Ca^{++}-SENSITIVE BINDING OF AMPPNP TO RABBIT SKELETAL MYOFIBRILS IN 50% ETHYLENE GLYCOL. R.E. Johnson, Department of Biochemistry, University of Arizona, Tucson, Arizona 85721.

It was previously shown that removal of Ca^{++} tightens the binding of Mg^{++}AMPPNP to rabbit skeletal myofibrils by a factor of 3 or 4 in the presence of 50% ethylene glycol. This was interpreted to mean that, since cross-bridge dissociation was coupled to Mg^{++}AMPPNP binding, the cross-bridges were attached 3 or 4 times more weakly in the absence of Ca^{++} than in its presence. In order to determine the relevance of this Ca^{++}-sensitivity to the Ca^{++} control of muscle contraction, the binding experiments were repeated with myofibrils from which the troponin C had been selectively extracted. It was found that troponin C removal completely eliminated the Ca^{++}-sensitivity of Mg^{++}AMPPNP binding in 50% ethylene glycol, with the extracted myofibrils now exhibiting the binding properties of intact myofibrils in the absence of Ca^{++}.

Other laboratories have found a similar effect with regulated acto-S1 or acto-HMM and excess ATP or AMPPNP in which the binding of S-1 or HMM to regulated F-actin was 3 or 4 times stronger in the presence of Ca^{++} than in its absence. This effect, however, is too small to fully account for the Ca^{++} control of regulated actomyosin ATPase activity, and other steps in the ATPase reaction mechanism besides reattachment of the cross-bridge (i.e. the steric blocking model) were suggested to be involved. I have shown here that the same effect is seen in intact myofibrils.

(Supported by a grant-in-aid from the American Heart Association, Arizona Affiliate.)

T-Pos234 ALTERED ACTIN AND TROPONIN BINDING OF ENGINEERED NH_2-TERMINUS MUTANTS OF STRIATED α-TROPOMYOSIN. S.E. Hitchcock-DeGregori and R.W. Heald, Dept. of Anatomy, UMDNJ-Robert Wood Johnson Med. Sch., Piscataway, N.J.

In order to investigate the role of the NH_2-terminus of tropomyosin (TM) on actin and troponin binding we have engineered mutants of chicken striated muscle αTM and expressed them in E. coli. One variant is identical to the muscle protein (AcαTM) except that the NH_2-terminal methionine is unacetylated ($NH_2\alpha$TM). The other is a fusion protein with 81 amino acids of a non-structural influenza virus protein on the NH_2-terminus (fαTM). We labeled the cysteines of the three TMs with ^{14}C-NEM and measured their apparent K_as to rabbit skeletal muscle F-actin by cosedimentation in an airfuge in 150mM NaCl, 2mM $MgCl_2$, 10mM TrisHCl, pH 7.5, 0.5 mM DTT, 0.1mMATP, 0.1mM $CaCl_2$ or 0.2mM EGTA, 25°C. The affinity of fαTM ($\sim 4\times10^6$ M^{-1}) is similar to that of AcαTM (5×10^6 M^{-1}) and binding is cooperative. The fusion peptide itself does not bind to F-actin. $NH_2\alpha$TM binds to F-actin only weakly ($\approx 5\times10^5$ M^{-1}). Troponin increases the affinity of $NH_2\alpha$TM for actin ($+Ca^{2+}$, 6×10^6 M^{-1}; $-Ca^{2+}$, 3×10^7 M^{-1}) but not to level of AcαTM binding ($+Ca^{2+}$, 7×10^7 M^{-1}; $-Ca^{2+}$, 3×10^8 M^{-1}). Troponin has no effect on the affinity of fαTM for F-actin though it does cosediment in the absence of Ca^{2+}. We conclude: 1. that close proximity of the NH_2- and COOH- ends of TM on the thin filament (prevented by the presence of a fusion peptide in fαTM) is not required for tight, cooperative binding to F-actin 2. but it is important for troponin binding. 3. Acetylation of the NH_2-methionine is critical for binding of normal TM to actin, but it is not necessary for troponin binding.

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T-Pos235 PROXIMITY OF SULFHYDRYL GROUPS TO THE SUBUNIT INTERACTION SITES OF RABBIT AND BOVINE CARDIAC TROPONIN. R.H. Ingraham and R.S. Hodges, Department of Biochemistry and MRC Group in Protein Structure and Function, University of Alberta, Edmonton, Alberta, Canada T6G 2H7.

Labelling of Cys residues with [1-^{14}C]iodoacetamide was performed in denaturing (6 M urea) and benign media (50 mM Tris, pH 7.5, 0.3 M KCl, 1 mM DTT, and either: a) 1 mM $CaCl_2$; b) 3 mM $MgCl_2$, 1 mM EGTA; or c) 2 mM EDTA) on native rabbit (RC) Tn and bovine cardiac (BC) Tn, rabbit cardiac TnC-TnI, TnI, and TnC, and BC-TnT. After labelling the Tn subunits were purified using reversed-phase HPLC and subjected to CNBr (TnC) or tryptic (TnI and TnT) cleavage. Fragments containing Cys or [^{14}C] CMC were isolated using RP-HPLC and the labelling determined by analysis and radioactivity measurements. Cys75 and 92 of TnI located in tryptic fragment 75-93, are completely accessible to iodoacetamide (>90% labelled) both when TnI is labelled in benign or denaturing media, or when TnC-TnI is labelled in the three benign media. Both residues are unreactive (<1% labelled) when either species of native Tn is labelled under benign conditions, suggesting burial in the TnI-TnT interface. Cys35 and 84 of TnC are located in CNBr fragments CB2 (2-45) and CB7 (82-85), respectively. Cys35 labelled in the two troponins is partially exposed with average labelling values of 30%, 40%, and 35% for the three buffers respectively. Partial exposure of this residue is consistent with its location in the non-functional Ca^{2+}-binding loop I of cardiac TnC and the surface profile values predicted using a computer program written in our laboratory (Parker, J.M.R. and Hodges, R.S., Biochemistry, in press). In contrast, Cys84, adjacent to the inter-domain helical-linker of TnC, is highly accessible to iodoacetamide, yielding average values of 81%, 69% and 81% for the three buffers respectively. Labelling of the Cys in BC-TnT either in native Tn or by itself in benign or denaturing media was essentially stoichiometric.

T-Pos236 FURTHER EVIDENCE THAT CYCLING CROSSBRIDGES INCREASE THE Ca^{2+} AFFINITY OF TnC. K. Guth, K. Winnikes, and James D. Potter, Dept. of Pharmacology, Univ. of Miami School of Medicine, Miami, FL

Previously (Guth, K. et al., Biophys. J. 49, 270a, 1986) we presented evidence that cycling crossbridges increase the affinity of the Ca^{2+}-specific (CS) sites of TnC for Ca^{2+} by a factor of at least ten using the technique of TnC replacement with TnC_{DANZ} in skinned fibers followed by simultaneous measurements of TnC_{DANZ} fluorescence (F) and tension (Zot, H. et al., (in press) J. Biol. Chem, 1986). We have extended these studies to include measurements of F in the rigor state at different sarcomere lengths and have found that on going from the small overlap state (3.8 μm) to the fully overlapped state (2.3 μm) we observed a two fold increase in the Ca^{2+} affinity of the TnC CS sites. Thus the affinity change brought about by the formation of rigor bridges cannot account for the observed ten fold increase in TnC affinity seen during activation. In an attempt to increase the population of cycling S1.ADP crossbridges we increased the ADP/ATP ratio (see Kerrick and Hoar Biophys. J. 47, 296a, 1985) and found a six fold increase in the Ca^{2+} affinity of the TnC CS sites. Under these conditions tension was maximal and independent of Ca^{2+}. These results suggest that the strongly attached and cycling (S1.ADP) bridges are primarily responsible for the large increase in affinity seen upon Ca^{2+} activation. We also observed that the steep Ca^{2+} dependence of F in the presence of ATP at short (2.3 μm) and long (3.8 μm) sarcomere lengths were not significantly different, suggesting that the cooperative effects of crossbridge binding extends over the entire length of the thin filament (Brandt, et al., J. Mol. Biol. 180, 379, 1984). Supported by NIH AR37701-01 and HL22619-3A.

T-Pos237 RECONSTITUTION OF THE CALCIUM REGULATORY RESPONSES IN TnC DEPLETED SKINNED MUSCLE FIBRES FROM THE RABBIT AND BARNACLE BALANUS NUBILUS. C.C. Ashley, W.G. Kerrick+, T.J. Lea, R. Khalil+, and J.D. Potter+, Univ. Lab. of Physiology, Parks Rd. Oxford, UK, +Univ. of Miami Dept. of Pharmacology, Miami, FL

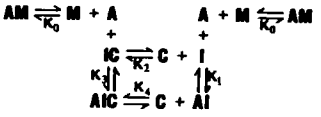
Single muscle fibres from the barnacle and rabbit were skinned and were tested at pCa ~3.8 before extracting the calcium regulatory proteins in a K_2 EDTA (pH 7.6) solution. Force responses after extraction were less than 10% at pCa ~3.8. Parallel extractions of barnacle myofibrils showed that BTnC (1 or 2) were extracted equally. The protocol followed for reconstitution was such that reassociation of the TnC (R or B) with TnI via Ca^{2+}-Mg^{2+} site dependent binding, or force activation (FA) via Ca^{2+}-specific site dependent interactions (see Fig. M=Ca/Mg, C=Ca spec. interactions) could be assessed. BTnC (1 or 2) or STnC were able to bind in pCa ~8 relaxing solutions which contain $+Mg^{2+}$ (did not wash out in protein free pCa ~8 solution) and to activate in pCa ~3.8 when reincorporated into B and R fibers respectively (see Table). RSTnC neither bound to B fibers in pCa ~8 solution nor activated in pCa ~3.8. BTnC did not bind to R fibers in pCa ~8 solution but in contrast to rabbit could activate in the pCa ~3.8 solution. These results suggest that the necessary TnC/TnI recognition sequences are sufficiently different between these species to prevent these interactions.

	Barnacle Fibers		Rabbit Fibers	
	Ca^{2+}-Mg^{2+} (Binding)	Ca^{2+}-Specific (FA)	Ca^{2+}-Mg^{2+} (Binding)	Ca^{2+}-Specific (FA)
RSTnC	-	-	+	+
BTnC	+	+	-	+

Supported by grants from the MDA.

T-Pos238 TnC_{DANZ} FLUORESCENCE CHANGE MAY INDICATE AN UNCOUPLED Ca^{2+} BINDING STEP IN THE REGULATION OF ACTOMYOSIN. Henry G. Zot, Dept. of Pharmacology, Univ. of Miami Sch. of Medicine, Miami, FL, 33101.

Recently the fluorescent derivative of TnC (TnC_{DANZ}) was incoporated into skinned fibers and myofibrils and steady-state Ca^{2+} dependent fluorescence and activity changes were recorded simultaneously (Zot et al., J. Biol. Chem., in Press). The fluorescence change, which responds to Ca^{2+} binding to the Ca^{2+}-specific sites of TnC, was found to be more sensitive to Ca^{2+} than the change in activity. These data can be explained (without proposing that TnC has separate conformational states for the fluorescence and activity changes) if Ca^{2+} binding to TnC is uncoupled from the actomyosin interaction at some point in the cycle. A canonical model that expresses coupled and uncoupled Ca^{2+} binding is shown in the figure where A and M have their usual meanings; C and I stand for Ca^{2+} and troponin-tropomyosin respectively; M, total A, and total I are fixed; $K_1 \gg K_0 \gg K_2$ (all units = M^{-1}). If activity correlates with AM then activity increases only when A and I become uncoupled. Since Ca^{2+} binding is a function of total I in this model both coupled (K_4) and uncoupled (K_2) binding reactions occur. If fluorescence intensity increases whenever Ca^{2+} binds to TnC_{DANZ} then a fluorescence signal is measured from both coupled (AIC) and uncoupled (IC) I states but an activity signal only from uncoupled A states. This work supported by HL22619-3A and HL07188.



T-Pos239 THE TIME-RESOLVED INTRINSIC FLUORESCENCE OF TROPONIN I AND TROPONIN C. Chien-Kao Wang[a], Iain Johnson[b], Dan Harris[b], Bruce Hudson[b], and Herbert C. Cheung[a]. (a) Department of Biochemistry, Univ. of Alabama at Birmingham, Birmingham, AL 35294 and (b) Department of Chemistry, Univ. of Oregon, Eugene, OR 97403

The emission decay of the single tryptophan of skeletal troponin I (TnI) was previously studied (Wang, et al., 1985, Biophys. J., 47, 472a) by using a YAG laser to synchronously pump a cavity-dumped dye laser. The decay excited at 300 nm and determined at 360 nm was best described by three exponential terms. The anisotropy decay showed two correlation times. The short one (ca. 0.9 ns) was not affected when TnI was complexed with TnC in the presence of EGTA, but was reduced by over 50% in the presence of Ca^{2+}. These studies have been extended to two additional emission wavelengths (380 nm and 420 nm). We also have measured the emission decay of the tyrosyl residues of troponin C (TnC) at 300 nm with 280 nm excitation. Three decay times (0.35, 1.37, and 2.71 ns) were extracted from the observed decay by both the method of moments and nonlinear least squares. The corresponding anisotropy decay was biphasic with a short correlation time of 0.67 ns and a long correlation time of 9.23 ns. The anisotropy at zero time was 0.225. (Supported in part by AM-25193 from the NIH).

T-Pos240 THE BIOLOGICAL IMPORTANCE OF EACH AMINO ACID RESIDUE OF THE TnI INHIBITORY SEQUENCE [104-115] TO THE INTERACTIONS WITH TnC AND TM-ACTIN. J.E. Van Eyk and R.S. Hodges. Department of Biochemistry and MRC Group in Protein Structure and Function, University of Alberta, Edmonton, Alberta, Canada T6E 2H7.

To systematically evaluate the contribution of each amino acid residue of the TnI inhibitory region [104-115], fourteen synthetic analogs were synthesized by the solid-phase method. The analogs consisted of either single glycine or multi-glycine replacements. The importance of the substituted amino acid(s) was determined from extent of inhibition of the acto-S1 ATPase activity and the strength of binding to a TnC HPLC affinity column. Every residue of the TnI sequence [104-115] is necessary to achieve maximum inhibition of the ATPase activity. However, the analogs quanitatively differed in the amount of inhibition induced. The least effective inhibitors were the analogs in which Val 114, Arg 112 and Arg 115 were replaced, suggesting that these are the most essential residues for inhibition. The TnI analogs bound less tightly to the TnC affinity column than the native synthetic peptide indicating that all residues in the TnI sequence contribute to the binding of TnC. In the presence of Mg^{2+}, the essential residues for TnC binding are Lys 105, Phe 106, Arg 108, 112, 113 and 115. In the presence of Ca^{2+}, there is a definite increase in the strength of the interaction between most analogs and TnC. This is accompanied with a shift towards a more specific interaction with the C-terminus of the TnI inhibitory sequence, especially residues Leu 111, Arg 112 and Arg 115. These results support the concept that in the absence of Ca^{2+} the TnI inhibitory region (especially the C-terminal region) binds TM-actin causing inhibition. While in the presence of Ca^{2+}, there is a conformational change such that this region strongly interacts with TnC.

T-Pos241 THE INTERACTION OF Tn-T WITH F-ACTIN AT PHYSIOLOGICAL IONIC STRENGTH. D.H. Heeley and L.B. Smillie, MRC Group in Protein Structure and Function, Department of Biochemistry, University of Alberta, Edmonton, Alberta, T6G 2H7 Canada.

Cosedimentation experiments with radiolabelled proteins, carried out at 150 mM KCl, have shown that Tn-T is able to form a direct linkage to F-actin. Under these salt conditions at a constant F-actin concentration of 7 μM and with increasing concentrations of Tn-T (0 → 5.0 μM), the binding of Tn-T to F-actin exceeds stoichiometric levels and does not attain saturation. Similar results were obtained by the densitometric analysis of pellets containing F-actin and unlabelled Tn-T, subsequent to the electrophoresis of these samples on SDS polyacrylamide gels. While no interaction has been observed between radiolabelled fragment T1 (residues 1-158) and F-actin, unlabelled and radiolabelled fragment T2 (residues 159-259) behaved in a similar manner to the parent molecule, indicating that the F-actin binding properties of Tn-T can be attributed to this portion of its structure. We have extended the investigations to mixtures of Tn-C + Tn-T (or T2) ± Ca^{2+}, Tn-I + Tn-T (or T2) and Tn-I + Tn-C + Tn-T (or T2) ± Ca^{2+} and found that in the presence of Tn-C the interaction between Tn-T (or T2) and F-actin is calcium sensitive, being reduced slightly in the absence of calcium and largely eliminated when calcium is included in the binding buffer. Binding was partially restored in the presence of Tn-C (+Ca^{2+}) by the addition of Tn-I. When Tn-C was excluded from the assay medium, Tn-I was also found to slightly accentuate the binding of Tn-T (or T2) to F-actin to levels above what was observed with Tn-T alone.

T-Pos242 TROPONIN T ISOFORM EXPRESSION IN SINGLE PHYSIOLOGICALLY INTACT VENTRICULAR MYOCYTES. Rashid N. Nassar[+], Gudrun E. Moore[*], and Page A. W. Anderson[‡]. Departments of Physiology[+‡] and Pediatrics[‡], Duke University Medical Center, Durham, N.C.; and Department of Biochemistry[*], St. Mary's Hospital Medical School, University of London, England.

Troponin T (TnT) expression in single cardiac cells is described for the first time. TnT has been shown in skeletal muscle to have multiple isoforms, which are considered to affect the sensitivity of the myofilaments to calcium. To investigate the presence of multiple isoforms of TnT in cardiac muscle, with its implications for physiological differences among cells, we have developed a method for characterizing TnT of single cardiac cells. Isolated cells were obtained enzymatically from rabbit ventricular myocardium. The cells were quiescent in millimolar [Ca], and responded to electrical field stimulation with synchronous sarcomere shortening. After the contraction waveform of a cell was recorded, the cell was chemically skinned, removed from the bath, and prepared for SDS gel electrophoresis. Purified rabbit ventricular TnT, troponin I (TnI), and tropomyosin were used as standards. Electrophoresis of the TnT standard yielded five bands which migrated differently from those of actin, TnI, and tropomyosin. Electrophoresis of individual cells revealed proteins which migrated to the same positions as those of the TnT standard. This is a new finding: multiple forms of TnT are present in each cardiac cell, and the relative amounts of the isoforms vary among the cells. These observations suggest that the sensitivity of the myofilaments to calcium varies among cardiac cells. The single cell should be the best preparation for testing the relationship between TnT expression and the physiological characteristics of the myofilaments.

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T-Pos243 EFFECTS OF CROSSLINKING OF THIN FILAMENT PROTEINS BY EDC ON THE THIN FILAMENT-MYOSIN INTERACTION. T. William Houk and Sondra Karipides, Department of Physics, Miami University, Oxford, Ohio 45056.

The "zero-length" crosslinker 1-ethyl-3-[3-(dimethylamino)propyl] carbodiimide (EDC) has been employed to crosslink tropomyosin to actin and to treat fully reconstituted thin filaments. Thin filaments reconstituted with tropomyosin and actin crosslinked by EDC show deceases in Ca^{++} sensitivity of activated myosin ATPase proportional to the degree of crosslinking. Fully reconstituted thin filaments containing actin, tropomyosin, and troponin can be crosslinked with EDC. In the absence of Ca^{++} the decrease in Ca^{++} sensitivity again depends on the degree of crosslinking between tropomyosin and actin. However, the effects are not identical to those obtained when troponin is not present during the crosslinking reaction. This effect may be due to the crosslinking which occurs between troponin and tropomyosin with EDC. In the presence of Ca^{++}, EDC crosslinking of the fully reconstituted filaments tends to destroy the CA^{++} sensitivity of the thin filament's ability to regulate the myosin ATPase activity, "freezing" the filament into the "switched on" configuration. This effect is similar to that which has been observed with other non-specific crosslinkers, but is again proportional to the degree of crosslinking effected by EDC.

T-Pos244 RESOLUTION AND PROPERTIES OF TWO BARNACLE TnC ISOFORMS. James D. Potter, Christopher C. Ashley[+], Karen Machado, John Collins[*], and Janet L. Theibert[*], Univ. of Miami, Dept. of Pharmacology, Miami, FL, [+]Univ. Laboratory of Physiology, Parks Rd. Oxford, UK, *Clarkson University, Dept. of Biology, Potsdam, NY.

Previous studies (Potter et al., 1986, Biophys J. 49, 249a) have shown that the giant barnacle (Balanus nubilus) contains tropomyosin and a troponin complex which together are capable of regulating rabbit acto-HMM ATPase in a Ca^{2+} dependent manner. The BTnC fraction isolated from the Tn complex contained one major band of Mr≅ 15,000 ($BTnC_2$) and a minor band of Mr≅ 18,000 ($BTnC_1$) in a 3:1 ratio. These TnC's have now been separated on HPLC using a modification of the method of Klee et. al. (Biochem. Int. (1981) 2, 485) on a C18 reversed phase column in the presence of EDTA. Cyanogen bromide cleavage, peptide isolation and subsequent a.a. sequence analysis have shown that $BTnC_2$ is not a breakdown product of $BTnC_1$. Only $BTnC_2$ incorporates dansylaziridine when the mixture is labeled while both $BTnC_1$ and $BTnC_2$ can equally restore Ca^{2+} dependent force to TnC depleted skinned barnacle muscle fibers. The Ca^{2+} dependence of $BTnC_{2DANZ}$ fluorescence consists primarily of a fluorescence increase with a midpoint at ~pCa=5.9, virtually unchanged by 5mM Mg^{2+} indicating that $BTnC_2$ contains at least one Ca^{2+}-specific site. Since BTnC (1 or 2) binds to TnC depleted barnacle fibers in low Ca^{2+} (pCa 8) Mg^{2+} containing solutions, this also suggests the presence of at least one Ca^{2+}-Mg^{2+} type site. SDS PAGE of single fibers from the lateral, scutal and tergal depressor muscles show a variable distribution of the two isoforms of BTnC. Since both forms appear to be biologically equivalent, the physiological significance of the presence of the two proteins in a single muscle fiber remains to be elucidated. (Supported by grants from the MDA and NIH).

T-Pos245 STRUCTURE OF CO-CRYSTALS OF TROPOMYOSIN AND TROPONIN.

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Recent studies have indicated that both globular and fibrous domains of troponin interact with various sites on the tropomyosin molecule. We are visualizing these interactions using X-ray crystallography. The structure of the tropomyosin filaments has been determined to 15 Å resolution. The crystals are unusually labile and are stabilized by lightly fixing with glutaraldehyde. Troponin and its fragments can then be diffused into the tropomyosin lattice and the structure of the resulting co-crystals can be solved. Whole troponin and two regions of its tail domain (T1, residues 1-158 of troponin-T; and CB2 residues 71-151) have been incorporated and visualized to 20 Å resolution.

These electron density maps reveal aspects of the interactions between tropomyosin and troponin. The T1 fragment lies along a considerable length (90 Å) of the carboxy end of tropomyosin. It is also interacts with a short segment of the adjacent tropomyosin molecule. Residues 1-71 of troponin-T are involved in binding to the termini of tropomyosin and residues 71-151 extend toward the middle of the tropomyosin molecule. The globular domain of troponin binds near amino acids 150-180 of the tropomyosin molecule. The visualization of these interactions will be useful in defining the roles of these proteins in the regulation of muscle contraction.

Supported by NIH grants AM32764 (G.N.P.) and AM17346 (C.C.) and the Muscular Dystrophy Association (C.C.). G.N.P. is an Established Investigator of the American Heart Association.

T-Pos246 FLUORESCENCE PROBE STUDIES OF THE STATE OF TROPOMYOSIN IN RECONSTITUTED THIN FILAMENTS.

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The monomer fluorescence of pyrene maleimide-labeled tropomyosin bound to F-actin (PTm-actin) increases by ~18% when myosin subfragment 1 (S1) binds and the change is 1/2 complete when only ~1 S1 is bound to the 1Tm-7 actin unit under tight S1 binding conditions, i.e., in the absence of nucleotide and low ionic strength (Ishii & Lehrer, Biochem. 1985). We now report that S1 binding to acrylodan-labeled ααTm (ACTm) produced a ~15% increase with a similar stoichiometry. Troponin (Tn) increased the peak fluorescence of ACTm-actin by ~25 ± 2% and PTm-actin by ~20± 2% (± Ca). S1 further increased the fluorescence of PTm-actin-Tn by 17% ± 2% independent of the presence of ADP. In the absence of nucleotide, the pyrene monomer fluorescence change was 1/2 complete when ~0.5 S1 (+Ca) and ~1.5 S1's (-Ca) were bound to the 7 actin unit, respectively. In the presence of MgADP, where S1 binding is weakened, the S1 binding profiles as measured by light scattering were highly cooperative, with the cooperative transition occurring at lower [S1] in the presence of Ca as first shown by Greene & Eisenberg (PNAS, 1980). The fluorescence of PTm-actin-Tn increased sharply at each transition [S1] and no further change occurred over the range where S1 bound strongly in agreement with the 2-state allosteric model of Hill et. al. (PNAS, 1980). These studies indicate that; i) these Cys-specific labels probe the S1-induced change of state of Tm in the Tm-actin and Tm-actin-Tn thin filament in the absence as well as the presence of Ca, ii) in the absence of S1, Ca does not significantly change the AC or P probe environment on Tm, iii) the S1-induced change in state is the same in the absence or presence of ADP. Supported by NIH, NSF and the MDA.

T-Pos247 ISOLATION OF A RABBIT SKELETAL MUSCLE TROPONIN C cDNA CLONE.

Anita S. Zot, James D. Potter and William L. Strauss. Department of Pharmacology, University of Miami School of Medicine, Miami, FL 33101.

Troponin C (TnC) is the Ca^{2+}-binding subunit of the protein troponin which is responsible for the regulation of muscle contraction. In order to better understand the molecular events underlying the divalent cation binding and regulatory properties of TnC, we have isolated a cDNA corresponding to the mRNA for rabbit skeletal muscle TnC. A cDNA library prepared from rabbit skeletal muscle mRNA was obtained from Dr.'s C. Brandl and D. MacLennan. Approximately 5000 recombinant clones carried in the mammalian expression vector pCD were screened with a nondegenerate synthetic oligonucleotide (50-mer) corresponding to amino acid residues 101-116 of rabbit skeletal muscle TnC. Thirty-four positive clones were identified suggesting that the abundance of TnC mRNA reflects that of the protein (0.7%). Eight clones with inserts ranging from 650 to 870 bp have been selected for further characterization. According to a published estimate (Garfinkel et al., JBC 257: 11078), the mRNA for TnC is 920 nucleotides long with a 5'-noncoding region of 293 nt and a 3'-noncoding region of 150 nt. We expect, therefore, that the longest clones which have been isolated contain the entire protein coding region. This conclusion is being confirmed by chain termination DNA sequencing in M13 mp18/19. A full-length clone and cDNA sequence information will enable us to produce site-specific mutants of TnC which can be tested for their Ca^{2+}-binding properties and ability to regulate the development of tension in skinned muscle fibers. Supported by a grant from the Lucille P. Markey Charitable Trust, Miami, FL (ASZ).

T-Pos248 STRUCTURE/FUNCTION OF TROPONIN SUBUNITS PROBED BY MONOCLONAL ANTIBODIES. Kimbrough D. Warber, Priscilla F. Strang, and James D. Potter. Dept. of Pharmacology, Univ. of Miami School of Medicine, Miami, FL 33101.

To probe region-specific interactions among the three subunits of rabbit skeletal muscle troponin (RSTn), we have developed panels of monoclonal antibodies (McAb's) directed against each subunit (RSTnI, RSTnT, RSTnC). Both IgM and IgG McAb's have been isolated which show I-band specific staining (indirect immunofluorescence) in skinned myofilaments. To begin mapping epitope specificity of the McAb's, crossreactivity with TnI's from muscle of phylogenetically distinct vertebrates and one invertebrate has been assessed via enzyme-linked immunosorbent assay (ELISA). Data suggest that certain of the anti-RSTnI McAb's recognize epitopes common to vertebrate skeletal muscle TnI's also shared by bovine cardiac TnI. The McAb's did not bind to a functionally homologous protein from muscle of the giant barnacle. A more complex pattern of crossreactivity for the anti-RSTnT McAb's suggest certain vertebrate TnI's may share sequence or structural epitopes with RSTnT. One epitope on RSTnI recognized by anti-RSTnI McAb's may be involved in TnI-TnC interaction. In a competitive inhibition ELISA, added STnC interfered with McAb binding to adsorbed STnI in a calcium-sensitive manner, as did added STnI.STnC complex; inhibition by STnI alone was Ca^{2+}-independent. The effect of McAb binding on subunit association and function in reconstituted thin filament constructs is being examined as well as the fine specificity of these McAb's by comparison of known subunit sequences. Supported by NIH RO1 AM33427. K.D. Warber is a Research Fellow of the American Heart Association, Florida Affiliate, Inc.

T-Pos249 TROPONIN I BINDS TO THE N-TERMINAL TWELVE-RESIDUE SEGMENT OF ACTIN. Z. Grabarek and J. Gergely. Dept. of Muscle Research, Boston Biomedical Research Institute; Depts of Biol. Chem. and Neurology, Harvard Medical School and Dept. of Neurology, Mass. Gen. Hospital, Boston MA 02114

The binding of troponin I to actin has been regarded as an important step in the inhibition of myosin activation by the actin-tropomyosin-troponin filament. Since in the absence of tropomyosin TnI binds to actin in the F-form in a 1:1 ratio with full inhibition of actomyosin ATPase, we have used the TnI-actin complex to identify the region of the actin sequence involved in interaction with TnI. Actin labeled at Cys-374 with 7-diethylamino- 3-(4'-maleimidylphenyl) 4-methylcoumarin has been crosslinked with TnI by incubation of the 1:1 TnI-actin complex with 1-ethyl-3-[3-(dimethylamino) propyl]-carbodiimide (1mM, 30 min, 22°C). The yield of the complex was 20-30% while the actin-actin crosslinking was marginal. The uncrosslinked actin and the crosslinked actin-TnI complex were separated on an SDS-polyacrylamide gel and the slices containing the proteins were subjected to proteolysis with CNBr, 2-[(2-nitrophenyl) sulfenyl]-3-bromoindolenine, 2-nitro-5-thiocyanatobenzoic acid and hydroxylamine at Met-X, Trp-X Cys-X and Asn-Gly bonds, respectively (Sutoh, Biochemistry 21,3654,1982). Based on comparison of the digestion patterns obtained for actin and the actin-TnI complex we conclude that TnI has been crosslinked to the N-terminal 12 residue fragment of actin. The same sequence has been previously recognized as the primary interaction site for myosin-S1 and for some other actin-binding proteins (depactin, fragmin) (cf. Sutoh, Biochemistry 25,435,1986). Supported by NIH (HL07266, HL05949) and MDA.

T-Pos250 COUPLING BETWEEN THE TWO DOMAINS OF MUSCLE FIBER TnC. T. Allen, L.D. Yates and A.M. Gordon. Dept. Physiology and Biophysics, University of Washington, Seattle, WA 98195.

We have used two fluorescence probes to study the calcium induced changes in the low and high affinity sites of fiber incorporated labeled TnC. TnC was labeled either at Met-25 with 5-dimethylaminonaphthalene-1-sulfonyl aziridine (DANZ) or at Cys-98 with tetramethylrhodamine-5-iodoacetamide (Rhod), and further was purified using a TnI affinity column. Labeled TnC was incorporated into partially TnC depleted fibers. With TnC-DANZ, fluorescent emission was followed, while with TnC-RHOD the dichroism of the emitted fluorescence, probe order, was measured. Beyond overlap of the thick and thin filaments, the probe's response to calcium was similar: over the range of pCa 9.2 to 5.0, both at 3 mM and 50 μM free magnesium in the absence of MgATP, the response of both probes was best fit with two transitions (Hill coefficients at 3 mM Mg: $pK_1 \approx 7.2$, $n_1 \approx 1.0$, $pK_2 \approx 5.9$, $n_2 \approx 1.6$; and at 50 μM Mg: $pK_1 \approx 8.1$, $n_1 \approx 2.0$, $pK_2 \approx 6.4$, $n_2 \approx 1.7$). Both probes reported a two fold increase in n_2 upon the addition of 3 mM MgATP. However, a striking difference existed when rigor cross-bridges were added by allowing overlap of the thick and thin filaments. The addition of calcium in the presence of rigor cross-bridges yielded a change in disorder of RHOD comparable to that due to cross-bridges alone, yet the DANZ fluorescence enhancement due to calcium was two orders of magnitude less than that due to rigor cross-bridges. Activation resulted in a further increase in DANZ fluorescence but a slight decrease in the rigor-calcium change observed with RHOD. Our results indicate that both probes are sensitive to calcium binding at the low and high calcium affinity domains demonstrating that the conformational changes associated with calcium binding to one domain are sensed at the other domain either through the molecule itself or through the calcium dependent interactions with the other proteins of the thin filament. Yet, cross-bridges readily affect the low affinity sites, leading to a loss of the tight coupling between the two TnC domains as observed in the absence of cross-bridges. This is consistent with a model in which TnC and, at least, TnI interact strongly in the presence of calcium, but even more so in the presence of cross-bridges, a conclusion consistent with the results obtained with TnI (Trybus and Taylor, 1980, Proc.Natl.Acad.Sci. 77:7209). The tight coupling observed between cross-bridges and DANZ fluorescence (see also Schultie et al., 1987, this volume) indicates that the low affinity sites, as opposed to the high affinity sites, are an integral part of the actomyosin ATPase scheme. This was supported by grants from NIH (NS 08384 and HL 31962) and MDA.

T-Pos251 PEPTIDIC INHIBITORS OF SMOOTH MUSCLE MYOSIN LIGHT CHAIN KINASE. S. Moreland, J.T. Hunt, and D.M. Floyd. Departments of Pharmacology and Chemistry, The Squibb Institute, Princeton, NJ.

Phosphorylation of the 20Kd myosin light chain (MLC) by the calcium-calmodulin dependent MLC kinase is thought to be a prerequisite to force development in smooth muscle. However, the study of the relationship between MLC kinase activity and force development has been slowed by the lack of a potent, specific inhibitor of MLC kinase. The amino acid sequence of MLC near the phosphorylation site (S_{19}) is -K_{11}-K-R_{13}-P-Q-R_{16}-A-T-S_{19}-N-V-F-A_{23}- (11-23). Synthetic peptides corresponding to this sequence, with A substituted for P_{14} and Q_{15}, were synthesized and tested for their ability to inhibit swine carotid MLC kinase. Activity in the presence of the inhibitor was compared to that in its absence and plotted against inhibitor concentration to obtain an IC_{50} value. Peptide 11-13 was a weak inhibitor (730μM). Peptides longer than 11-16 were not significantly more potent than 11-16 itself; IC_{50} values for peptides 11-16, 11-19, and 11-23, were 27, 14, and 18μM, respectively. In an effort to improve inhibitory potency, similar compounds were prepared in which the free amino terminal (contained in a peptide bond in the MLC) was capped by an acetyl (Ac) group. Interestingly, the Ac capped peptides were weaker inhibitors than the corresponding peptides with free amino terminals. The IC_{50} values for the Ac11-13, Ac11-16, Ac11-19, and Ac11-23 were 2300, 205, 290, and 900μM, respectively. Thus, the free amino terminal seemed to improve the binding interaction of the peptide with MLC kinase. This additional binding interaction became more important as the length of the peptide increased as suggested by a comparison of IC_{50} values. Ac11-23 was also a poorer substrate than 11-23 (K_m/V_{max} was 153 and 40, respectively) due to an increase in the K_m value. In summary, peptides which include only the two basic recognition sites on the MLC (residues 11-13 and 16) inhibit the MLC kinase as potently as previously reported peptides which are extended to include the phosphorylation site and the carboxy terminal hydrophobic residues. Capping the amino terminal with Ac decreases the ability of the peptides to act both as inhibitors and as substrates.

T-Pos252 Mn^{2+} ACTIVATES SKINNED SMOOTH MUSCLE CELLS DIRECTLY WITHOUT MYOSIN LIGHT CHAIN PHOSPHORYLATION AND BY REVERSIBLE OXIDATION. Phyllis E. Hoar[+] and W. Glenn L. Kerrick[+#], Depts. of [+]Physiology & Biophysics and [#]Pharmacology, Univ. of Miami School of Medicine, Miami, FL 33101.

Freshly skinned gizzard smooth muscle cells contracted when exposed to 1 mM Mn^{2+} and 2 mM MnATP. The Mn^{2+}-activated cells did not relax when exposed to normal relaxing solutions which contain MgATP and no Ca^{2+}, except when mM DDT was added to the solutions. The cells then acted like control cells which could be reversibly activated by Ca^{2+} and MgATP. Thus it appears that smooth muscle cells activated by Mn^{2+} are in part activated by protein oxidation. In contrast, skeletal muscle cells which were activated by Mn^{2+} did not need to be treated with DTT in order for them to relax. Mn^{2+} activation of smooth muscle is not entirely due to oxidation, since the skinned gizzard cells contracted rapidly in the presence of Mn^{2+} and DTT and subsequently relaxed rapidly in a normal relaxing solution without DTT. Both this contraction and this relaxation were more rapid than a normal Ca^{2+}-activated contraction and relaxation. Mn^{2+} activation is not due to activation of myosin light chain kinase as judged by the lack of ^{32}P incorporation into myosin light chains. Therefore it seems likely that Mn^{2+} acts at sites not associated with myosin light chain kinase. Likewise Mn^{2+}-activation of skinned smooth muscle cells can be achieved in Mn^{2+}-activating solutions in which CTP has been substituted for ATP. In contrast to cells activated in MnATP solutions, cells activated in MnCTP solutions did not require exposure to DDT to be reversibly relaxed, suggesting that oxidation may be dependent upon the presence of ATP or inhibited by CTP. Thus Mn^{2+} can activate by two mechanisms, directly and through protein oxidation. Supported by American Heart Assn. (National and Florida Affiliate), and MDA.

T-Pos253 Mg^{2+} AFFECTS MAXIMUM TENSION AND RELAXATION RATES IN SKINNED SMOOTH MUSCLE CELLS, BUT NOT THE pCa-TENSION RELATIONSHIP. Phyllis E. Hoar[+], R. Emmet Kenney[+], W. Glenn L. Kerrick[+#], Depts. of [+]Physiology & Biophysics and [#]Pharmacology, Univ. of Miami School of Medicine, Miami, FL 33101.

Skinned gizzard and rabbit pulmonary artery cells were used to study the the effect of Mg^{2+} on smooth muscle. Varying pMg from 4.3 to 2.0 in rabbit pulmonary artery skinned cells had little effect on the relationship between pCa and percentage of maximum Ca^{2+}-activated tension. This finding is consistent with the known inability of Mg^{2+} to bind strongly to or alter the activity of calmodulin, an activator of the myosin light chain kinase system in smooth muscle. In contrast low levels of Mg^{2+} caused a dramatic decrease in the maximum tension the cells could develope. This decrease in tension at low levels of Mg^{2+} could be partially overcome by addition of exogenous calmodulin. This suggests that low levels of Mg^{2+} are affecting the myosin light chain kinase-phosphatase system of smooth muscle activation. However, when the skinned cells were maximally activated by thiophosphorylation of the myosin light chains with ATPγS, low levels of Mg^{2+} still decreased maximum tension slightly. Therefore, in addition to affecting the myosin light chain kinase-phosphatase system, Mg^{2+} also affects actin-myosin cross-bridge interactions. This conclusion is further supported by the observation that lower Mg^{2+} levels cause a dramatic increase in the rate of relaxation in the skinned cells. Supported by the American Lung Assn., the American Heart Assn. (National & Florida Affiliate), and the Muscular Dystrophy Assn.

T-Pos254 MG-INDUCED TENSION IN "SKINNED" CORONARY ARTERIES. Elias J. Khabbaza and J. David Johnson. Dept. of Physiological Chemistry, The Ohio State University Medical Center, Columbus, Ohio.

The most widely accepted mechanism for tension production in smooth muscle is the phosphorylation of myosin light chain (MLC) by the calcium-calmodulin-dependent myosin light chain kinase. MLC phosphorylation increases with calcium-induced tension but falls to nearly basal levels while force is maintained. We have examined the role of Mg in modulating the contraction-relaxation cycle of "skinned" porcine coronary arteries in the absence of activating Ca^{2+}. The addition of 10mM $MgCl_2$ to coronaries equilibrated in low [$MgCl_2$] produced tension which was reversed with EDTA. Unlike Ca-induced tension this Mg-induced tension was insensitive to calmodulin antagonists drugs. Mg-induced tension was augmented by Ca^{2+}, and Ca^{2+} removal reduced tension back to the level produced by Mg alone. Ca-induced tension could be maintained after Ca^{2+} removal by raising [Mg^{2+}]. ATP but not ADP, CTP or ITP was necessary for Mg-dependent tension. Determination of MLC phosphorylation levels indicated that the initial phase of Mg-induced tension correlated with an increase of the phosphorylation of MLC, but that this phosphorylation declined while tension remained. Our studies provide evidence that Ca^{2+} and Mg^{2+} can produce tension by distinct mechanisms both of which involve phosphorylation of MLC. The possible role of Mg^{2+} in maintaining tension after the fall of the Ca^{2+} transient is discussed. This work was supported by grants from The American Heart Association (84-792) and its East Central Ohio and Central Ohio Affiliates.

T-Pos255 **REGULATION OF GLYCOGEN UTILIZATION IN VASCULAR SMOOTH MUSCLE.** **Ronald M. Lynch and Richard J. Paul, Department of Physiology and Biophysics, University of Cincinnati College of Medicine, Cincinnati, OH 45267-0576.**

Despite exhaustive stimulation, glycogen generally cannot be depleted in vascular smooth muscle. To further study the regulation of glycogen utilization during contraction, we measured the activities of phosphorylase, debrancher enzyme (amylo-1,6-transglucosidase) and glycogen content in porcine carotid artery. Elevation of medium K^+-concentration to 85 mM elicits a contraction and activates phosphorylase *a* (activity ratio -/+ AMP, 0.16 ± 0.01, n = 16 to 0.37 ± 0.03, n = 6). After an initial period of rapid glycogenolysis, glycogen content remains constant at approximately 50% of control despite a continued elevation in phosphorylase *a* activity. In the absence of exogenous substrate, however, glycogen content continues to decrease at a slow rate, ~ 10 nmol glucosyl unit per min·gm. We measured the activity of glycogen debrancher enzyme using the same assay as for phosphorylase except with a limit dextran as substrate. The limit dextran was produced by exhaustive digestion of glycogen with phosphorylase. The tissue activity of glycogen debrancher averaged 18.7 ± 5.5 (n = 6) times less than the total phosphorylase activity (0.175 ± 0.029 µmol/min·g, n = 6). This is similar in magnitude to the secondary rate of glycogen breakdown in the intact tissue. Our studies also indicate that glycogen is utilized as a primary substrate for mitochondrial oxidative phosphorylation during tension generation. However, the utilization of glycogen as a metabolic substrate during prolonged tension maintenance is limited by the activity of the glycogen debrancher enzyme. Supported in part by NIH 23240 and an Established Investigatorship of the American Heart Association (RJP).

T-Pos256 MYOSIN LIGHT CHAIN KINASE- AND PROTEIN KINASE C- MEDIATED CONTRACTIONS IN ARTERIAL AND VENOUS SMOOTH MUSCLE. M. Chatterjee, M. Tejada and P.J.S. Chiu, Schering Corporation, 60 Orange St., Bloomfield, NJ 07003. (Intr. by B.J.R. Pitts)

We have compared the roles of myosin light chain kinase and protein kinase C in regulation of contraction in intact and skinned preparations of dog saphenous vein and porcine carotid artery. [Ca^{++}] required for half-maximal and maximal stress development (6.29 ± 0.33 x 10^4 N/m^2, n=4) in Triton X-100 skinned saphenous veins were $2.9x10^{-7}$ M and 10^{-5} M, respectively. Myosin light chain phosphorylation exhibited a similar Ca^{++}-dependence and increased from basal values of 0.29±0.02 mole P_i/mol light chain (n=4) in 0 Ca^{++} (0 added Ca, 5 mM EGTA) to 0.66 ± 0.03 mol P_i/mol light chain (n=4) in 10^{-5} M Ca^{++}. Thus, force and MLC phosphorylation in response to changes in intracellular [Ca^{++}] were qualitatively similar in artery (Chatterjee and Murphy, Science 221: 464-466, 1983) and vein. Phorbol 12,13-dibutyrate (PDBu), an activator of protein kinase C, elicited dose-dependent contractions in the intact vein. Steady-state contractions in response to 10^{-6} M PDBu were inhibited by 50% upon pretreatment with 10^{-6} M nifedipine with an associated 80% reduction in Ca^{++} influx. Under identical conditions, Ca^{++} influx in the artery was reduced by 96% with no apparent effect on steady-state stress. Skinned veins did not respond to PDBu under conditions in which the skinned arteries did. Thus phorbol ester-dependent contractions in the dog saphenous vein, unlike the porcine carotid artery, demonstrated great dependence on external Ca^{++}, presumably via activation of the voltage-dependent Ca^{++} channel associated with the plasma membrane.

T-Pos257 **EFFECTS OF MYOSIN KINASE INHIBITING PEPTIDE ON ISOMETRIC FORCE (F_o) AND UNLOADED SHORTENING VELOCITY (V_{us}) IN CHEMICALLY SKINNED GUINEA PIG TAENIA COLI.** **John D. Strauss, Primal deLanerolle and Richard J. Paul, Depts. of Physiology and Biophysics, Univ. of Cincinnati (45267-0576) and Illinois, Chicago (60612).**

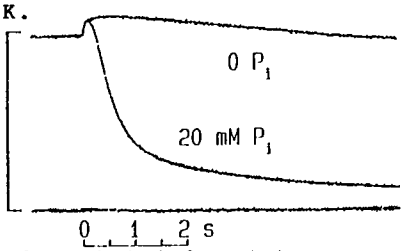
A nine residue peptide analog of the phosphorylation site of smooth muscle myosin light chains has been reported to be a competitive inhibitor of the myosin light chain kinase (K_i = 10 μM) in free solution (Pearson et al., J. Biol. Chem. 261, No. 1 25-27, 1986). Triton X-100 "skinned" fibers in solutions containing between 100 and 400 μM of this peptide produced normal maximum isometric forces at pCa ≤ 5.2 and relaxed at pCa > 8.0. Solutions are described in Circ. Res. 53:342. Using the "slack test" to measure unloaded shortening velocity and series elasticity (S.E.), we found that the V_{us} in the presence of inhibitor to that in bracketing control contractions was 0.62 ± .03 (n = 9). No consistent changes in S.E. were observed with inhibitor. Force ratios averaged 0.96 ± .04 (n = 9) indicating that velocity was decreased with no concurrent decrease in force. Our results suggest that myosin peptide inhibitor can depress cross bridge cycle rate but with no apparent change in number of interacting crossbridges. Further interpretation of these results awaits measurement of myosin phosphorylation levels (in progress).

Supported by NIH HL 22619, NIH HL 35808, and NIH HL 07571.

T-Pos258 CALCIUM-INDEPENDENT PHOSPHORYLATION OF THE 20,000-D MYOSIN LIGHT CHAIN IN PERMEABILIZED RABBIT TAENIA COLI DOES NOT LEAD TO ACTIVE FORCE OUTPUT. T.M. Butler, M.J. Siegman and S.U. Mooers, Department of Physiology, Jefferson Medical College, Philadelphia, PA 19107 USA

In the permeabilized rabbit taenia coli (RTC), the 20,000 dalton myosin light chain (MLC) is 39±3% phosphorylated in a relaxing solution containing 20 mM EGTA and no added [Ca^{++}]. In contrast, only 9.2±1.5% of the MLC is in the low pI form in resting intact RTC. The MLC phosphorylation in relaxing solution is not associated with an increase in force even though phosphorylation exceeds that of the intact RTC under all conditions of full activation. At pCa 4.5, the RTC develops maximum force with 49±2% of the MLC monophosphorylated and 20±1% doubly phosphorylated. In relaxing solution, permeabilized chicken gizzard shows very little of the low pI form of the MLC (5.6±0.2%); in pCa 4.5, maximum force occurred, and the monophosphorylated form of the MLC increased to 41±1%, with little double phosphorylation (1.5±1.5%). Similar results were obtained for the rat anococcygeus. Thus, an increase of 0.4 to 0.5 mole phosphate/mole MLC was associated with maximum force in all muscles. Under activating conditions with ATPγS, the RTC shows almost 100% monothiophosphorylation of the MLC; subsequent incubation of the muscle in relaxing solution results in additional phosphorylation of the thiophosphorylated MLC. Trifluoperazine (300 μM) almost totally inhibits both force production and the increase in MLC phosphorylation at pCa 5.0, but has no effect on the basal MLC phosphorylation in relaxing solution. Therefore, the phosphorylation present in low [Ca^{++}] cannot support force generation at high [Ca^{++}]. It is likely that this calcium-independent MLC phosphorylation is at a site different from that normally phosphorylated by MLC kinase, and although it does not lead to force generation, it might play a role in regulation of crossbridge cycling rate. (Supported by HL 15835 to the Pennsylvania Muscle Institute)

T-Pos259 COOPERATIVITY AND NEGATIVELY STRAINED CROSS-BRIDGES IN VERTEBRATE SMOOTH MUSCLE. A.V.Somlyo, Y.E.Goldman, T.Fujimori, M.Bond, D.R.Trentham* & A.P.Somlyo, U. PA Sch. of Med., Phila., PA; *MRC London, U.K.



Laser pulse photogeneration of ATP^1 was used to rapidly initiate tension transients in permeabilized guinea pig portal vein strips (PAMV). In 0 Ca^{2+}, liberation of ~20μM ATP into rigor muscles caused a tension increase to 20-40% maximal active tension and a decrease in stiffness. Above ~40μM, ATP relaxed the rigor tension following a lag phase(~400ms).In striated muscle, a similar biphasic effect of ATP, in 0 Ca^{2+}, is thought to be due to thin filament cooperativity[2]. Decreasing or increasing the length of PAMV in rigor, prior to the laser trigger, revealed an initial quick tension rise ($t_{1/2}$~50ms) or fall$_3$(~100s^{-1}) respectively, upon release of 0.5-1mM ATP. 10mM P_i in the photolysis medium[3] had no effect on this quick tension rise or fall, but reduced or abolished the lag phase and markedly accelerated final relaxation (Fig.1). The rapid rate and lack of P_i-effect on the quick tension rise are consistent with detachment of negatively strained cross-bridges after length decreases and positively strained cross-bridges after length increases. The maintained tension and slow time course of final relaxation in the absence of P_i may indicate cycling due to cooperativity among rigor cross-bridges in a "myosin-regulated" muscle. 1.Goldman et al., Nature 300:701, 1982; 2. Bremel & Weber, Nature new Biol. 238:97,1972; 3. Arner et al., J. Mus. Res. Cell Motil.7:381, 1986. Supp by NIH grant HL15835 to the PMI.

T-Pos260 MULTIPLE SITE PHOSPHORYLATION OF THE 20,000 DALTON LIGHT CHAIN OF SMOOTH MUSCLE MYOSIN (LC20): REGULATION OF GLYCERINATED SMOOTH MUSCLE CONTRACTION. Joe R. Haeberle, Timothy A. Sutton and Brett A. Trockman, Krannert Institute of Cardiology, Department of Physiology/Biophysics, and Medicine, Indiana University School of Medicine, Indianapolis, IN

We have previously reported multiple site phosphorylation of LC20 associated with contraction of glycerinated porcine carotid artery smooth muscle. The purpose of the present study was to determine if multiple site phosphorylation alters the mechanics of smooth muscle contraction. The unphosphorylated (LC20), monophosphorylated (LC20P), and diphosphorylated (LC20PP) forms of LC20 from contracted muscles were separated by urea-glycerol gel electrophoresis. Two-dimensional peptide maps of limit, tryptic digests of both LC20P and LC20PP indicated the presence of a single phosphopeptide. LC20P contained phosphoserine while LC20PP contained both phosphoserine and phosphothreonine. Muscles which were phosphorylated in the presence of thio-ATP contained only phosphoserine and were phosphorylated to a maximum stoichiometry of 1.0 mol PO_4/mol LC20. Thiophosphorylated muscles could be further phosphorylated, in the presence of ATP, calcium, and calmodulin to 1.5 mol PO_4/mol LC20, with the additional phosphate incorporated exclusively into phosphothreonine. Isometric force and lightly-loaded shortening velocity were measured (pCa 8) for muscles phosphorylated to 1.0 mol PO_4/mol LC20 with thio-ATP. Muscles were then phosphorylated to 1.5 mol PO_4/mol LC20 and isometric force and shortening velocity were measured (pCa 4.7) subsequent to phosphorylation of the second site. There was no significant change in either isometric force (11.9 N/cm^2) or lightly-loaded shortening velocity (0.008 Lo/sec) associated with phosphorylation of a second site (thr). These studies indicate that phosphorylation of LC20 (ser) to 1.0 mol PO_4/mol LC20 is adequate for full activation of contraction.

T-Pos261 **CALCIUM-DEPENDENCE OF SHORTENING VELOCITY IN CANINE TRACHEAL SMOOTH MUSCLE.** W.T. Gerthoffer, K. Murphey and M. McGinnis. Dept. Pharmacol., U. Nevada School of Med., Reno, NV 89557.

Maximum shortening velocity (Vmax) was calculated from force-velocity curves obtained at several extracellular calcium concentrations. Tracheal muscle strips were stimulated with 10^{-6} M carbachol, Ca^{2+}-free solution, and contracted by increasing the $CaCl_2$ concentration in the bath. Other muscles were stimulated in 1.6 mM $CaCl_2$ and then relaxed by reducing the $CaCl_2$ concentration. Muscle shortening was measured following quick-release to afterloads ranging from 0.05 to 0.4 F/Fo, where F is the clamped force and Fo is the force generated at the moment of quick-release. Vmax was calculated from the linearized form of the Hill equation. The increase in Vmax upon increasing $CaCl_2$ was not significant (P = 0.21, ANOVA). However, the decrease in Vmax following reduction of extracellular $CaCl_2$ was significant (P = 0.01, ANOVA). The results suggest there is some hysteresis in the apparent calcium-dependence of shortening velocity that correlates with a similar hysteresis in the calcium-dependence of myosin phosphorylation reported previously (Gerthoffer Am. J. Physiol. 256: C597, 1986). Supported in part by grants from the Pharmaceutical Manufacturers Association Foundation, American Lung Association and NIH (HL35805).

	Increasing $CaCl_2$ (N = 9)			Decreasing $CaCl_2$ (N = 7)	
	Vmax (Lo/sec)	Fo (10^5 N/m^2)		Vmax (Lo/sec)	Fo (10^5 N/m^2)
0.025 mM	0.051 ± 0.003	0.86 ± 0.012	0.05 mM	0.032 ± 0.006	0.69 ± 0.09
0.1 mM	0.057 ± 0.007	1.30 ± 0.17	0.1 mM	0.037 ± 0.004	1.23 ± 0.22
0.4 mM	0.067 ± 0.003	1.87 ± 0.21	0.4 mM	0.051 ± 0.007	1.71 ± 0.26
1.6 mM	0.072 ± 0.010	2.55 ± 0.20	1.6 mM	0.052 ± 0.009	2.28 ± 0.18

T-Pos262 STRUCTURAL CHANGES IN CONTRACTING, SKINNED SINGLE SMOOTH MUSCLE CELLS, G.J. Kargacin P.H. Cooke and F.S. Fay Dept. of Physiology Univ. of Mass. Med. Ctr., Worcester, Mass.

Smooth muscle lacks an easily visualized sarcomere-like structure. Thus, it is not well understood how the contractile proteins are arranged in resting muscle nor is it known what changes in organization occur during contraction. We have begun to study these questions in functional, skinned, isolated smooth muscle cells that, as a result of skinning, display optically resolvable internal structures. Single cells, isolated from the stomach of the toad, Bufo marinus, were exposed to saponin (25 µg/ml) for 5 min under rigor conditions (0 ATP, 5mM EGTA). These cells shortened to < 1/3 L_i in the presence of ATP at a rate dependent on $[Ca^{++}]$. Under darkfield or phase contrast optical microscopy, skinned cells displayed well resolved dense bodies and a system of fine fibrils. To determine the three dimensional organization of these structures, optical sections of cells were made at 0.25 µm intervals and digitized images of these sections were subject to a constrained deconvolution with the point spread function of the optical system. Movement of dense bodies during contraction was analyzed from 2-D digital images acquired at intervals during cell shortening. Displacement of the dense bodies was not passive during contraction because the extent of their radial movement was less than would be expected for the observed increases in cell diameter. Over long periods of time (30 - 50 sec) the average axial movement of dense bodies (0.3 µm/sec) was uniform. However, over shorter intervals (3 - 6 sec) movement of closely spaced dense bodies or groups of dense bodies was not uniform suggesting that independent contractile elements are present within the cells. Supported by NIH grants AM07341, HL14523 and a grant from MDAA.

T-Pos263 A 31P-NMR STUDY OF SUBSTRATE USE AND HYPOXIA ON ISOLATED PORCINE CAROTID ARTERIES. M. J. Fisher & P. F. Dillon, Depts. Physiol. & Radiol., Mich. State U., E. Lansing, MI.

The metabolic parameters determined by 31P-NMR of hog carotid arteries show a substantial substrate dependence. Segments of porcine carotid arteries were dissected clean, cannulated, and placed in a 10 mm NMR tube. They were perfused at 5 ml/min with low phosphate (Pi=0.12 mM), high potassium (100 mM) PSS at room temperature. The perfusate contained either 15 mM glucose (G) or 5 mM glucose + 10 mM pyruvate (GP) and was equilibrated first with 95%O2-5%CO2 and then with 95%N2-5%CO2 (hypoxia) via an artificial lung. NMR measurements were made using a Bruker AM-400 WB spectrometer and a 10 mm 31P-dedicated probe. The spectra from oxygenated arteries perfused for > 1 hr with either G or GP were characterized by a prominent phosphomonoester peak (PME = 4.2 ppm), a small phosphodiester peak (PDE = 0.49 ppm), and a phosphocreatine (PCr)-to-ATP ratio of 0.58. The PME and PDE peaks constituted 26% and 4%, resp., of the total NMR-detectable phosphate. Intracellular pH (pHi) and free Mg++ were 7.10 $\pm$ 0.02 and 0.52 $\pm$ 0.02 (n = 3), resp., for G-perfused arteries, and 7.04 $\pm$ 0.03 and 0.42 $\pm$ 0.03 for those perfused with GP. After 2 hrs of hypoxia, G-perfused arteries had a lower pHi than GP-perfused arteries (6.67 $\pm$ 0.01 vs. 6.83 $\pm$ 0.06) and developed a distinct ADP peak (= -6.1 ppm). GP-perfused arteries showed a greater decrease in PCr (80% vs. 60%) and increase in Pi (75% vs. 50%) than G-perfused arteries. ATP, PME, and free Mg++ were not detectably changed during hypoxia with either substrate. One hour of reoxygenation restored pHi in both G- and GP-perfused arteries and returned PCr and Pi toward control levels. These data show that porcine carotid arteries can maintain stable phosphagen levels during prolonged stimulation with high potassium provided oxygenation is adequate. Further they suggest that ADP levels achieved during hypoxia may be a function of substrate. (Supp. by NIH AM34885 & Whitaker Fdn.)

T-Pos264 A GEOMETRIC 3-DIMENSIONAL THERMODYNAMIC MODEL FOR CROSSBRIDGE (XB) ATTACHMENT IN SMOOTH MUSCLE (SM). M. Li* and D.M. Warshaw, Biometry and Physiol & Biophys, Univ. of Vermont, Burlington, VT, 05405 (Intr. by N.R. Alpert)

SMs generate active stress comparable to that in skeletal muscle but with less myosin. An increased probability of XB attachment followed by a long-lived force producing XB state may in part account for the enhanced stress generating capabilities in SM. We therefore developed a 3-dimensional thermodynamic model for XB attachment based upon the models of Schoenberg (Biophys J 30:51, 1980) and Eisenberg et al. (Biophys J 29:195, 1980). The model assumes a single myosin XB capable of interacting with multiple actin sites in a plane normal to the filament's longitudinal axis. Assuming a S1 length of 16nm and a S2 of 40nm, the model predicts that XB attachment to actin is restricted to azimuthal angles between $\pm$ 60^{o} due to geometric limitations. Since in SM 12-15 actin filaments surround each myosin filament as compared to 6 in skeletal muscle, the possibility that an increased probability of attachment exists in SM was assessed by the model. The model predicts that XB's in SM and skeletal muscle have similar probabilities of attachment contrary to the proposed hypothesis. In addition, the XB in SM distributes its attachment amongst all available actin sites. Therefore the availability of additional actin sites in SM may not enhance XB attachment. However the model response is dependent upon the geometrical assumptions which could lead to the present findings. (Supported by NIH AR34872)

T-Pos265 HELICAL SHORTENING IN SINGLE ISOLATED SMOOTH MUSCLE CELLS (SMC). D.M. Warshaw, S. Work*, W.J. McBride*, Physiol. & Biophysics, Univ. of Vermont, Burlington, VT 05405

All estimates of shortening velocity in smooth muscle have been determined from end-to-end measurements of cell length (Lcell). To characterize the shortening process in smooth muscle, we studied freely shortening SMC isolated from the toad, Bufo marinus, stomach muscularis (Warshaw and Fay, 1983). To detect cell motion, a SMC was held at one end only to a micropipette and the cell surface uniformly decorated with 1 um resin beads. The cell image was viewed at 400X through a video camera. Cells were electrically stimulated to contract and video images digitized every 500 ms. Upon shortening, relative bead position along the cell length remained constant suggesting uniform cell shortening. However, beads were observed to rotate around the cell during cell shortening. Thus cells shorten in a helical, corkscrew-like fashion with a pitch of 1.4 Lcell. The helical shortening suggests that shortening velocity may be underestimated using end-to-end measurements by a factor of 2. In addition, the contractile apparatus within the cell must be arranged in a helical manner to allow corkscrew-like shortening to occur in SMC which was predicted by the model of Small (1977). (Supported by AR34872 & HL35684)

T-Pos266 CHANGES IN LENGTH-TENSION AND FORCE-VELOCITY RELATIONSHIPS OF RAT UTERINE SMOOTH MUSCLE DURING PREGNANCY. Janet L. Smart and Fred J. Julian, Dept. Anesthesia Research, Brigham & Women's Hospital, Boston, MA 02115.

The influence of pregnancy related hypertrophy and hyperplasia on contractile properties of uterine smooth muscle was examined in the longitudinal and circular layers of rat myometrium. Length-tension and force-velocity relationships were studied in muscle strips from nonpregnant rats and at 20 days gestation. Experiments were performed at 37°C and tissues were activated by periodic electrical field stimulation. Active and passive tension were recorded at several muscle lengths between 40 and 110% of Lmax, the length at which the active tension generated is maximal. Force-velocity characteristics were determined by releasing activated muscle strips to shorten against several loads ranging from 2-50% of the active tension at the time of release. All releases were made from a muscle length of 95% Lmax at a time when the rising tension reached 60% of maximal force. The passive tension level at 95% Lmax set the limit for lightest loads. Velocities were measured 50-75 ms after release. Vmax, the speed of unloaded shortening, was estimated by extrapolating to zero force along a hyperbola fitted to the force-velocity data. Passive tension (as percent active force at Lmax) and Vmax (muscle lengths/sec) respectively, from each layer were as follows: longitudinal nonpregnant (n=4), 2.9% $\pm$ 0.4, 0.76 $\pm$ 0.09; longitudinal pregnant (n=3), 21.0% $\pm$ 7.7, 0.29 $\pm$ 0.06; circular nonpregnant (n=5), 54.2% $\pm$ 11.6, 0.36 $\pm$ 0.03; and circular pregnant (n=3), 38.2% $\pm$ 12.5, 0.24 $\pm$ 0.01. These data suggest that speeds of shortening of both smooth muscle layers are reduced at late stages of pregnancy. However, the possible contribution of increased passive tension to the decrease in Vmax seen in longitudinal muscle of pregnant rats requires further investigation. Supported by NIH grant HL 35032 (FJJ)

T-Pos267 ACTIVATION OF CALMODULIN-SENSITIVE PHOSPHODIESTERASE AND MYOSIN PHOSPHORYLATION IN SMOOTH MUSCLE. J.R. Miller, J.N. Wells, J.T. Stull* and K.E. Kamm*, Dept. Pharmacol. Vanderbilt Univ.,Nashville, TN 37232 and *Dept.Physiol.,U.Tx.Hlth.Sci.Ctr.Dallas, Dallas,TX 75235.

Activation of smooth muscle contraction is associated with phosphorylation of the 20,000 dalton myosin light chain (LC), however, the sequence of biochemical events leading to phosphorylation, including calcium release and activation of myosin light chain kinase, have not been completely defined. Because measurements of myosin light chain kinase activation were technically unfeasible, we measured another calmodulin-dependent process, calmodulin-sensitive cyclic nucleotide phosphodiesterase (PDE), by a modified method of Saitoh *et al.* (Biochemistry 24:1613, 1985). Bovine trachealis was quick-frozen with a rapid-release freezing device after field stimulation. Fractional activation of PDE by calcium-calmodulin and LC phosphorylation were measured. PDE was maximally activated by 500 msec and was sustained for a least 4 sec. In contrast, monophosphorylated LC (LCP) was unchanged from the basal value of 5 percent for at least the first 500 msec, but increased thereafter to a maximum value of 65 percent at 2 sec. Diphosphorylated LC (LCP2) increased from 0 to 2.5 and 4.5 percent at 2 and 4 sec, respectively. Isometric force followed LC phosphorylation and PDE activation. With 10 min stimulation, there were decreases in PDE activation, LCP and LCP2. Thus, it appears that neural stimulation results in activation of calmodulin-sensitive PDE (and presumably myosin light chain kinase) before the onset of LC phosphorylation and that PDE activation is maintained for several seconds. The decrease in LC phosphorylation with sustained stimulation is associated with a decrease in the extent of calmodulin activation of PDE. (Supported by HL19325, GM21220, HL26043 and HL32607.)

T-Pos268 MONOPHOSPHORYLATED AND DIPHOSPHORYLATED MYOSIN LIGHT CHAIN IN CONTRACTING SMOOTH MUSCLE. C.H. Michnoff, S.B. Rybicki, T. Myint, K.E. Kamm and J.T. Stull, Dept.Physiol., Univ. Tx. Hlth. Sci. Ctr. Dallas, Dallas, TX 75235.

It has been recently reported that myosin light chain kinase phosphorylates myosin light chain (LC) rapidly at serine-19 and slowly at threonine-18 (Ikebe *et al.*(1986) J. Biol. Chem. 261:36.) Purified LC from bovine tracheal smooth muscle was phosphorylated with bovine tracheal muscle myosin light chain kinase to 1.2 mol ^{32}P/mol LC by 5 min and 2.0 mol ^{32}P/mol LC by 180 min. Monophosphorylated LC (LCP) contained ^{32}P-phosphoserine, whereas diphosphorylated LC (LCP2) contained both ^{32}P-phosphoserine and ^{32}P-phosphothreonine. Bovine trachealis was incubated in ^{32}P-PO_4 and subsequently stimulated with 10 uM carbachol for 1 or 30 min and quick-frozen. Nonphosphorylated and phosphorylated forms of LC were separated by urea-glycerol PAGE. After 1 min in carbachol, myosin was 59 $\pm$ 4% LCP and 9 $\pm$ 2% LCP2. At 30 min, the distribution was 45 $\pm$ 4% LCP and 4 $\pm$ 1% LCP2. Myosin heavy chain was not phosphorylated as determined by SDS-PAGE and autoradiography. SDS-PAGE of the urea-glycerol gel showed that a 28 kDa ^{32}P-labelled protein comigrated with 20 kDa LCP2. The amount of ^{32}P in the 28 kDa protein was greater at 30 min compared to 1 min exposure to 10 uM carbachol. At both 1 and 30 min ^{32}P-LCP contained ^{32}P-phosphoserine, whereas ^{32}P-LCP2 contained both ^{32}P- phosphoserine and ^{32}P-phosphothreonine. The 28 kDa protein contained ^{32}P-phosphoserine. Thus, stimulation of bovine tracheal smooth muscle cells with a high concentration of carbachol results in the formation of both LCP and LCP2 with phosphoamino acid compositions consistent with biochemical observations obtained with purified myosin light chain kinase. The amount of LCP2 formed is substantially less than the amount of LCP. (Supported by HL26043).

T-Pos269 INHIBITION OF FORCE GENERATION BY CYTOCHALASIN D DOES NOT PREVENT MYOSIN PHOSPHORYLATION AND DEPHOSPHORYLATION IN INTACT SMOOTH MUSCLE. K.E. Kamm, Department of Physiology, University of Texas Health Science Center at Dallas, Dallas, Texas 75235.

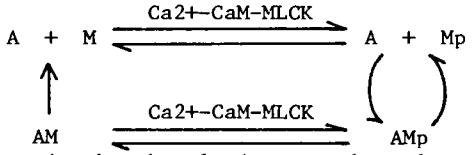
The present experiments were initiated to investigate whether presumed alterations in myosin conformation associated with force generation affect the properties of myosin as a substrate for either myosin light chain kinase or phosphatase. Cytochalasin D (CD), a drug which inhibits actin polymerization, has been reported to inhibit force development in smooth muscle (Adler, K.B. et al., Cell Motility 3:545, 1983). When trachealis muscle was incubated for 2 hrs in 10 uM CD, isometric force obtained with either 1 uM carbachol (CCh), 65 mM KCl (KCl) or electrical stimulation of intrinsic nerves (NS) was reduced to less than 0.1 of pretreatment values. The concentration for half-maximal inhibition (1 uM CD) was the same for each stimulus. The inhibition was slowly reversible (5 hrs for recovery from 0.05 to 0.5 pretreatment values). Under control conditions tracheal muscle myosin light chain (LC) phosphorylation reaches maximal values after 60 sec exposure to CCh or KCl or after 5 sec NS. Normally, initial phosphorylation of LC is followed closely by the development of isometric force. Tissues treated 2 hrs in 10 uM CD were frozen after 60 sec CCh or KCl or after 5 sec NS, or were frozen at different times after removal of the stimulus. With each stimulus, force was significantly reduced by CD treatment, while values of LC phosphorylation were not different from values in untreated muscles. After removal of the stimulus, LC was dephosphorylated to resting values, as is observed for untreated tissues. It is concluded that both myosin light chain kinase and phosphatase remain active and that neurotransmitter release and action are not overtly compromised at concentrations of CD that inhibit isometric force. (Support by HL32607.)

T-Pos270 DISSOCIATION OF AEQUORIN-ESTIMATED MYOPLASMIC [Ca2+] AND MYOSIN PHOSPHORYLATION IN HISTAMINE INDUCED TONIC CONTRACTIONS OF SWINE CAROTID MEDIA. CM Rembold and RA Murphy, Depts. of Physiology and Medicine (Cardiology), University of Virginia School of Medicine, Charlottesville, VA 22908, USA.

We examined the hypothesis that changes in myoplasmic [Ca^{2+}] are the primary determinant of myosin light chain phosphorylation in smooth muscle. The time course of aequorin light production, myosin phosphorylation, shortening velocity at zero load (V_o), and stress were measured after stimulation of swine carotid media with 10 uM histamine, 10 uM phenylephrine, and depolarization with 109 mM KCl or 20 mM KCl. Myosin phosphorylation was proportional to aequorin estimated [Ca^{2+}] after depolarization at 37 C (Circ Res 58:803, 1986). A similar proportionality was found with depolarization at 22 C, with phenylephrine at 37 C, and during the initial light and phosphorylation transient seen with histamine at 22 C. However, during tonic histamine contractions (3 - 30 min), light fell to near basal levels while phosphorylation, V_o, and stress remained elevated. The observed fall in light was not attributable to the consumption of aequorin as a typical depolarization light response was elicited by adding KCl to a histamine contraction. Phosphorylation during tonic histamine contractions occurred at a lower light-estimated myoplasmic [Ca^{2+}] than during depolarization or phenylephrine stimulation. These data suggest histamine stimulation in the swine carotid is associated with modulation of the relationship between myoplasmic [Ca^{2+}] and myosin light chain phosphorylation. Supported by the Markey Foundation (86-025), NIH 5PO1-HL19242 and 5T32 HL07355.

T-Pos271 CROSSBRIDGE PHOSPHORYLATION MAY BE NECESSARY AND SUFFICIENT TO REGULATE CONTRACTION IN SMOOTH MUSCLE: A MODEL OF CROSSBRIDGE KINETICS. Chi-Ming Hai and Richard A. Murphy, Department of Physiology, School of Medicine, University of Virginia, Charlottesville, VA 22908.

Smooth muscles exhibit unique properties including (1) regulation of shortening velocity with zero external load (proportional to crossbridge phosphorylation), and (2) stress maintenance with low levels of phosphorylation and reduced crossbridge cycling rates (latch). We tested the following model:



In this model, Ca2+–activated myosin phosphorylation was the only postulated regulatory mechanism. The illustrated rate constants were resolved by fitting experimental data obtained from homogeneously activated (field stimulation) bovine trachealis and swine carotid media. The model fitted the time courses and steady state levels of stress development and crossbridge phosphorylation with and without an initial phosphorylation transient. We conclude that Ca2+-activated myosin phosphorylation may be both necessary and sufficient to explain activation and maintenance of the latch state in smooth muscle with the assumption that dephosphorylation of an attached crossbridge gives rise to a long lasting, non-cycling latch-bridge. Supported by 5-PO1-HL19242 and The Am. Heart Assoc., Va Affiliate.

T-Pos272 Sr2+ SUBSTITUTES FOR Ca2+ IN THE ACTIVATION AND MAINTENANCE OF THE LATCH STATE IN SWINE CAROTID MEDIA. Chi-Ming Hai and Richard A. Murphy, Department of Physiology, School of Medicine, University of Virginia, Charlottesville, VA 22908.

Sr2+ induced stress development in both K+-depolarized Ca2+-depleted intact tissues and in skinned tissues.The EC50 for Sr2+-induced stress development and myosin phosphorylation (37 uM) was greater than that for Ca2+ (0.86 uM) in skinned tissues. However, the stress-myosin phosphorylation relationship was similar. Ca2+-depleted tissues with intracellular stores previously refilled with Sr2+ were K+-depolarized in the presence of extracellular Sr2+. The kinetics and final steady state of stress development and myosin phosphorylation induced by 5 mM Sr2+ were similar to that induced by 1.6 mM Ca2+. Stress developed monotonically (T1/2 = 0.70 min) to a steady state of 89.7 $\pm$ 2.0 % K-PSS (1.6 mM Ca2+) induced stress, whereas myosin phosphorylation increased from a basal level of 10.7 $\pm$ 3.3 % to a peak of 53.2 $\pm$ 5.1 % at 1 min and then decreased to a steady state of 21.7 $\pm$ 2.0 %. Without prior refilling of intracellular stores with Sr2+, stress developed slowly (T1/2 = 2.9 min) to a steady state of 95.9 $\pm$ 1.5 % K-PSS induced stress, whereas myosin phosphorylation increased monotonically to a steady state of 28.8 $\pm$ 2.7 %. This suggested that intracellular Sr2+ release was responsible for the myosin phosphorylation transient. The initial myosin phosphorylation transient only accelerated stress development; steady state levels of stress development depended only on the steady state myosin phosphorylation level. These results suggest that Sr2+ substitutes for Ca2+ in the activation and maintenance of the latch state, and is consistent with the hypothesis that myosin phosphorylation regulates crossbridge interactions in the latch state. Supported by 5-P01-HL19242 and Am. Heart Assoc., Va Affiliate.

T-Pos273 MECHANICAL PROPERTIES OF FERRET AORTA. Frank V. Brozovich and Kathleen G. Morgan. Department of Medicine and Cardiovascular Division, Beth Israel Hospital, Harvard Medical School, Boston, MA 02215.

Muscle stiffness and the maximum unloaded shortening velocity (V_{max}) were measured in small strips of ferret aorta; strips of aorta (7x0.5 mm) were dissected, and small foil T-clips were attached to the ends. The preparations were mounted between a force transducer (resonant at 750 Hz) and length driver (step response of 1.5 msec). Preparation length was stretched to the point just before passive force developed, and then warmed to 33 C. The strips were activated with 10 µM phenylephrine. Stiffness and V_{max} were measured during the rising phase of force development and also, during the force steady state. The maximum unloaded shortening velocity was computed using the slack test, and stiffness measured from either the force response to a small (<1%) 100 Hz oscillation in muscle length or, alternatively, from the force responses to rapid, small (<1%) muscle length changes. Both stiffness measurements gave similar results. After warming, muscle force rose to 13 ± 10% ($\bar{x}$ ± sd) while stiffness increased to 43 ± 15% of their maximum values, respectively; suggesting a shift in the population of cross-bridges to a non or low force producing state. After activation, muscle force and stiffness both rose to a plateau in 3-5 min, where they remained for the duration of contraction. V_{max}, on the other hand, rose rapidly to peak in approximately 1 min and then fell to 40% of its maximum level, indicating that the cross-bridge cycling rate falls during force maintenance. The rise in stiffness lead that of force, consistent with the existence of an attached non or low force producing cross-bridge state prior to force production. (Supported by grants from the NIH #31704 and an AHA EI).

T-Pos274 MEASUREMENTS OF HYDRAULIC CONDUCTIVITY AND VOLUME REGULATORY DECREASE FROM NON-PIGMENTED CILIARY EPITHELIAL CELLS. N.A. Farahbakhsh and G.L. Fain, Jules Stein Eye Institute, UCLA School of Medicine, Los Angeles CA 90024 USA.

The ciliary epithelium lies at the margin of the eye adjacent to the lens and is responsible for secreting the aqueous humor. If a single ciliary process from the rabbit is isolated and viewed at its edge using Hoffman modulation contrast optics, it is possible to record changes in the dimensions of the non-pigmented epithelial (NPE) cells in response to changes in tonicity. From measurements of the rate of volume increase in response to hypotonic shock, we have determined a minimum value for the hydraulic conductivity (P_{OS}) of the basal membrane of the NPE cells of about 100 um/sec. Although this is likely to be an underestimate, it is still large enough to suggest that the aqueous humor is secreted by a standing gradient across the interdigitations of the NPE basal membrane. We have also used this preparation to show that the NPE cells reduce their volume after swelling in response to exposure to hypotonic solutions (volume regulatory decrease or VRD). The extend of this VRD depended upon the composition and osmolarity of the bathing solutions. The initial rate of VRD (measured as a change in NPE cell width) in response to a 33% hypo-osmotic shock was 8 $\pm$ 1 um/min (mean $\pm$ S.E.) and could be reduced to 2.1 $\pm$ 0.4 um/min by addition of 2 mM $BaCl_2$. Large increases (4X or 8X) in extracellular K also reduced the rate of volume reduction. These observations suggest that at least part of the VRD is caused by exit of K through Ba-sensitive K channels in the basal membrane. In addition, we found that pretreatment of the epithelium with 100 uM ouabain before the (33%) hypotonic shock reduced the initial rate of VRD to 1.8 $\pm$ 0.4 um/min. Although it is possible that the effect of ouabain is solely the result of a decrease in K gradient, we think it more likely that active Na pumping also makes a significant contribution to the VRD in these cells.

T-Pos275 DIFFERENT PARACELLULAR RESISTANCES ARE ASSOCIATED WITH SURFACE AND OXYNTIC CELLS IN THE GASTRIC MUCOSA. J.R. Demarest and T.E. Machen, University of California, Berkeley, 94720

Microelectrode and vibrating probe techniques were used to measure total paracellular shunt, R_S, and the paracellular shunt resistance associated specifically with the surface epithelial cells $_{sec}R_S$ of the isolated Necturus gastric mucosa. R_S was determined under open circuit condtions using microelectrodes to measure responses of the basolateral membrane potential to sudden increases in serosal $[K^+]$. Apical impalements of surface cells, SECs, and basolateral impalements of oxyntic cells, OCs, indicated that R_S calculated from measurements from the two cell types were not significantly different. Further, R_S was independent of the size of the step change in $[K^+]_S$ over the range of 5 - 50mM/L. R_S was estimated to be~1300 Ω-cm^2 in resting mucosae and was unaffected by stimulation with histamine (10^{-4} M). The ratio of the transepethelial resistance, R_t, to R_S was 0.6 under resting conditions and 0.4 after stimulation. $_{sec}R_S$ was determined using a vibrating probe to record the change in local transepithelial current (measured above the surface epithelium between crypts) that resulted from voltage clamping the short circuited epithelium to -20 mV, mucosal negative, in the presence of the 10^{-6} M mucosal amiloride. Since mucosal amiloride blocks the apical Na^+ conductance of the SECs, the local resistance of the surface epithelium in the presence of amiloride reflects $_{sec}R_S$. $_{sec}R_S$ was up to 12 times R_S. These data indicate that: 1) R_S is lower than previously thought, 2) the paracellular shunt resistance associated with the OCs was only one tenth of $_{sec}R_S$ and 3) the tight junctions associated with the SECs and OCs are different. (Supported by NIH grant AM-17328.)

T-Pos276 HOMEOSTATIC EFFECTS OF Na:K:Cl COTRANSPORT IN A MATHEMATICAL MODEL OF TOAD URINARY BLADDER, J. Strieter, J.L. Stephenson, L.G. Palmer, A.M. Weinstein, Cornell Univ. Medical Coll., New York, NY

The toad urinary bladder epithelium is represented as compliant cellular and paracellular compartments bounded by mucosal and serosal bathing media. Model variables include the concentrations of Na, K, and Cl, hydrostatic pressure, and electrical potential, and the mass conservation equations have been formulated for both steady-state and time-dependent problems. Two membrane transport equations for ionic conductance are considered: the Goldman constant field equation (Civan and Bookman, J.Mem.Biol. 15:63) and that of linear nonequilibrium thermodynamics (NET). Results of these two formulations seem virtually indistinguishable over a broad range of bath conditions. A basolateral cotransporter of Na, K, and Cl with 1:1:2 stoichiometry, represented by the linear NET description of Geck and Heinz (Ann.NY. Acad.Sci. 341:57), has been added to the model. The effects of the cotransporter are marked, giving (1) elevation of intracellular chloride concentration to experimentally reasonable levels, and (2) protection against large increments in cell volume during increases in transepithelial sodium flux. An increase in the number of basolateral sodium pumps also provides cell volume homeostasis but does not yield elevated cell chloride. With the inclusion of the cotransporter, it is also found that a decrease in apical Na conductance may yield an increase in cell volume, due to enhanced KCl uptake when cell Na falls.

T-Pos277 MECHANISMS OF RENAL BASOLATERAL MEMBRANE ANION TRANSPORT. Pei-Yuan Chen and A.S. Verkman. Cardiovascular Research Institute, Univ. Cal., San Francisco, CA 94143.

Anion transport pathways in basolateral membrane vesicles (BLMV) isolated from rabbit renal cortex were characterized by $^{35}SO_4$ uptake, acridine orange (AO) fluorescence, and quenching of the Cl-sensitive probe 6-methoxy-N-(3-sulfopropyl) quinolinium (SPQ). $^{35}SO_4$ uptake was driven by cis H and Na gradients, and trans anion gradients (HCO_3>PO_4>Cl>SO_4, at 10 mM). Each transport system is neutral and inhibited by stilbenes, furosemide, probenecid and chlorothiazide, but not by amiloride and acetazolamide. Using the pH gradient sensitive probe AO, inwardly directed SO_4 gradients (K_D = 10 mM) stimulated HCO_3 efflux, causing BLMV acidification, AO accumulation and fluorescence quenching. SO_4/HCO_3 exchange was neutral and inhibited by stilbenes (K_I < 5 μM). Outwardly directed Na gradients also caused HCO_3 efflux, except that transport was enhanced by a negative membrane potential and was inhibited only at higher stilbene concentrations (50 μM). Using the entrapped Cl-sensitive probe SPQ, there was no effect of K/valinomycin gradients on Cl flux, indicating absence of Cl conductance and K/Cl cotransport. Voltage insensitive Cl flux was driven by cis H gradients (4-fold stimulation for 2 unit pH gradient) indicating neutral Cl/H(OH) transport inhibited by H_2DIDS (K_I=15 μM) with activation energy 5.4 kcal/mole. Stilbene inhibitable neutral Cl flux was also trans stimulated by SO_4 and HCO_3. In contrast, under similar assay conditions (in the absence of organic anions) the renal brush border membrane contains a voltage-dependent Cl conductance, but no stilbene inhibitable neutral anion exchange. Fluorescence enhancement studies using DBDS (4,4'-dibenzamido-2,2'-disulfonic stilbene) suggest that the BLMV anion transporter(s) constitute approximately 20% of total membrane protein. Our findings are incorporated into a mathematical model of Cl absorption in the renal proximal tubule.

T-Pos278 DETERMINATION OF REFLECTION COEFFICIENT OF UREA IN RENAL BRUSH BORDER MEMBRANE VESICLES. Eric G. Holmberg, Michael B. Singer and James A. Dix, Department of Chemistry, State University of New York, Binghamton, NY 13901.

The reflection coefficient (σ) of a solute is a parameter from irreversible thermodynamic theory which describes the interaction between solute and solvent flow across a membrane. A value of σ significantly less than unity indicates coupling between solute and solvent flow. We have used two methods to determine σ of urea in brush border membrane vesicles isolated from rabbit kidney proximal tubule. The "zero-time" method (Chasan and Solomon, BBA **821**, 56, 1985) determines σ as the ratio of initial volume flow subsequent to osmotic shock with permeant (urea) to that with impermeant (mannitol) solutes. A large correction was necessary with this method because the index of refraction of the permeant urea solution differed significantly from that of the impermeant mannitol solution. The initial volume flux induced by urea was corrected by using initial volume fluxes obtained at constant urea osmotic shock and varying index of refraction. σ determined by this method was 0.87 ± 0.07. The reflection coefficient was also determined by fitting the entire time course of volume change to the Kedem-Katchalsky equations as described previously (Smith, Myslik and Dix, Biophys. J. **49**, 273a, 1986). In this method, the urea permeability coefficient and reflection coefficient are varied while the water filtration coefficient is held fixed. σ determined by this method was 0.94 ± 0.01. These values for σ indicate that there is little interaction between urea and water in permeating BBMV. Supported in part by the American Heart Association, Broome County, New York State Chapter.

T-Pos279 MONOCLONAL ANTIBODIES AGAINST THE PURIFIED EPITHELIAL Na^+ CHANNEL PROTEIN Mary Ann Accavitti and Dale J. Benos. Departments of Medicine and Physiology and Biophysics, University of Alabama at Birmingham, Birmingham, Alabama 35294

Three hybridoma cell lines secreting antibodies against the purified amiloride-inhibitable Na^+ channel protein from bovine renal papilla were produced. A solid phase enzyme-linked immunosorbant assay (ELISA) utilizing the purified channel protein as bound antigen was developed for screening. Ascites fluids (containing 1.5-5 mg antibody/ml) were produced by injecting 10^7 channel protein-specific hybridoma cells into pristane primed BALB/c mice. The isotype of each antibody was determined by ELISA using goat antiserum to mouse IgG subclasses or μ heavy chains. Clone 13.10 secretes IgG_{2B} antibody, while clones 9.6 and 12.12 secrete IgM. The purity of each monoclonal antibody was assessed by sodium dodecylsulfate polyacrylamide gel electrophoresis. All three monoclonal antibodies, when bound to rabbit anti-mouse IgG plus IgM Staphylococcus aureus cell complex, can specifically immunoprecipitate ^{125}I-labelled channel protein from a mixture of solubilized bovine papillary proteins. Thirteen additional cell lines secreting antibody against the purified Na^+ channel have been produced and frozen. These antibodies, however, have not yet been characterized. All of these antibodies will be useful as probes for further refinements in the channel purification scheme, for immunocytochemical localization studies of Na^+ channel location and distribution and, ultimately, as molecular biological reagents necessary to screen a cDNA library to assist in defining the points of biosynthetic and genetic regulation of this ubiquitous transport protein.
Supported by NIH Grant DK37206 and AM20614.

T-Pos280 IDENTIFICATION OF SECOND MESSENGER-SPECIFIC PROTEIN KINASES (PKs) IN EPITHELIAL TISSUES. Mrinalini C. Rao and Nancy T. Nash (Intr. by Kate Barany), Dept. of Physiology and Biophysics, University of Illinois College of Medicine at Chicago, Chicago, IL 60612.

Sodium and chloride transport across a variety of epithelia are regulated by Ca, cAMP(cA) and/or cGMP(cG). Second-messenger specific protein phosphorylation may play an important role in these effects. Therefore, we surveyed the distribution of Ca-, cA- and cG-specific PKs in particulate and cytosolic fractions of a variety of epithelia including those with largely absorptive (flounder and trout intestine), secretory (bovine trachea) or both absorptive and secretory properties (chick intestine, rabbit ileum and colon). The Ca-Calmodulin (CaM)PKs I and II were assayed by their relative ability to phosphorylate the neuronal protein Synapsin I and Ca-CaM PK III by its ability to phosphorylate its specific 100K substrate. PK-C activity was assayed by the ability of phorbol esters to stimulate phosphorylation in the presence of phosphatidyl serine. [^{32}P]-8-azido-cA and [^{32}P]-8-azido-cIMP were used to identify cA and cG binding proteins. All the epithelia possess Ca-CaM PK II and III activities; mammalian but not flounder epithelia possess Ca-CaM PK I. cA-specific binding proteins could be detected in all the epithelia; the piscean forms appear to be of smaller M.W. (46 and 44 kd) than their mammalian counterparts (52 and 48 kd). Peptide maps of the cA binding proteins reveal that the larger and smaller forms are identical in different tissues. A minor, 80 kd, cG-specific binding protein was seen in the rabbit ileum and was barely detectable in flounder intestine but not in any other tissue. Under conditions where PK-C activity could be detected in chick intestinal cytosol, no activity of this enzyme was seen in flounder intestine. We conclude that the distribution of the Ca-, cA- and cG-specific PKs parallel the effects on ion transport of these mediators. (Work supported in part by NIH BRSG 84431 & AM 34270 to MCR.)

T-Pos281 ARGININE RESIDUES AT OR NEAR THE INTESTINAL NA/PHOSPHATE COTRANSPORTER PHOSPHATE SITE. Brian E. Peerce. Department of Physiology, UCLA School of Medicine, Los Angeles, CA 90024.

An arginine residue has been identified at, or near to, the phosphate binding site of the Na/phosphate cotransporter of rabbit intestinal brush border membranes. Three arginine group specific reagents were used to evaluate the role of arginine residues in Na/phosphate cotransport. These included: glyoxal, phenylglyoxal, and 2,3 butanedione. All three reagents inhibited Na/phosphate cotransport from 70% to 85% with $K_{0.5}$'s for inhibition ranging from 35uM to 80uM. The effect of substrates on phenylglyoxal inhibtion of phosphate uptake was used to evaluate the site of phenylglyoxal action. The presence of Na + phosphate afforded complete protection against phenylglyoxal inhibition of Na-dependent phosphate uptake up to 250uM reagent. At 500uM phenylglyoxal protection was less than 75%. The apparent $K_{0.5}$ for phosphate protection against phenylglyoxal-induced inhibition of phosphate uptake was 200uM similiar to the apparent K_M for phosphate uptake. The addition of Na, Li, or K in the absence of phosphate afforded no protection against phenylglyoxal inhibition. These results suggest that arginine residues are located at, or near to, the phosphate binding site of the intestinal Na/phosphate cotransporter.

(Supported by AM 34807 and AM 19567)

T-Pos282 STOICHIOMETRY OF THE INTESTINAL NA/PHOSPHATE COTRANSPORTER. Brian E. Peerce, Department of Physiology, UCLA School of Medicine, Los Angeles, CA 90024

The substrate stoichiometry of the intestinal Na/phosphate cotransporter of rabbit brush border membranes was investigated. Na activation of phosphate uptake was found to be sigmoidal with a Hill coefficient of 1.9, indicating multiple Na binding sites on the cotransporter. The valence state of transported phosphate was examined using mono-, di-, and tri- methyl phosphate derivatives as competitive inhibitors of Na/phosphate cotransport. In the presence of 1mM theophylline, a potent inhibitor of alkaline phosphatase, only dimethyl phosphite was found to have significant inhibitory effect on Na gradient driven phosphate uptake. Maximum inhibition was 65% in the presence of 100uM phosphate. Dixon plots indicate that inhibition was competitive with respect to phosphate with an apparent K_I of 160uM. Differentiation between dimethyl phosphite inhibition of phosphate binding and phosphate transport was performed by preloading the vesicles with 100mM Na, 100mM Na + variable concentrations of phosphate, or 100mM Na + variable concentrations of dimethyl phosphite. Na alone causes 65% inhibition of Na/phosphate cotransport, which is 93% relieved by phosphate. Dimethyl phosphite also relieves the Na$_i$ inhibition of Na/phosphate cotransport with a $K_{0.5}$ of 50uM. These results suggest that dimethyl phosphite is a competitive inhibitor of Na-dependent phosphate transport and binding, and that the valence state of phosphate which is transported is 2. On the basis of these studies and the results of other laboratories suggesting that Na/phosphate cotransport is electroneutral, I suggest that the Na/phosphate cotransporter has a stoichiometry of 2 Na's per HPO_4. (supported by AM 34807, AM 19567)

T-Pos283 INTRACELLULAR CA^{2+} AND CAMP ACTIVATE K^+ CHANNELS IN THE BASOLATERAL MEMBRANE OF NECTURUS OXYNTIC CELLS. D.D.F. Loo, S. Ueda and G. Sachs. Dept. of Physiology, UCLA and CURE, VA Wadsworth Los Angeles, Ca. 90024.

Acid secretion involves increases in the K^+ permeability of the parietal and oxyntic cells. We have applied patch clamp methods to study single channel events in isolated oxyntic cells and gastric glands prepared from Necturus gastric mucosa by pronase digestion. In cell-attached patches from the basolateral membrane, with the pipette solution containing (in mM): 110 K, 1 Na, 1 Mg, 115 Cl, 1 Ca, 10 EGTA and 10 Hepes/KOH, and bath solution (in mM): 110 Na, 2 K, 1 Mg, 116 Cl, 1 Ca and 10 Hepes/NaOH, channels with linear I-V relations and with conductances of 67±21 pS(n=7) were observed in 24% of the patches and channels of smaller conductance 33±6 pS(n=14), in 56% of the patches. Reversal potential measurements indicated that both classes were highly selective for K over Na (P_K/P_{Na}>>10). The 67 pS channel was activated by intracellular Ca^{2+}. In excised inside-out patches, increasing bath Ca^{2+} from 5 nM to 500 nM increased open probability P_o by more than 1000 fold (pipette potential = -60 mV), and in cell-attached patches, addition of A23187 (2 μM) to the bath solution increased P_o by 3.1±1.3 fold (n=3). The 33 pS channel was activated by cAMP. In cell-attached patches, addition of 1 mM dbcAMP to the bath solution increased P_o by 3.2±1.9 fold (n=6). The 67 pS channel was unaffected by cAMP while the 33 pS channel was unaffected by Ca^{2+}. The presence of K^+ channels in the basolateral membrane regulated by these second messengers can account for the hyperpolarisation of the membrane observed with stimulation by the secretagogues carbachol and histamine.

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T-Pos284 BASOLATERAL POTASSIUM, AMPHOTERICIN, CYANIDE AND OUABAIN EFFECTS ON SINGLE-CHANNEL DENSITY AND CURRENT IN R.PIPIENS SKIN. T.Hoshiko and R.A.Grossman Dept.of Physiology and Biophysics, School of Medicine, and S.Machlup Dept.of Physics, Case Inst.of Technology, Case Western Reserve University, Cleveland OH 44106.

Single-channel density and current were estimated from voltage effects on amiloride noise (see Machlup & Hoshiko, Abstr.Bioph.Soc.Mtg.,Feb.1987). Amiloride (2 μM) spectra were averaged and fitted as a Lorentz function plus a 1/f component. In each of six experimental conditions, 1) basolateral $[Na]_i$=115 mM; 2) Na plus 10 mg/ml amphotericin B; 3) Na, amphotericin and 0.1 mM ouabain; 4) basolateral potassium $[K]_i$=120 mM ;5) K plus amphotericin; and 6) K, amphotericin and 5 mM KCN, spectra were obtained at clamp voltages from +50 mV and to -50 mV altered in 10 mv steps. Shunt conductances increased slightly in potassium but were relatively untouched by the metabolic inhibitors. EMF's were 75,82,36,44,45 and 26 mV for the 6 conditions. Channel densities were 3.3,5.6,.04,3.3, 7.7,3.7 /μm^2. Single-channel currents were 105,82,331,99,76,59 fA. Amphotericin enhanced channel density but left single-channel current unchanged. Cyanide depressed channel density slightly but changed single-channel current very little. On the other hand, ouabain depressed channel density by 140x and increased single-channel current 4x. This calculation of single-channel density and current was made upon the assumption that amiloride kinetics were unchanged by amphotericin, ouabain and cyanide, an assumption supported by our previous finding that amiloride corner frequency is unaffected by these inhibitors. Supported by NIH grant AM 5865.

T-Pos285 LANTHANIDES MIMIC Na ON THE RENAL BRUSH BORDER PROLINE CARRIER Bryndis Birnir, Bruce Hirayama & Ernest Wright. Physiology Dept., UCLA, Los Angeles, Ca 90024.

Lanthanum supports electrogenic substrate transport across renal brush border membranes(J. Physiol. 360:95,1985). In the present study we have used rabbit renal brush border membrane vesicles to examine the specificity and kinetics of lanthanide cotransport. Experiments were carried out under voltage clamped, zero-trans conditions using a rapid-mix/filtration procedure. Initial experiments confirmed that lanthanum produced the classical overshoot phenomenon. The initial rates of 0.85mM proline uptake relative to Na were Eu~Tb~Nd~Pr~Ho(3.3) > Na(1.0) > La(0.86) > choline (0.1). Tb activated proline uptake with a Hill coefficient of 1.2 and a $K_{0.5}$ of 41mM, while Na activated with a Hill coefficient of 1.5 and a $K_{0.5}$ of 20mM. The K_m and V_{max} were 0.05mM and 16pmoles/(mg x s) in 140mM Na and 0.28mM and 73pmoles/(mg x s) in 140mM Tb. These results suggest that Tb has a lower affinity for the cotransporter cation sites than Na, and that in Tb proline has a lower affinity for the substrate binding site than in Na. The higher V_{max} in Tb may mean that the mobility of the Tb loaded carrier is greater than the Na loaded carrier. We conclude that lanthanide ions are able to substitute for Na on the brush border proline carrier, and that the lanthanides may serve as useful probes for the sodium sites.

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T-Pos286 TWO TYPES OF Ca-ACTIVATED CHANNELS IN ISOLATED TURTLE COLON EPITHELIAL CELLS. Neil W. Richards and David C. Dawson, Dept. of Physiology, Univ. of Michigan Medical School, Ann Arbor, MI 48109

The patch clamp technique was used to identify the ion channels responsible for a specific basolateral K conductance induced by osmotic swelling in colonic epithelial cells (Richards and Dawson, AJP 251:C85-C89, 1986). In addition, we have identified two types of Ca-activated channels in these cells: 1) a large conductance K-selective channel and 2) a smaller conductance channel selective for monovalent cations. The Ca-activated K channel had a conductance of approximately 90 pS and an extrapolated reversal potential of -50 mV in inside-out patches with Na-Ringers (112mM NaCl; 2.5mM $KHCO_3$; 10mM glucose; 10mM HEPES; 1mM $CaCl_2$; pH 7.4) in the pipet and K-Ringers (NaCl replaced with KCl) in the bath. Changing the bath to Ca-free K-Ringers + 1mM EDTA abolished the single-channel currents, which were immediately restored by replacing the bath with K-Ringers + 1mM Ca. Currents were almost completely blocked by 20 uM $BaCl_2$ on the cytoplasmic side (inside-out patches) while at the extracellular face, even 5mM $BaCl_2$ did not produce complete block. In both inside-out and outside-out patches, quinidine (200uM) caused a flickery block and reduced the current amplitude. The smaller conductance channel (30 pS) had a reversal potential near 0 mV in inside-out patches with Na-Ringers in the pipet and K-Ringers in the bath. Isosmotic replacement of KCl in the bath with sucrose shifted the reversal potential to positive cell potentials, indicating that this channel is cation selective. This channel was also reversibly inactivated in inside-out patches by Ca-free solutions in the bath. In outside-out patches, inclusion of 1mM Ni in the bath completely and reversibly blocked single-channel currents. (NIH support)

T-Pos287 APICAL AND BASOLATERAL MEMBRANE CHLORIDE TRANSPORT CHARACTERIZED IN BOVINE TRACHEAL EPITHELIAL CELLS. P. Fong, Nicholas P. Illsley, J.H. Widdicombe, and A.S. Verkman. Dept. Physiol. and Cardiovascular Research Institute, Univ. of Calif., San Francisco, CA 94143.

The intact tracheal epithelium secretes Cl actively, with Na-linked Cl entry across the basolateral membrane and conductive Cl exit across the apical membrane. We examined Cl transport mechanisms in bovine tracheal epithelial cells by using apical (AMV) and basolateral (BMV) membrane vesicles isolated by scraping, homogenization, differential centrifugation, and Mg precipitation. AMV were enriched 60-fold in alkaline phosphatase and BMV 5-fold in Na/K ATPase compared to homogenate with <1-fold enrichment of contaminant membrane markers. Vesicles were loaded with the halide-sensitive fluorescent probe 6-methoxy-N-(3-sulfopropyl) quinolinium (SPQ) at 4°C for 36 h in Cl-free buffer and washed 3x to remove external SPQ. Vesicles were voltage and pH-clamped with K/valinomycin and nigericin (10 µg/mg vesicle protein). Cl influx measured from the time course of SPQ fluorescence upon addition of external Cl was 17 pmole/cm^2s for a 50 mM inwardly directed Cl gradient in AMV. Diphenylamine-2-carboxylate (100 µM) decreased Cl influx by 45%, whereas furosemide (100 µM) and H_2DIDS (50 µM) had no effect. Iodide influx was 4-fold greater than Cl influx in AMV. In BMV, Cl influx was inhibited >90% by 50 µM H_2DIDS and enhanced 4-fold by a 2 pH unit inwardly directed H gradient (pH_{in}=7, pH_{out}=5), indicating the presence of a Cl/OH(H) transporter. Furosemide (100 µM) also reduced Cl influx in BMV, consistent with the Na/K/2Cl cotransporter proposed in current models for Cl transport. These studies support the presence of apical membrane Cl conductance and neutral basolateral membrane Cl transport in bovine tracheal epithelial cell membranes measureable by a Cl-sensitive fluorescent probe.

T-Pos288 CHLORIDE CONDUCTANCE IN HUMAN PLACENTAL MICROVILLOUS MEMBRANE VESICLES. C.S. Glaubensklee, Nicholas P. Illsley, B. Davis and A.S. Verkman. Cystic Fibrosis Research Center, Cardiovascular Research Institute, Univ. of California, San Francisco, CA 94143.

Apical membrane vesicles from human placenta were examined for chloride conductive, exchange and co-transport activities. Vesicles isolated by shearing and differential centrifugation were loaded with the chloride-sensitive fluorescent probe SPQ (6-methoxy-[3-sulfopropyl]quinolinium) by incubation with 10 mM dye for 24 hr at 4°C. Vesicles were washed immediately prior to use to remove external dye. Ion and pH gradients, K^+/valinomycin-induced membrane potentials and a series of inhibitors were used to determine the mechanisms of transmembrane chloride movement. For all experiments an inwardly directed 50 mM chloride gradient was imposed with a K^+/valinomycin voltage clamp, at 37°C. The initial rate of chloride influx was 21±1 nmol/s·mg membrane protein (mean ± s.d.; n=4) which increased to 42±3 nmol/s·mg in the presence of a +60 mV membrane potential. Influx was inhibited 75% by 0.5 mM diphenylamine-2-carboxylate but was not affected by 0.1 mM H_2DIDS. Chloride influx was not affected by pH gradients (pH_{in}=7.0; pH_{out}=5.5, 25±5 nmol/s·mg; pH_{out}=8.5, 22±2 nmol/s·mg). Chloride influx was not affected by a 50 mM Na gradient directed either inwardly (22±2 nmol/s·mg) or outwardly (18±2 nmol/s·mg). These results demonstrate the existence of a voltage-sensitive, inhibitable, chloride conductance pathway in the placental apical membrane but absence of both chloride/hydroxyl exchange and sodium-coupled chloride transport mechanisms. This profile of chloride transport mechanisms closely resembles that observed in other epithelial tissues including kidney, lung and trachea. These findings provide support for the use of placental vesicles for investigation of the chloride conductance defect in cystic fibrosis.

T-Pos289 ANOTHER METHOD FOR ESTIMATING CHANNEL DENSITY FROM AMILORIDE NOISE LEVEL IN ISOLATED FROG SKIN. T. Hoshiko, Dept. of Physiology and Biophysics, and Stefan Machlup, Dept. of Physics, Case Western Reserve University, Cleveland, OH 44106

The low-frequency plateau value S_o of the Lorentzian spectrum of the current fluctuations in the presence of amiloride blocker is proportional to the square of the single-channel sodium current i: $S_o = ki^2$. The constant k is a measure of the channel density. If dependence of clamp current I on clamp voltage V is linear, then S_o is a concave quadratic in V (three coefficients A_0, A_1, A_2) and, consequently, a concave quadratic in I (three coefficients). Combinations of these coefficients yield values of the conductance g_o of the cellular pathway via the sodium channels, the conductance g_s of the leakage pathways, and the skin emf E. The linear fit to the I-V curve, on the other hand, gives only slope g_o+g_s and intercept g_oE. But the method -- treating the V-dependence and the I-dependence as if they were independent -- also provides a validation of the channel model and an internal check on the consistency of the data. Selfconsistency requires that the ratio $A_0A_2/A_1^2 = \frac{1}{4}$ for both sets. Experiments whose curves are not concave (positive A's) are rejected; so are those for which the dimensionless ratio is too far from $\frac{1}{4}$. Out of 58 experiments (Hoshiko, Grossman, and Machlup, Abstr. Biophys. Soc. Mtg., Feb. 1987), 38 passed this test. The 6 A-coefficients give 6 values of the channel density for each experiment; standard deviations were between 5 and 10%. For Rana pipiens in 115 mEq sodium sulfate Ringer's, using corner-frequency data and a simple 2-state model gives a channel density of 3 channels per square micron. [Supported by NIH Grant AM 5865.]

T-Pos290 FUNCTIONAL SUBTYPING OF THE LUMINAL TACHYKININ RECEPTOR ON CANINE TRACHEAL EPITHELIUM. P.K.Rangachari, D.McWade and B.Donoff, Intestinal Disease Research Unit, McMaster University, Hamilton, Ontario, Canada, L8N 3Z5.

We have reported earlier that luminal addition of tachykinins to the open-circuited canine tracheal epithelium produces a bi-phasic response - a rise in P.D., preceded by a rapid, transient decrease, the "dip". Concentration-response curves demonstrated the following orders of potency: SP>P>E=K for the tachykinins (denoting an SP-P receptor), and SP>Octa>Hexa using SP fragments; both sequences are similar to those reported for the dog carotid artery. These observations were confirmed by cross-tachyphylaxis experiments. SP-O-methyl ester, a selective agonist for the SP-P receptor, elicited identical responses, and exhibited cross-tachyphylaxis to SP. Bradykinin produced similar luminal responses. The dips observed bore a linear relation to the pre-existing P.D., and were also dependent upon luminal Cl^-. Different receptors are involved, however, since no cross-tachyphylaxis was observed between bradykinin and the tachykinins. Since the dip appears to be a passive event, dependent solely upon existing electrochemical gradients, we have previously argued that the tight junctions would be an appropriate locus for this phenomenon. Thus, various classes of peptides, acting on different receptors, could modulate junctional permeability. (Supported by MRC Canada and Cdn. Cystic Fibrosis Fdn.).

T-Pos291 ACTIVATION OF K^+AND Cl^- CHANNELS IN KIDNEY EPITHELIAL (MDCK) CELLS. W. V. Breuer, E. Mack, and A. Rothstein. Department of Cell Biology, Research Institute, The Hospital for Sick Children, Toronto, Canada M5G 1X8.

In dissociated MDCK cells, activators of the cyclic AMP system cause depolarization detectable by changes in fluorescence of the membrane potential-sensitive dye bis-oxonol. The resting potential was -20 to -25 mV. Addition of forskolin (60 uM), vasopressin (2uM), 8 - Bromo-cyclic AMP (0.5 mM) depolarized the cells by 15 to 25 MV in low Cl- (5 mM), but only by 3 to 6 mV in high Cl- (140 mM) medium. The depolarizing action is also present in high K^+ - low Cl^- medium. These results are consistant with cyclic AMP-activation of Cl^- channels.

With L- epinephrine (10 uM) a biphasic response was observed, first hyper-polarization (by -6 mV) followed by depolarization (by 5 mV). The epinephrine-hyperpolarization requires the presence of Ca^{++} in the medium, and is associated with an increase in cellular Ca^{++} (detected by the Ca++ indicator, indo-I). The effect appears to be associated with opening of Ca^{++} dependant K^+ channels resulting from interaction of L- epinephrine with α-adrenergic receptors. Isoproterenol, on the other hand, does not cause an increase in Ca^{++} and does not produce the hyperpolarizing effect (supported by the Medical Research Council of Canada).

T-Pos292 **STRUCTURAL, FUNCTIONAL AND ENZYMATIC RELATIONSHIPS BETWEEN THE TRANSVERSE TUBULE MgATPase AND THE SR CaATPase.** **J.J. Kang, E. Damiani, K.C. Norton, D. Hammi, H.B. Cunningham, R.A. Sabbadini, and A.S. Dahms. Molecular Biology Institute, San Diego State University, San Diego, CA 92182-0328**

The transverse tubule (TT) of chicken skeletal muscle contains a very active (c. 250 umol/hr/mg) MgATPase (Mr 102 kD) which comprises 85% of the integral membrane protein. The enzyme exhibits a host of features which clearly distinguish it from the SR Ca-pump, among which are its unusual kinetic and thermodynamic properties, its activation by Con-A and its insensitivity to FITC and vanadate (Moulton et al., JBC 261, 12244); data will be presented which show that the TT MgATPase, in marked contrast to the SR CaATPase, is incapable of binding ^{45}Ca, as monitored by ^{45}Ca blotting procedures; further, the data show the absence (<1%) of any contaminating SR proteins (CaATPase and calsequestrin) in current TT membrane preparations. On the other hand, despite these and other prominent functional and enzymatic differences, Western immunoblots, Cleveland maps and ELISA assays clearly demonstrate that the TT Mg-ATPase and the SR Ca-ATPase are closely related and share many common epitopes and structural domains (Damiani et al., JCB, in press); data will be presented which show that interrupted trypsinolysis of TT-membrane vesicle Mg-ATPase produces an A/B fragmentation identical to that produced by chicken skeletal muscle SR-membrane vesicle CaATPase. Further, during the monitoring of the onset of non-thiol dischargeable fluorescent labeling concurrent with inactivation of the TT MgATPase by NBD-fluoride, we have observed specific crosslinking (XL) of the TT Mg-ATPase with formation of high molecular weight oligomers; identical results have been obtained with the SR-CaATPase. NBD-F promoted XL of TT, SR, RBC and SMP membrane proteins show covalent oligomerization of only the TT MgATPase, the SR CaATPase, spectrin and Band 3; of a large number of oligomeric soluble proteins, only myosin exhibits NBD-mediated XL. The rarity of this event obtained with a large number of oligomeric proteins suggests that NBD specifically and covalently bound to interfacial subunit regions of these proteins may be mediating the XL event. The near identity of the responses of the TT MgATPase and the Ca-ATPase to NBD-mediated XL lends further support to our contention that these two proteins are very closely-related and share not only many common 1° and 3° structural features but 4° features as well. **Supported in part by NSF PCM 8405007, NIH EYO 531001 and the California Metabolic Research Foundation.**

T-Pos293 IMMUNOCYTOCHEMICAL AND ELECTRON MICROSCOPIC LOCALIZATION OF THE T-TUBULE Mg-ATPASE. E. Damiani, A. Margreth, P. Yazaki, A. S. Dahms, P. Paolini and R. Sabbadini. Institute of General Pathology, University of Padova, via Loredan 16, Padova, Italy and Molecular Biology Institute, San Diego State University, San Diego, CA 92182.

Polyclonal antibodies were raised against the 102 kDa Mg-ATPase purified from chicken T-tubule membranes which were devoid of SR contamination (i.e. no Ca-ATPase activity and lack of SR proteins in one or two-dimensional slab gels). Western immunoblots performed on electrophoretically-resolved TT proteins stained with rabbit anti-(chicken) TT Mg-ATPase 102 kDa protein indicated that the antibody reacted only with the TT 102 kDa protein. ELISAs also demonstrated that the antibody did not cross react to SR proteins.

Indirect immunofluorescence of cryostat-sectioned muscle showed that the anti Mg-ATPase antibodies could be localized exclusively in the TT-rich I-Band region of the muscle cell. No significant labeling was seen either on the sarcolemma or in the SR-rich A-Band regions. Antibody localization was determined by (1) computer-assisted image analysis which overlaid the fluorescent images on phase contrast micrographs taken from identical regions of the tissue and (2) by comparing Texas-Red labeled anti Mg-ATPase antibody staining with fluorescein-labeled anti-myosin. The immunofluorescence results were confirmed by the indirect colloidal gold label immuno EM technique on ultrathin cryosections. Colloidal gold labeled anti TT Mg-ATPase antibodies were associated with the surfaces of the TT membranes. These results confirm previous biochemical data indicating that the Mg-ATPase is exclusively a TT protein and that Mg-ATPase activity is not present in SR or sarcolemma membranes. Supported by NSF DMB8405007 and INT8515846.

T-Pos294 **CHANGES IN THE SARCOPLASMIC RETICULUM MEMBRANE PROFILE UPON E~P FORMATION AT ~15 A RESOLUTION.** D. Pascolini, L. Herbette[1], V. Skita, A. Scarpa, J.K. Blasie, Depts. of Chemistry and Biochemistry/Biophysics, U. of Pennsylvania, Philadelphia, Pa 19104-[1]Depts. of Medicine and Biochemistry, U. of Connecticut, Farmington Ct 06032.

Time-resolved x-ray diffraction studies of the isolated SR membrane at 7.5°C with a time resolution of 0.2-0.5 seconds, utilizing the flash-photolysis of caged ATP, have provided the difference electron density profile for the SR membrane for which the Ca^{2+} ATPase is predominately in the first phosphorylated intermediate state under conditions of turnover versus that prior to ATP-initiated phosphorylation of the enzyme. Analogous studies at 0° to -2°C with a time resolution of 2-5 seconds have provided the difference electron density profile for the SR membrane for which E~P is transiently trapped in absence of detectable turnover versus that prior to enzyme phosphorylation. The two difference profiles are somewhat similar but present features distinct from each other which, given the low resolution (~50 A) of the profiles, have been analyzed by subjecting them to a model refinement analysis. This analysis utilized step function models for electron density profiles obtained from static diffraction studies of the SR membrane at 7.5° and -2°C at the same relatively high resolution (~15 A). The model refinement analysis indicates that the difference profiles at both temperatures arise from a net loss of density in the outer monolayer of the SR membrane lipid bilayer, but the conserved redistribution of this electron density in the membrane profile in the two cases is significantly different, suggesting a distinct structure for the trapped E~P. Unlike previous models, this analysis treats the difference profiles at 7.5° and -2°C independently and only in terms of the respective static high resolution step function models for the membrane profile without any utilization of the protein profile itself. Supported by NIH HL-18708

T-Pos295 MODERATE RESOLUTION PROFILE STRUCTURE OF THE SARCOPLASMIC RETICULUM MEMBRANE UNDER "LOW" TEMPERATURE CONDITIONS. D.Pascolini, F. Asturias, J.K.Blasie, Depts. of Chemistry and Biochemistry/Biophysics University of Pennsylvania, Philadelphia Pa 19104

The calcium uptake reaction kinetics of isolated SR is at least biphasic over a range of temperatures (26° to -10°C), with a fast phase identified with the formation of E~P and Ca^{2+} "occlusion" and a slow phase with the Ca^{2+} translocation across the membrane and turnover of the Ca^{2+} ATPase ensemble. At temperatures of 0°C or lower E~P is transiently trapped in absence of detectable turnover for 10-20 seconds, as indicated by the presence of only the fast phase over this time interval. Time-resolved x-ray diffraction studies at 0° to -2°C with a time resolution of 2-5 seconds have provided the low resolution (~50 A) profile structure for the SR membrane for which E~P is transiently trapped, however at these temperatures the structure of the SR membrane profile in the "resting" state is significantly different, as a reversible, temperature-induced structural transition occurs at about 3°C for the isolated SR membrane. We have investigated the nature of this structural transition utilizing static lamellar and equatorial x-ray diffraction studies of SR membrane multilayers in the range of temperature between 7.5° to -2°C. The lamellar diffraction has provided the profile structure for the SR membrane at the highest versus the lowest temperatures at the same relatively high resolution of 15 A, the equatorial diffraction has provided information on the average lipid chain configuration in the two cases. In order to identify the contribution of each membrane component in producing the differences between the profile structures at 7.5° and -2°C, step function models have been fitted to the high resolution electron density profiles at the two temperatures. Supported by NIH HL-18708.

T-Pos296 LIGAND EFFECTS ON THE ROTATIONAL DYNAMICS OF THE CALCIUM ATPase IN CARDIAC SARCOPLASMIC RETICULUM.

Woubalem Birmachu, Franz Nisswandt and David D. Thomas, Department of Biochemistry, University of Minnesota Medical School, Minneapolis, Minnesota 55455.

We have used time resolved optical anisotropy techniques to study the rotational dynamics of the Calcium ATPase isolated from cardiac sarcoplasmic reticulum. The ATPase was labeled with Eosiniodoacetamide at a unit stoichiometry with retention of enzymatic activity. We have resolved motions ranging from hundreds of nanoseconds to several microseconds. Very fast motions in the submicrosecond time scale result in the initial decrease in phosphorescence anisotropy and are characteristic of motions of segments of the protein. The high residual anisotropy indicates that the ATPase as a whole undergoes restricted rotational motion, characterized by two correlation times, one near 10 microseconds and the second near 100 microseconds. Comparison of the motion of cardiac Calcium-ATPase to that of the skeletal enzyme indicates that the two enzymes undergo similar types of motions. Our goal is to correlate the functional aspects of the cardiac enzyme with its motion. To this end we will present studies of the effect of various ligands on the phosphoresence anisotropy of this enzyme.

T-Pos297 CHARACTERIZATION OF THE Ca^{2+} OCCLUDED STATE OF SARCOPLASMIC RETICULUM Ca-ATPase BY TRYPTIC CLEAVAGE AND HYDROPHOBIC LABELING

Bente Vilsen[1], Peter L. Jørgensen[1], John H. Collins[2] and Jens P. Andersen[1].
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Ca^{2+} occlusion in membranebound and in soluble monomeric Ca-ATPase was studied with CrATP as phosphorylating substrate (cf. Vilsen and Andersen, Biochim. Biophys. Acta 855 (1986) 429-431). The Ca^{2+} concentration dependence of the rate of Ca^{2+} occlusion was similar in the two enzyme preparations with Hill numbers of 2 and 4, at pH 7.0 and 8.0, respectively, indicating cooperative interactions between Ca^{2+} occlusion sites on the same peptide chain. Mg^{2+} was found to enhance the rate of Ca^{2+} occlusion. The conformational state of the Ca^{2+} occluded phosphorylated complex belonged to the E_1 class as indicated by tryptic cleavage patterns, showing an exposed T_2 cleavage site (at Arg 198). Hydrophobic labeling from the membrane phase by the photoactivable reagent trifluoromethyl-[^{125}I]iodophenyl-diazirine (TID) showed that E_1 forms (including the Ca^{2+} occluded state), are less exposed to the hydrophobic label than E_2 states (with and without vanadate). The preferential hydrophobic labeling (about 20%) in E_2 was found to be localized on the A_1 tryptic fragment by HPLC in SDS. We conclude that the conformational change resulting in Ca^{2+} occlusion ($E_1Ca_2^{2+} \rightarrow E_1 \sim P[Ca^{2+}]_2$) is of limited extent whereas the structure change corresponding to $E_1Ca_2^{2+} \rightarrow E_2$ and $E_1Ca_2^{2+} \rightarrow E_2V$ transitions involve transposition of peptide mass from the polar to the hydrophobic phase.

T-Pos298 MAPPING THE NUCLEOTIDE DOMAIN OF SARCOPLASMIC RETICULUM ATPase BY PHOTOLABELING. Sergio T. Ferreira and Sergio Verjovski-Almeida. Department of Biochemistry, Federal University of Rio de Janeiro, 21910 Rio de Janeiro, RJ, Brazil.

Sarcoplasmic reticulum ATPase was photolabeled with [^{3}H]UTP by irradiation of the nucleotide with ultraviolet light in the presence of the enzyme. The photolabeled enzyme was washed by acid precipitations and centrifugations and it was dissolved in SDS. The soluble protein was fractionated by gel filtration in a column and radioactivity was detected in the protein fractions (50-60%) as well as in the more included detergent-lipid mixed micelles (40-50%). The stoichiometry of photolabeling was determined under the eluted protein peak. The levels of photolabeling obtained were 0.8-1.0 nmol [^{3}H]UTP/mg protein at the saturating concentrations of UTP (3-5 mM). The UTP concentration dependence for photolabeling was similar to the UTP concentration dependence for activation of UTPase activity. ATP protected against photolabeling by [^{3}H]UTP. The labeled enzyme was digested by trypsin and the photolabeled peptides were analyzed by SDS-PAGE and autoradiography. Controls in the absence of illumination did not show any radioactivity. Photolabeled ATPase showed a predominant photoincorporation of UTP in the A fragment (55 kdaltons). Further digestion of the ATPase reveled that the photolabel was in the A2 fragment (26 kdaltons). This peptide sequence has been postulated by MacLennan et al. (Nature 316:696,1985) to correspond to the "transduction" domain of the ATPase and it has been shown to contain the proposed Ca^{2+} binding stalk. Our data supports the postulated interaction of the nucleotide domain with the other globular extramembranous domains of the ATPase.

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T-Pos299 INOSITOL-1,4,5-TRISPHOSPHATE-INDUCED CALCIUM RELEASE FROM CANINE AORTIC SARCOPLASMIC RETICULUM VESICLES

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Regulation of inositol-1,4,5-trisphosphate (IP_3)-induced calcium release from canine aortic smooth muscle sarcoplasmic reticulum (SR) vesicles was examined using the calcium indicator antipyrylazo III. Calcium release was initiated by addition of IP_3 to aortic vesicles 7 min after initiation of ATP-supported calcium uptake. Half-maximal calcium release occurred at 1 uM IP_3, with maximal calcium release amounting to 25$\pm$ 2% of the intravesicular calcium (n=12, 9 preparations). Sodium azide (10 mM) did not inhibit either calcium uptake or IP_3-induced calcium release. Ruthenium red (10-20 uM), which has been reported to block IP_3-induced calcium release from skeletal muscle SR, did not block aortic SR IP_3-induced calcium release. Elevation of Mg^{2+} concentration from 0.06 to 7 mM markedly inhibited aortic IP_3-induced calcium release while stimulating IP_3 hydrolysis. Inclusion of 3 mM 2,3-diphosphoglycerate, an inhibitor of IP_3 hydrolysis, did not alter this Mg^{2+}-inhibition of IP_3-induced calcium release. Thus, aortic SR vesicles contain an IP_3-sensitive calcium channel which is inhibited by millimolar concentrations of Mg^{2+}, but which is not inhibited by ruthenium red and so differs from the IP_3-sensitive channel in skeletal muscle SR.

T-Pos300 DEMONSTRATION OF MULTIPLE NUCLEOTIDE BINDING PROTEINS IN CARDIAC SARCOPLASMIC RETICULUM BY DIRECT PHOTOAFFINITY LABELING. C.A. Tate[1] and T.F. Walseth[2], [1]Sec. of Cardiovas. Sci., Dept. of Med., Baylor College of Med., Houston, TX 77030; [2]Dept. of Pharmacology, Uni. of Minnesota, Minneapolis, MN 55455.

Multiple nucleotide binding proteins in native cardiac sarcoplasmic reticulum (SR) were identified by direct photoaffinity labeling with 50-200 nM [α-^{32}P]ATP and [α-^{32}P]GTP. Six peptides specifically interacted with ATP (in particular, the Mr=130,000 and 95,000 components), and 11 with GTP (notably the Mr=175,000; 130,000; 95,000; 84,000; 76,000; and 33,000 components). Of the two peptides labelled with ATP and GTP, excess, unlabelled ATP eliminated the labeling of the 130,000-Da peptide by [α-^{32}P]GTP and vice versa, suggesting that labeling occurred at the same nucleotide binding site. In contrast, the labeling of the Mr=95,000 ATPase by [α-^{32}P]GTP was markedly reduced by excess GTP, but not ATP, (and vice versa) even though both nucleotides labelled equally, indicating that the binding site for GTP differs from the ATP site in the ATPase of canine cardiac SR. These results cannot be explaind by cross-contamination by either cardiac sarcolemma (SL) or mitochondria since (1) the (Na^++K^+)ATPase of isolated cardiac SL was not labelled with either nucleotide under the conditions utilized; and (2) the nucleotide binding pattern of isolated cardiac mitochondria differed markedly from either cardiac SR or SL. Additionally, in both SR and SL there are minor GTP binding proteins in the 38,000-45,000-Da region which may represent G-regulatory proteins (see Scherer et al., this meeting). Supported by NIH (HL 13,870, AG 06,221 and HD 18,247).

T-Pos301 MODIFICATION OF HISTIDINE UNCOUPLES COOPERATIVITY OF Ca BINDING TO THE Ca•Mg•ATPase OF SR
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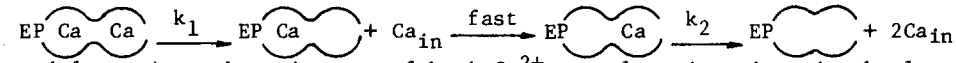
Modification of the histidyl residues by ethoxyformic anhydride inhibits enzyme activity, but does not inhibit the binding of either ATP or Ca^{2+} to the enzyme. However, the cooperativity of the Ca^{2+} binding is eliminated along with the ability of ATP to transfer the phosphate to the enzyme. In addition, we find that the binding affinity of the two Ca^{2+} sites has not changed on modification, but rather that the two sites have simply become independent of each other. This suggests that the cooperativity observed with the native enzyme is due to the exposure of the second site rather than a change in binding affinity per se. On the modified enzyme both sites are apparently exposed. It has long been established that Ca^{2+} is required for enzyme phosphorylation and it seems possible that a conformational change associated with cooperativity may link Ca^{2+} activation to Ca^{2+} binding through a mechanism involving a histidyl residue. We are currently pursuing experiments designed to specifically label and identify the histidyl residue(s) involved.

T-Pos302 SARCOPLASMIC RETICULUM Ca^{2+}-ATPase: SEQUENTIAL INTRAVESICULAR DISSOCIATION OF TWO Ca^{2+}-IONS FROM E~P·Ca_2.
D. Khananshvili, W. P. Jencks, Grad. Dept. of Biochemistry, Brandeis University, Waltham, MA 02254.

ATP-dependent ^{45}Ca-uptake, in the presence or absence of intravesicular Ca^{2+}, was examined by quenching with ADP + EGTA within 3-360 msec. Passively loaded SRV(20-40 mM Ca^{2+}) take up ~1 Ca^{2+}/mol of EP with a first-order rate constant of 34 s^{-1}. The same initial rate was observed for "empty"-SRV, but the overall rate constant for uptake of 2 Ca^{2+}/mol of EP is ~17 s^{-1} after correction for a slow increase after the first turnover. Inhibition of ~1 mol Ca^{2+}/mol of EP by $[Ca^{2+}]_{in}$ is concentration dependent showing K_I ~ 2-3 mM. The absence of inhibition of transport of the first Ca^{2+} by 20-40 mM internal Ca shows that the initial step does not involve transport from a high-affinity site to an empty low-affinity site, such as might be expected for some "E_1-E_2" models. The data are consistent with a model describing sequential intravesicular dissociation of $2Ca^{2+}$ from E~P·Ca_2, in which release of 1 mol of Ca^{2+} with k_1 ~ k_2 ~ 34 s^{-1} accounts for the rate constant for release of 2 moles of Ca^{2+} with k_{obs}~17 s^{-1}:



This model requires the existence of both Ca^{2+}-translocating sites in the low affinity state; it is consistent with a single form of E~P·Ca_2, in which both ~P and Ca_2 are mutually destabilized. This mechanism is different from a "flip-flop" destabilization of ~P and Ca_2, in which E_1~P·Ca_2 (destabilized ~P and stabilized Ca_2) is converted into "E_2-P·Ca_2" (stabilized-P and destabilized Ca^{2+}).

T-Pos303 INFLUENCE OF VARIOUS FACTORS ON THE INTERACTION OF CALMODULIN WITH PHOSPHOLAMBAN
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Wheat germ calmodulin (CaM) was monoiodinated with ^{125}I, followed by incorporation of the photoactivatable crosslinker, benzophenone-4-maleimide (^{125}I-Bz-CaM) at the single cysteine residue (Cys 27) (Strasburg et. al., Biophys. J. 49, 566a (1986)). Affinity labeling of cardiac sarcoplasmic reticulum vesicles with ^{125}I-Bz-CaM confirmed our previous work showing that the primary receptor for Ca^{2+} plus CaM is phospholamban (Louis and Jarvis, J. Biol. Chem. 257, 15187 (1982)). The optimal $[Mg^{2+}]$ for affinity labeling (maintaining constant ionic strength with KCl) was 4 - 10 mM. Affinity labeling of phospholamban was reduced by increasing ionic strength; eg., addition of KCl to 0.2 M reduced affinity-labeling by about 40%. Similar results were obtained using choline chloride or potassium methane sulfonate, suggesting that the reduced affinity-labeling results from electro-static interactions rather that a specific ion effect. Phosphorylation of phospholamban by the cAMP-dependent protein kinase substantially reduced affinity-labeling (~75%), while phosphorylation by the endogenous Ca^{2+} plus CaM-dependent protein kinase, which phosphorylates a different site on phospholamban, reduced labeling by approximately 25%. Simultaneous phosphorylation of phospholamban by both kinases, or phosphorylation by either kinase at high ionic strength (0.2 M), virtually eliminated affinity labeling, indicating that these effects are additive. Affinity labeling by ^{125}I-Bz-CaM was unaffected by pH over the range of 6.5-9.5. These studies suggest that the interaction of CaM with phospholamban in the cardiac cell may be differentially modulated by both phosphorylations and by local changes in ionic composition (Supported by NIH HL-25880).

T-Pos304 IMMUNOGOLD LOCALIZATION OF PHOSPHOLAMBAN IN CANINE CARDIAC HOMOGENATES AND MICROSOMES. Ellen F. Young*, Donald G. Ferguson+ and Evangelia G. Kranias*. Departs. of Pharmacology and Cell Biophysics*, and Physiology and Biophysics+, Univ. of Cincinnati.

Several lines of evidence indicate that phospholamban (PL) is the putative regulator for the Ca^{2+} pump in cardiac SR. However, the disposition of PL in the SR membrane and its possible interactions with other proteins have yet to be determined. A low resolution model for PL has been proposed, in which PL is a pentamer of identical subunits aligned to form an interior, largely hydrophilic channel (J Biol Chem, 261:13333, 1986). We have predicted a different molecular model for PL, using computer analysis of the recently determined complete primary sequence of PL (Bioch Biophy Res Comm, 138:1044, 1986). It appears that there is a single segment capable of forming the a-helix required for a hydrophilic channel but this region is only long enough to span at most half the bilayer. According to this model, PL would have to be inserted from both cytoplasmic and luminal surfaces to form a functional channel and antigenic sites would exist on both sides of the SR membrane. To test this possibility we immunolocalized phospholamban with an affinity-purified, polyclonal antibody. However, immunogold electron microscopic studies of canine cardiac muscle homogenates and triton-solubilized microsomes indicate that the PL antibody binds only to the cytoplasmic surface of the SR membrane. Furthermore, preliminary crosslinking studies indicate that PL exists as either a monomer or, at most, a dimer in the SR membrane. Although these findings do not completely exclude a channel arrangement for PL, they imply that PL may be regulating transport in SR by a more direct interaction with the Ca^{2+} pump. (Supported by NIH Grants HL26057, HL22619 (EGK) and AHA SW Ohio Chapter (DGF)).

T-Pos305 **IMMUNOGOLD LOCALIZATION OF PHOSPHOLAMBAN-LIKE POLYPEPTIDE IN CANINE VASCULAR AND VISCERAL SMOOTH MUSCLE HOMOGENATES.** **Donald G. Ferguson*, Ellen F. Young+ and Evangelia G. Kranias+, Depts. of Physiol./Biophysics*, Pharmacol./Cell Biophysics+, Univ. of Cincinnati.**

Phospholamban, an intrinsic protein of cardiac sarcoplasmic reticulum (SR), is the putative regulator of the SR Ca^{2+} ATPase. Phosphorylation of phospholamban at three distinct sites by cAMP-, Ca^{2+}/calmodulin- and Ca^{2+}/phospholipid-dependent protein kinases leads to increased rates of Ca^{2+} uptake by cardiac SR in vitro. We have applied an affinity-purified, polyclonal antibody raised against canine cardiac phospholamban to homogenates of canine vascular and visceral smooth muscle and have localized the binding sites using immunogold electron microscopy. We confirm that phospholamban antibody does bind specifically to SR and not to sarcolemma as previously suggested by immunolocalization studies on isolated membrane fractions in gels (Raeymaekers and Jones, BBA 882:258, 1986). However, the most intense labeling is associated with the outer membrane of the nuclear envelope. This phenomenon was also observed in cardiac muscle and may not be too surprising as the outer nuclear membrane is active in protein synthesis. The degree of labeling of the nuclear envelope is much higher than that of the subsarcolemmal SR in smooth muscle suggesting a lower density of phospholamban, but as yet we cannot completely rule out inadequate penetration of the antibodies. In addition, substantial label is associated with cytoplasmic structures; eg. glycogen granules and the contractile apparatus. This labeling is not likely to be nonspecific as the antibody is affinity purified, there is virtually no labeling when only the secondary immunogold probe is applied and there is no cytoplasmic labeling in canine cardiac muscle. These results imply that there is abundant, phospholamban-like polypeptide distributed widely throughout smooth muscle cells. Presumably, phosphorylation of this protein may play an important role in regulating a wide range of activities, including nuclear function.

Supported by an AHA Southwest Ohio Region grant-in-aid (DGF) and by NIH HL 26057, HL 22619 (EGK).

T-Pos306 CAFFEINE ACTIVATION OF THE Ca^{2+} RELEASE CHANNEL OF SKELETAL AND CARDIAC SARCOPLASMIC RETICULUM. GERHARD MEISSNER, ERIC ROUSSEAU and JEFFREY LA DINE. Departments of Biochemistry and Physiology, University of North Carolina, Chapel Hill, NC 27514

A Ca^{2+}-conducting, ligand-gated Ca^{2+} release channel has been identified in skeletal and cardiac muscle sarcoplasmic reticulum (*Nature* 316: 446, 1985; *Biophys. J.*, Nov. 1986). Activation of this channel by the Ca^{2+} releasing drug caffeine was studied by determining the Ca^{2+} release behavior of vesicles passively loaded with $^{45}Ca^{2+}$ and by incorporating the channel into planar lipid bilayers. At $\leq 10^{-6}$ M free external (cis) Ca^{2+}, caffeine increased the rate of $^{45}Ca^{2+}$ efflux in a dose-dependent manner. The frequency and duration of single channel events were increased without changing the unit conductance of the Ca^{2+}-activated channel. Caffeine mimicked the effects of external Ca^{2+} in that (i) micromolar Ca^{2+} and nanomolar Ca^{2+} plus 10 mM caffeine were 10 times more effective in stimulating $^{45}Ca^{2+}$ release from cardiac vesicles ($t1/2 = 0.02 - 0.05$ s) than skeletal vesicles ($t1/2 \cong 0.5$ s), (ii) Ca^{2+}- and caffeine-induced $^{45}Ca^{2+}$ release were less sensitive to inhibition by Mg^{2+} in cardiac vesicles relative to skeletal vesicles, and (iii) Ca^{2+}- and caffeine-induced $^{45}Ca^{2+}$ release in the presence of Mg^{2+} were potentiated to a similar extent by adenine nucleotides. These results suggest that Ca^{2+} release is activated by Ca^{2+} and caffeine through a common pathway which is modulated in a similar manner by adenine nucleotides and Mg^{2+}. Supported by NIH grants AM 18687 and HL 27430 and C.H.F. fellowship to E.R.

T-Pos307 GLYCOPROTEINS INVOLVED IN THE REGULATION OF CA^{2+} RELEASE Maria Eugenia Cifuentes, Cecilia Hidalgo, and Noriaki Ikemoto, Dept. Muscle Res., Boston Biomed. Res. Inst., Boston, Mass. 02114; Dept. Neurol., Harvard Med. Sch.; Dept. Phys. Biophys., Fac. Med., Univ. Chile

Our previous finding that binding of concanavalin A (ConA) to the T-tubule/SR complex alters the $[Ca^{2+}]$-dependence of Ca^{2+} release induced by ionic replacement without affecting that of (Ca^{2+} and caffeine)-induced Ca^{2+} release (Biophys. J. 49, 235a, 1986), suggested the involvement of glycoprotein(s) in the regulation of the T-tubule-mediated Ca^{2+} release . To identify such component(s) we have analyzed ConA binding proteins in both T-tubule and SR membranes of rabbit by several methods. The ConA binding proteins as shown by fluorescence staining of the electrophoretically separated membrane components with FITC-ConA are in kDa: (T-tubule) 130-150, 102; (SR) 300-350, 155-170, 90-95, 50-55. Intact vesicles were treated with photo-affinity ConA to label glycoproteins whose carbohydrate moiety is exposed to the cytoplasmic surface (Ji and Ji, Anal. Biochem. 121, 286, 1982). No such component was found in the T-tubule, but SR components with M_r of c. 300 kDa (presumably a component of the foot) and c. 160 kDa were found to be externally accessible glycoproteins. Furthermore, the foot protein from the solubilized junctional SR membrane was retained in a ConA-affinity column. Thus, the foot component appears to be a glycoprotein that is likely involved in the regulation of the T-tubule mediated Ca^{2+} release. (Supported by grants from NIH and MDA)

T-Pos308 CALCIUM CHANNELS IN HEAVY SARCOPLASMIC RETICULUM MEMBRANES FROM THE FROG. B.A. Suárez-Isla and Verónica Irribarra. Centro de Estudios Científicos de Santiago, P.O. Box 16443 & Dept. of Physiology and Biophysics, School of Medicine, U. de Chile, P.O. Box 70055, Santiago, Chile

Native SR membranes from rabbit skeletal muscle studied with patch-clamp techniques (Suárez-Isla et al. PNAS Oct. 1986), contain a small conductance Ca channel (5 pS in symmetrical 200 mM $CaCl_2$), that is activated by caffeine(2 mM) and blocked by dantrolene(50 μM) but not by nitrendipine(10^{-2}μM). In contrast, Ca channels present in native T-tubule membranes isolated from frog muscle, studied with the same techniques, are sensitive to dihydropyridines.

We studied the electrical properties of highly purified membranes of light and heavy SR obtained from leg muscles of the chilean frog Caudiverbera caudiverbera. Patch-clamp techniques were applied directly to native SR membrane suspensions (tip-dip method, Orozco et al. Biophys. J. 47, 57a, 1985), or after channel incorporation into POPE/PS or PE/PS bilayers(molar ratio 7:1)previously assembled at the tip of patch pipets. We found a spontaneously active cation channel that opens in bursts and is permeable to Ca and Ba. This channel opens at both positive and negative pipet potentials,in contrast with the rabbit small Ca channel that could be resolved only at positive voltages. In symmetrical 200 mM $CaCl_2$ the current-voltage relationship deviates from linearity and current amplitude does not increase further beyond -60 mV or +60 mV. Under these conditions the slope of the linear region of the I/V curve yields a single channel conductance of 14.5 pS. The fraction of time spent in the open state was voltage dependent and increased from 0.28 at -60 mV to 0.61 at +60 mV ($p < 0.001$). The possible role of this channel in Ca release from SR during activation of frog muscle is under investigation. Supported by NIH Grants HL23007 (to C.Hidalgo) and GM35981 and by a grant from the Tinker Foundation Inc. to the Centro de Estudios Científicos de Santiago.

T-Pos309 INFLUENCE OF TRIFLUOPERAZINE ON CALCIUM TRANSPORT ACROSS THE MEMBRANE OF SARCOPLASMIC RETICULUM IN SAPONIN SKINNED SMOOTH MUSCLE. M.A. Stout, K.M. Sullivan and L. Moyer (Intro. G.B. Weiss). Dept. Physiology, UMDNJ-New Jersey Medical School, Newark, NJ 07103-2757.

Phenothiazine antipsychotic drugs, such as trifluoperazine (TFP), specifically modify calmodulin (CaM) or CaM-regulated processes, but they may also act nonspecifically because of their ability to partition into the phospholipid component of membranes. The effect of TFP (0.01-0.5 mM) on 45-Ca uptake and efflux by sarcoplasmic reticulum (SR) of saponin skinned rat caudal artery was investigated. TFP concentrations less than 0.1 mM caused no significant change in the steady state Ca uptake or unidirectional Ca release. Higher concentrations produced a significant, dose-dependent inhibition of uptake and stimulation of Ca release. The TFP-stimulated Ca release qualitatively resembles the caffeine-stimulated Ca release. It consists of a single, slowly rising component which is sustained over several minutes. The release does not depend upon exogenous MgATP and it is not inhibited by procaine. In contrast to caffeine, the TFP-stimulated Ca release is inhibited by 17.5 uM ruthenium red, but low concentrations of TFP (0.05 mM) do not enhance the Ca-stimulated Ca release. The data indicate that micromolar concentrations of TFP which should specifically inhibit CaM do not affect Ca uptake or release. The decrease in Ca uptake appears to result from stimulation of Ca release through TFP interaction with membrane channels or receptors which are also activated by caffeine; however, an inhibition of the unidirectional Ca influx cannot be precluded. (Supported by NIH grant HL-31152 and AHA, NJ Affiliate grant #85-01).

T-Pos310 ELIMINATION OF AN ANIONIC CONDUCTANCE IN SARCOPLASMIC RETICULUM MEMBRANES BY HYPOSMOTIC TREATMENT. P.F. Heller and J.P. Froehlich, Gerontology Research Center, National Institute on Aging, Baltimore, Maryland 21224.

The linear steady state phase of ATP-dependent Ca^{2+} accumulation in sarcoplasmic reticulum (SR) membrane vesicles can be artificially prolonged by including a Ca^{2+} trapping agent such as oxalate or phosphate in the incubation medium. Although the mechanism of oxalate permeation obeys saturation kinetics, its exact mode of entry (carrier or channel) is unknown. We have found that exposure of the SR vesicles to a K^+-free, low ionic strength medium (10 mM Tris-HEPES, pH 7.0) following high salt extraction virtually eliminates Ca^{2+} accumulation due to the presence of oxalate. Similar results were obtained by replacing oxalate in the incubation medium with phosphate. As neither the transient accumulation of Ca^{2+} measured in the absence of oxalate or CaATPase activity were affected by the hyposmotic treatment, it appears likely that the disappearance of oxalate-facilitated Ca^{2+} transport is due to an inability of the precipitating anion to cross the SR membrane. Accompanying the loss of membrane permeability to anions in the energized system was the elimination of a 90 kilodalton protein from the membrane as determined by SDS PAGE. This protein, which has a molecular weight similar to that of the anion exchange protein in red cell membranes, was distinct from the 105 kilodalton Ca^{2+} pump protein. The positive correlation between the loss of oxalate-facilitated Ca^{2+} transport and the 90 kilodalton protein suggests that the latter may be a structural component of the anion conductance in SR that prevents charge accumulation during Ca^{2+} release and re-uptake <u>in vivo</u>.

T-Pos311 LOCALIZATION BY IMMUNO-GOLD LABELLING ELECTRON MICROSCOPY OF THE JUNCTIONAL SPANNING PROTEIN IN TERMINAL CISTERNAE/TRIAD VESICLES OF SKELETAL MUSCLE. J-P. Brunschwig, R.M. Kawamoto and A.H. Caswell., Dept. of Pharmacology, Univ. of Miami School of Medicine, P. O. Box 016189, Miami, FL 33101. (Intr.by N.Brandt.)

Recently we have isolated a protein of approximately 300,000 daltons from preparations of terminal cisternae (TC)/triads. This protein, referred to as the spanning protein (SP), has been identified biochemically and by immunoelectron microscopy as a constituent of junctional feet. Affinity purified polyclonal antibodies have been prepared against SP. By immunoelectron microscopy using gold probes, we have localized the SP antigenic sites in TC/triad vesicles from rabbit skeletal muscle using two protocols. In protocol 1, the organelles were treated with primary antibody (polyclonal vs. SP) followed by secondary antibody (goat antimouse IgG) tagged with 5 nm gold particles. Pellets were fixed and sectioned by conventional protocols. In protocol 2 pellets were lightly fixed with 0.5% glutaraldehyde and 3% paraformaldehyde and embedded in LR white resin which was polymerized at 0°C. Sections were then reacted with the primary antibody followed by gold tagged secondary antibody. Controls in both protocols in which specimens were not treated with primary antibody showed very few non-specific gold particles. Vesicles treated with antibody showed significant gold labelling on the cytoplasmic surface of the vesicles in the region of the triad junction using both protocols. However in protocol 2 some gold was seen on the inside of the vesicle. Trypsin was employed to hydrolyze exposed portions of the SP on the cytoplasmic side of the vesicles. Subsequent immunoelectron microscopy showed little gold in protocol 1 but considerable gold in protocol 2. This suggests the existence of antigenic sites of SP on the luminal side of SR. Supported by NIH grant AR21601. RMK was a Muscular Dystrophy fellow.

T-Pos312 CALCIUM FLUX IN LIPID VESICLES RECONSTITUTED WITH PURIFIED SPANNING PROTEIN FROM RABBIT SKELETAL MUSCLE. R.M. KAWAMOTO AND A.H. CASWELL, Dept. of Pharmacology, Univ. of Miami School of Medicine, P.O. Box 016189, Miami, FL 33101.

The junctional feet of triads have been implicated in the mechanism of excitation-contraction coupling in skeletal muscle. The spanning protein (SP), a protein of M_r 300,000, is believed to represent the principal constituent of the triad junction. We purified the SP from terminal cisternae sarcoplasmic reticulum/triads of rabbit skeletal muscle using affinity chromatography on a monoclonal antibody column eluted with Zwittergent 3-14 and NaSCN. The SP was passed through a molecular sieve column containing Triton X-100 and 50 mM K gluconate. Ca gluconate (1 mM) and 0.04% phospholipids extracted from longitudinal sarcoplasmic reticulum were added. A control containing no SP was passed through the molecular sieve and similarly treated. The SP was incorporated into lipid vesicles by removal of the Triton by overnight incubation with BioBeads. The supernatant was centrifuged for 2 hrs at 100,000xg. The resulting pellet was resuspended in 2 mM Tris Cl, pH 7.0, 50 mM K gluconate and 1 mM Ca gluconate. SDS-PAGE analysis confirmed that the SP was pelleted with the SR lipid vesicles. Ca^{++} movement in the reconstituted vesicles was assayed by tracing ^{45}Ca in an exchange experiment following the establishment of a calcium gradient and a membrane potential difference. The vesicle preparations were diluted 33 fold in incubation medium (2 mM Tris Cl, pH 7.0, 250 mM sucrose, 10 uM $CaCl_2$) containing 5 uCi/ml $^{45}CaCl_2$. The suspension was incubated 5 min at 22°C, quickly passed through a Chelex cation exchange column to remove free Ca^{++} and washed with 5 volumes of the incubation medium. Vesicles containing the SP accumulated Ca^{++} while the control vesicles lacking SP showed little Ca^{++} retention. Ryanodine (100 uM) caused a modest enhancement of the Ca^{++} uptake. Supported by NIH grant AR21601 and NIH training grant HL07188.

T-Pos313 SPECIFIC ASSOCIATION OF JUNCTIONAL PROTEINS IN TERMINAL CISTERNAE/TRIADS OF SKELETAL MUSCLE. Taehong Choi,Kyungsook C.Kim,Richard M.Kawamoto and Anthony H.Caswell, Department of Pharmacology,University of Miami,P.O.Box 016189,Miami,Florida 33101

We have recently described the isolation of the spanning protein (SP) which attaches T tubule to terminal cisternae (TC) using a monoclonal antibody column. An affinity column was constructed by coupling the purified SP to CNBr Sepharose. TC/triads were dissolved in detergent and eluted through the column. Retained proteins which were released with 1M NaCl had Mr 150,62 and 38 K daltons. 150 and 62 K proteins stained blue with stains all indicating that Mr 62 K protein was calsequestrin. TC/triads were preincubated with ^{32}P ATP in the presence of the catalytic subunit of c-AMP dependent protein kinase. The SP affinity column retained a labelled protein of Mr approximately 80 K. A similar affinity column of glyceraldehyde phosphate dehydrogenase retained proteins of Mr 150,80,62,55 and 38 K daltons. The 62 K dalton protein was similarly identified as calsequestrin. We also used Sulfosuccinimidyl 2-(p-azidosalicylamido) ethyl 1,3-dithiopropionate (SASD) to identify the association of the junctional proteins in situ. SASD has an amino active group separated by a disulfide linkage from a photoactivable moiety which can be labelled with ^{125}I. It was reacted in the dark with the purified SP. The SP was incubated with TC/triads for 30 min at 37^0C and then illuminated to photoactivate the azide moiety of SASD. Controls were treated identically except that illumination preceded incubation with TC/triads. The crosslinked products were identified by autoradiography after separating the proteins by SDS-PAGE which contained mercaptoethanol to break the S-S bond of the crosslinked proteins. Two labelled proteins with Mr 100 and 62 K were identified from the gel. This work was supported by NIH grant AR-21601; R.M.K was a Muscular Dystrophy fellow.

T-Pos314 CALCIUM BINDING PROTEINS OF JUNCTIONAL SARCOPLASMIC RETICULUM. DETECTION BY ^{45}Ca LIGAND BLOTTING. Francesco Zorzato and Pompeo Volpe. Centro di Studio per la Biologia e la Fisiopatologia Muscolare del CNR, Istituto di Patologia Generale dell'Universita' di Padova, via Loredan 16, 35131 Padova, Italy

In skeletal muscle, junctional sarcoplasmic reticulum (SR) plays a crucial role in E-C coupling and Ca^{2+} release. SR was fractionated into longitudinal SR (LSR) and terminal cisternae (TC). Junctional face membranes (JFM) were obtained by Triton X-100 treatment of the TC fraction (Costello et al., (1986) J. Cell Biol. 103, 741-753). Each fraction was comprised of a) unique proteins as detected by SDS/polyacrylamide gel electrophoresis, b) characteristic integral membrane proteins as shown by photolabeling with radioactive azidophospholipids (Volpe et al., submitted) and c) specific Ca^{2+} binding proteins as judged by ^{45}Ca ligand overlay on nitrocellulose blots. Ca^{2+} binding proteins of LSR were the Ca^{2+}ATPase (M_r of 115k) and the intrinsic glycoprotein (M_r of 160k); Ca^{2+} binding proteins of junctional SR were several polypeptides in the M_r range 350k-52k. Ca^{2+} binding was specific because the assay medium contained 5 mM $MgSO_4$, 60 mM KCl and 10 uM $CaCl_2$, was reversible and was not due to Ca-Ca exchange. Measurements of Ca^{2+} binding to several SR fractions by equilibrium dialysis indicated that 8-20 nmoles Ca^{2+}/mg protein were specifically bound. After extraction of calsequestrin with EDTA, JFM still bound Ca^{2+} (5-6 nmoles/mg protein) suggesting that junctional SR had specific Ca^{2+} binding sites. Some of these sites might be the Ca^{2+} binding sites of Ca^{2+}-gated Ca^{2+} channels localized in junctional TC (Chu et al., Biochemistry, in press) and might also be involved in transverse tubule-SR coupling where a Ca^{2+}-dependent step has been postulated (Volpe and Stephenson (1986) J. Gen. Physiol. 87, 271-298).

T-Pos315 INTERACTION OF CALCIUM AND RYANODINE WITH CANINE CARDIAC SARCOPLASMIC RETICULUM. Barbara H. Alderson and Joseph J. Feher, Dept. of Physiology, Med. Coll. of Virginia, Richmond, VA 23298.

The interaction of ryanodine and Ca with canine cardiac SR was studied using ^3H-ryanodine binding and the effect of ryanodine on Ca uptake. In the absence of Ca, ^3H-ryanodine binding showed no saturation up to 20μM ryanodine, and thus was taken to represent non-specific binding. The apparent Km for Ca depended on the ryanodine concentration, varying between 2 and 0.8μM over the ryanodine concentrations of 10 to 265nM. The Hill coefficient for Ca was near 2 for all concentrations of ryanodine studied. Scatchard analysis of ryanodine binding indicated the existence of a high-affinity site with a Bmax of 5.2 ± 0.4 pmol/mg and with a Ka of 6.8 ± 0.1nM. Binding to the high-affinity site was not correlated with the stimulation of oxalate-supported Ca uptake by ryanodine. Ca uptake was stimulated only with ryanodine concentrations in the micromolar range. The stimulation of Ca uptake presumably required occupation of a second, low-affinity binding site having an apparent Km of about 17μM. This suggests that the high-affinity site may be correlated with activation of the channel while the low-affinity site is involved with closure of the Ca release channel as indicated by stimulation of oxalate-supported Ca uptake. The stimulation of Ca uptake by ryanodine also required sub-micromolar concentrations of Ca. Thus, both the high-affinity and low-affinity sites interacted with Ca. Calculations indicate that there were some 450 pump units and only 4-5 channels per ryanodine-sensitive vesicle, indicating that these are distinct structures.

Supported by grant HL34681 from the National Institutes of Health.

T-Pos316 RYANODINE INCREASES TENSION AS WELL AS AEQUORIN LIGHT EMISSION IN SINGLE MUSCLE FIBER FROM BALANUS NUBILUS. K.S. Hwang[1], J.F. Godber[2], T.J. Lea[2], C.C. Ashley[2] and C. van Breemen[1], (Intr. by D.J. Adams) Dept. of Pharmacology, Univ. of Miami, Miami, Florida, 33101, USA[1], Univ. of Oxford, University Laboratory of Physiology, Park Road, Oxford, OX1 3PT, U.K.[2]
Single muscle fibers from the barnacle, Balanus nubilus were cannulated and axially microinjected with 50 μM aequorin to give a final concentration of 0.5 μM, assuming uniform distribution through diffusion for 3 h at 4°C (Ashley et al., 1974, J. Physiol. 242:255-272). Ryanodine alone, at both 50 μM and 500 μM, increased isometric tension and aequorin light emission in Ca^{2+} free physiological saline (with 2 mM EGTA and no added Ca^{2+}) at 20°C. 50 μM ryanodine produced 2% of the tension and 6% of the light induced by 50 mM caffeine by 70 min, while 500 μM ryanodine increased these to 8% tension and 20% light by 90 min. In Ca^{2+} containing physiological saline, 500 μM ryanodine showed a similar response, albeit enhanced to 31% tension and 56% of light of response induced by 50 mM caffeine in Ca^{2+} containing physiological saline. In myofibrillar bundles, 200 μm in diameter (Ashley and Moisescu, 1977, J. Physiol. 270:627-652), 100 μM ryanodine induced tension in 0.1 mM EGTA (pCa 6.7) at 14°C. Tension reached its peak, 47% of that induced by 5 mM caffeine by 130 s, compared to 15 s in 5 mM caffeine. The above data indicate that ryanodine releases Ca^{2+} from the intracellular stores, presumably sarcoplasmic reticulum (SR). The responses, however, were delayed and small compared to those induced by caffeine. These results are consistent with the observation that ryanodine releases Ca^{2+} from the SR (Hwang and van Breemen, Pflugers Archiev, in press) in the rabbit aortic smooth muscle. This work was supported by grants NIH-HL07188, NIH-HL29467 and MRC (U.K.).

T-Pos317 IMMUNOAFFINITY PURIFICATION OF RYANODINE RECEPTOR FROM RABBIT SKELETAL MUSCLE. Toshiaki Imagawa, C. Michael Knudson, Albert T. Leung, Steven D. Kahl and Kevin P. Campbell. Dept. of Physiology and Biophysics, University of Iowa, Iowa City, IA 52242.

Monoclonal antibody XA7 which is able to immunoprecipitate [^{3}H]ryanodine binding activity from digitonin solubilized triads has been used to immunoaffinity purify the ryanodine receptor from rabbit skeletal muscle. Monoclonal antibody XA7, which is an IgM immunoglobulin, was purified from ascites fluid or culture media by gel filtration chromatography and then covalently coupled to Sepharose 4B to form an immunoaffinity column. [^{3}H]Ryanodine binding activity was solubilized from isolated triads in 1% digitonin solution containing 0.5 M NaCl and applied directly to the immunoaffinity column. Solubilized [^{3}H]ryanodine binding activity was completely removed by the immunoaffinity column. Elution of the immunoaffinity column with a solution of 0.5 M NaSCN and 0.3% digitonin resulted in the co-purification of [^{3}H]ryanodine binding activity and the 350,000 Da junctional-specific protein. This result strongly suggests that the 350,000 Da junctional-specific protein is the ryanodine receptor of the junctional sarcoplasmic reticulum and possibly a component of the Ca^{2+} release channel. (Supported by NSF 85-17834 and MDA).

T-Pos318 LOCALIZATION OF RYANODINE RECEPTOR (RyR) IN A SUBFRACTION OF CARDIAC SARCOPLASMIC RETICULUM (CSR) Sherry Wang, Makoto Inui, Eunice Ogunbunmi, Akitsugu Saito and Sidney Fleischer, Department of Molecular Biology, Vanderbilt University, Nashville, TN 37235

Subfractions of CSR were prepared to characterize the mechanism of Ca^{2+} release from CSR. Subfractionation from microsomes was achieved by selective calcium phosphate loading followed by sucrose density gradient centrifugation. Two subpopulations were resolved. F1 has a high Ca^{2+} loading rate (0.9-1.0 $\mu mol.mg^{-1}.min^{-1}$) which is unchanged by addition of ruthenium red (RR), and is devoid of feet structures observed by electron microscopy as well as the high molecular weight protein (HMW) with M_r of 340K on SDS-PAGE. F2 has a low Ca^{2+} loading rate which is enhanced about 5-fold to 1.0 $\mu mol.mg^{-1}.min^{-1}$ by RR or high concentration of ryanodine (10 μM). F2 has feet structures and HMW. Thus, F1 and F2 have similar characteristics to longitudinal SR (L-SR) and junctional terminal cisternae of SR (JTC) from skeletal muscle, respectively. [^{3}H]-ryanodine binding study revealed that the RyR was enriched in cardiac JTC (~6-10 pmol/mg protein). Scatchard analysis of the binding data showed two different binding sites (high affinity site, Kd ~5 nM; low affinity site, Kd ~100-300 nM), contrasting with skeletal JTC which has one binding site with Kd of ~50 nM. The RR-enhanced Ca^{2+} loading rate in cardiac JTC was blocked by pretreatment with ryanodine as reported for skeletal JTC. The inhibition constant is lower (~5 nM) than that of skeletal JTC (~50 nM) and coincides with Kd for the high affinity site of the RyR. Calcium release obtained from cardiac JTC has similar characteristics to that from skeletal muscle in terms of RR and ryanodine action, but the calcium release channel from heart is much more sensitive to ryanodine. (NIH HL32711 to S.F. and Investigatorship of American Heart Association, Tennessee Affiliate to M.I.)

T-Pos319 A COOPERATIVE PROCESS MAY BE INVOLVED IN ELECTRICALLY-INDUCED MEMBRANE FUSION. Arthur E. Sowers, Cell Biology Laboratory, American Red Cross/Holland Laboratory for the Biomedical Sciences, Rockville, MD 20855.

Use of dielectrophoresis to induce membrane-membrane contact and electric pulses to induce membrane fusion permits the effects of (a) chemical characteristics of the medium, (b) fusion-inducing stimulus (pulses), and (c) membrane contact to be separated from one another in studies of fusion mechanisms. The fact that electrically-induced fusion in human erythrocyte ghosts occurs regardless of whether membrane contact is induced before or after the pulses (JCB,99:1989;JCB,102:1358) suggests that two mechanisms may be involved. Since pulse-induced electropores are unlikely to be part of at least one of these mechanisms (FEBS Let 205:179) it is possible that some other pulse-induced membrane structure or property may be involved. It has been rigorously determined that induced transmembrane voltage, V, in vesicular-shaped membranes is related to pulse strength, E (=V/mm), and radius, r, of the membranes according to the equation V=1.5Er (Fricke, 1953). Fusion between 5-day old chick embryo erythrocytes (r=4.9 um) show higher fusion yields than in adult chicken erythrocytes (r=3.7 um) which generally supports the prediction of this equation in terms of a voltage-induced fusogenic alteration. However, fusion between a large radius membrane and a small radius ghost generally requires an intermediate pulse strength which indicates a cooperative process.

T-Pos320 FUSION OF LIPOSOMES MEDIATED BY Lα-H_{II} INTERMEDIATES. H. Ellens,[a] D. P. Siegel,[b] D. R. Alford,[c] P. L. Yeagle[d] and J. Bentz[c]. Dept. [a]Pharmacology and [c]Pharmacy and Pharmaceutical Chemistry, UCSF, San Francisco, CA 94143, [b]Proctor and Gamble, Co. P.O. Box 39175, Cincinnati, OH, 45247, [d]Dept. of Biochemistry, SUNY, Buffalo, NY. 14214.

N-methylated dioleoylphosphatidylethanolamine (DOPE-Me) liposomes show mixing of aqueous contents in the temperature range, T_I, where the lipid at equilibrium shows isotropic ^{31}P-NMR resonances (Ellens et al., 1986 Biochem. 25, 4141). At lower temperatures, the lipid is in the Lα phase, and there is no mixing of contents. At higher temperatures, where the lipid is in the hexagonal H_{II} phase, there is contact-mediated lysis. We proposed that above T_H rapid accumulation of inter-membrane intermediates (IMI) leads to collapse of apposed liposomes towards H_{II} phase structures, accompanied by leakage, but no contents mixing. Calculations by Siegel (1986, Biophys. 49, 1171) showed that lipid systems with large equilibrium radii of curvature (usually large H_{II} tube diameters), like DOPE-Me and PE/PC mixtures, can form new structures leading to contents mixing between apposed liposomes. Based on these calculations we proposed that the contents mixing at T_I occurs through these structures. To test the generality of this result we have used mixtures of phosphatidylethanolamine (DOPE) and phosphatidylcholine (DOPC). The T_H's of PE/PC 2:1 3:1 and 4:1 mixtures at pH 4.5 were determined by DSC to be 78-81, 61 and 44 ± 4° C. All PE/PC mixtures were shown to have isotropic ^{31}P NMR resonances below T_H. We have correlated the initial kinetics of contents mixing and leakage with T_I and T_H and show that the PE/PC liposomes show a temperature dependent behaviour very similar in all respects to the DOPE-Me liposomes. Supported by NIH grants GM-31506 (J.B.) and HL-23853 (P.Y.).

T-Pos321 A 2-ARACHIDONYL DIGLYCERIDE IS PARTICULARLY EFFECTIVE IN INDUCING L_α/H_{II} TRANSITIONS AND MEMBRANE FUSION --- D.P. Siegel[a], D. Alford[b], H. Ellens[c], L. Lis[d], P.J. Quinn[e], P.L. Yeagle[f], and J. Bentz[b]. --- [a]Procter & Gamble Co., P.O. Box 39175, Cin. OH 45247; [b]Depts. of Pharm. & Pharm. Chem. and [c]Pharmacol., Univ. of California, San Francisco, CA 94143; [d]Dept. of Physics, Kent State Univ., Kent, OH 44242; [e]Dept. of Biochem., Kings College London, Campden Hill Rd., Kensington, London W8 7AH, U.K., [f]Dept. of Biochem., SUNY at Buffalo, Buffalo, NY, 14214

In the PI cycle, phosphatidylinositol hydrolysis produces 2-arachidonyl-diglyericdes at transient concentrations of ca. 1 mole % of membrane lipid. Various diglycerides have been shown to lower the L_α/H_{II} transition temperatures (T_H) of PE systems by about 8°C per mole % diglyceride [1]. Here we show that only 2 mole % of 1-oleoyl, 2-arachidonyl glycerol (OArG) reduces T_H by 20-30°C in POPE, monomethylated DOPE (DOPE-Me) and equimolar DOPE/DOPC/CHOL systems. We have verified this phase behavior with ^{31}P-NMR, DSC, and time-resolved X-ray diffraction experiments. It was previously shown that liposomes of lipids whose bulk systems show isotropic ^{31}-P-NMR resonances at temperatures below T_H undergo fusion at those temperatures [2]. Above T_H, the liposomes only undergo contact-mediated lysis. Here, using liposome contents mixing assays, we show that fusion is also observed in OArG-doped DOPE-Me systems in the vicinity of the (reduced) T_H. The effectiveness of different diglycerides in inducing H_{II} phases will be compared. The lipid systems studied here are somewhat similar to biomembranes (similar head group compositions). Many biomembrane preparations or lipid extracts adopt the H_{II} phase upon dehydration or heating (refs. in [3]). We speculate that one role of 2-arachidonyl diglycerides produced during stimulus-secretion coupling may be to transiently reduce T_H of apposable membranes so that fusion can occur via L_α/H_{II} transition intermediates [2,3]. --- 1) Epand, Biochem. 24:7092 (1985). 2) Ellens, et al., Biochem. 25:4141 (1986). 3) Siegel, Biophys. J. 49:1171 (1986).

T-Pos322 APPARENT VS. TRUE FUSION OF SENDAI VIRUS WITH ACIDIC LIPID VESICLES AT LOW pH MEASURED BY FLUORESCENCE ENERGY TRANSFER. Ruby I. MacDonald, Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, Evanston, IL 60201.

To test whether the apparent fusion of Sendai virus with acidic lipid vesicles at low pH (MacDonald, Biophys. J., 49, 133a, 1986) were due to acceptor quenching by virus and not to acceptor dilution by virus-vesicle fusion, phosphatidylserine (PS) or phosphatidylcholine (PC) vesicles containing NBD-PE, Rh-PE, pyrene-PC or no probe were incubated with or without Sendai virus at pH 4.2-7.2 before raising the pH to 7 and measuring the fluorescence. Probes in PS vesicles, particularly Rh-PE, were partially quenched by Sendai virus as the pH was decreased to 4.1. The fluorescence of probes in PC vesicles, particularly NBD-PE, was slightly enhanced by the virus towards pH 7. The apparent fusion between virus and PS vesicles measured by fluorescence energy transfer was corrected for virus quenching of Rh-PE. This was done by re-setting the 0% and 100% lipid mixing limits of the standard curve, which gave the % lipid mixing or fusion of unknown samples by their NBD-PE fluorescence in the presence and absence of Rh-PE. Corrected % lipid mixing or fusion between virus and PS vesicles was no more than 20%--instead of an uncorrected 60%--at pH 4.2, 80%--instead of an uncorrected 100%--at pH 5.1, remained about 15% at pH 6.4 and about 0% at pH 7.2. Correction had little effect on PC vesicle fusion, which was a maximal 25% at pH 7.4. The correction procedure is valid insofar as 1) pH 4, but not pH 5, treatment inactivates viral hemolysis and 2) virus-vesicle fusion hybrids were seen in electron micrographs after incubation at pH 5 but not at pH 7. Thus, spurious fusion measured by fluorescence energy transfer can be corrected for by a relatively simple adjustment of the standard curve of lipid mixing. Supported by NIH AI20421.

T-Pos323 H+ - INDUCED FUSION OF PHOSPHATIDYLSERINE LIPOSOMES: EFFECTS OF TWO PHASE TRANSITIONS. Aeri Kim, Harma Ellens*, Joe Bentz and Francis C. Szoka. Department of Pharmacy and Pharmaceutical Chemistry, School of Pharmacy and *Department of Pharmacology, University of California, San Francisco, CA 94143.

The phase behavior of bovine brain phosphatidylserine (PS) was examined as a function of pH using differential scanning calorimetry. The gel to liquid-crystalline phase transition temperature (Tc) shows an upward shift of about 15°C as the pH changes from 7 to 3. Below pH 3, a second transition appears with an onset temperature of 33°C. The ^{31}P-NMR spectra of the second transition has a line shape characteristic of H_{II} phase. X-ray spectra will be obtained to establish the identity of this phase. We have studied the temperature dependence of fusion and leakage of PS LUV at low pH, utilizing the ANTS/DPX fusion assay at an excitation wavelength (λ_{ex} = 306 nm) where the fluorescence is independent of pH. ANTS does not bind to PS down to pH 2. PS LUV show very rapid aggregation, and aqueous contents mixing and leakage below pH 2.7. Fusion and leakage at pH 2.1 were observed from 10°C, near Tc, up to 40°C, above T_H. The initial rate of fusion increases with temperature. The effect of the gel to liquid-crystalline phase transition on this initial rate is not dramatic. The initial rate of fusion does not appear to be strongly affected at temperatures near T_H. Supported by GM 29514 (FCS) and GM 31506 (JB).

T-Pos324 La^{3+} INDUCED FUSION OF PHOSPHATIDYLSERINE LIPOSOMES: CLOSE APPROACH, INTERMEMBRANE INTERMEDIATES AND THE ELECTROSTATIC SURFACE POTENTIAL. Joe Bentz[a,b], Dennis Alford[a], Joel Cohen[d] and Nejat Düzgüneş[b,c]. Departments of [a]Pharmacy and [b]Pharmaceutical Chemistry and [c]Cancer Research Institute, University of California, San Francisco, CA 94143 and [d]Department of Physiology, University of the Pacific, San Francisco, CA 94115.

The fusion of phosphatidylserine liposomes (PS LUV) induced by La^{3+} has been monitored using the ANTS/DPX fluorescence assay for the mixing of aqueous contents. The fusion event is extensive and non leaky, with up to 95% mixing of contents within the fused liposomes. However, addition of excess EDTA leads to disruption of the fusion products in a way which suggests the existence of intermembrane contact sites. The maximal fusion activity occurs with between 10-100 μM La^{3+} and fusion can be rapidly terminated, without loss of contents, by the addition of 1 mM La^{3+}. This is explained by surface charge reversal due to a very large binding constant (~ 10^5 M^{-1}) of the La^{3+} to the PS headgroup, as measured by microelectrophoresis. La^{3+} binding to PS releases a proton; however, both fusion and microelectrophoresis are substantially affected when the pH is below 5.0. We propose that La^{3+} binds to PS at two sites, with one site being the primary amino group. The fusogenic capacity of La^{3+}, i.e., the amount of bound cation required to induce rapid fusion, is less than that of Ca^{2+}, even though La^{3+} binds much more avidly to the PS headgroup. These results establish that the general mechanism of membrane fusion must include two kinetic steps: close approach, or thinning of the hydration layer, and the formation of intermembrane intermediates which determine the extent to which membrane destabilization leads to the biologically relevant fusion event with mixing of aqueous contents. Supported by NIH Grants GM-31506 and GM-28117.

T-Pos325 PHYSICAL PROPERTIES AND FUSION OF LIPID VESICLES CONTAINING HEAD GROUP-MODIFIED ANALOGUES OF PHOSPHATIDYLETHANOLAMINE. Pamela M. Brown, S.W. Hui, P.L. Yeagle and J.R. Silvius. Department of Biochemistry, McGill University, Montréal, Québec, Canada.

Three classes of structural analogues of dioleoyl and dielaidoyl phosphatidylethanolamine (PE) have been synthesized, with varying patterns of N-alkylation, alkylation of C-2 of the ethanolamine moiety, or varying numbers of methylene groups separating the phosphoryl and amino groups. The physical properties of aqueous dispersions of these PE analogues have been studied by calorimetry, ^{31}P-nuclear magnetic resonance, and freeze fracture electron microscopy. These studies have shown that the abilities of PE analogues to form nonlamellar phases are enhanced by modifications of the PE head group that increase the hydrophobicity without compromising the hydrogen bonding capacity of the amino group. We have also examined the divalent cation-triggered fusion and destabilization of large unilamellar vesicles composed of various PE analogues together with 25 mole % of phosphatidylserine (PS). Vesicles containing PE analogues with enhanced abilities to form nonlamellar phases fuse and destabilize in the presence of divalent cations at rates that are comparable to or faster than those observed for vesicles containing PE. The relative rates of divalent cation induced leakage from vesicles containing PS plus various PE analogues correlate very well with the lamellar-to-nonlamellar transition temperatures (T_H) of the different PE analogues. The rates of contents mixing between such vesicles are not perfectly correlated with T_H, suggesting that the physicochemical determinants of vesicle fusion may be different from, and perhaps more complex than those that determine vesicle destabilization as reflected in the release of vesicle contents. (Supported by the Medical Research Council of Canada).

T-Pos326 CONTACT-DEPENDENT LIPID MIXING BETWEEN PHOSPHOLIPID VESICLES WITH OPPOSITE SURFACE CHARGES. Leonidas Stamatatos, Rania Leventis and John R. Silvius. Department of Biochemistry, McGill University, Montréal, Qué., Canada.

Phospholipid vesicles with positive or negative surface charges have been prepared using neutral phospholipids together with small molar proportions of a cationic lipid analogue, 1,2-diacyl-3-(trimethylammonium)-1,2-propanediol, or phosphatidylserine, respectively. Mixtures of these two types of vesicles show substantial aggregation, and lipid mixing (monitored by fluorescence assays) that is strongly affected by the neutral lipid compositions of the two types of vesicles. These interactions are highly dependent on the presence of at least a small surface charge (> 2.5 mole % charged lipid) in both vesicle populations, but curiously, lipid mixing between vesicles of opposite charge is enhanced rather than antagonized by increasing ionic strength. Perhaps the most useful application of our system is to examine interactions between two vesicle populations with different neutral lipid compositions under conditions where interactions between vesicles of like composition are suppressed. As an example, we have found that vesicles rich in choline phospholipids, which normally do not show lipid mixing even when aggregated, can show extensive (but incomplete) mixing of lipids with vesicles containing phosphatidylethanolamine (PE). These results are of particular interest since PE-containing liposomes have recently been used to deliver liposome-associated molecules to animal cells, a process that has been suggested to occur by fusion between the PE-rich vesicles and the choline phospholipid-rich surfaces of cellular membranes. (Supported by the Medical Research Council of Canada).

T-Pos327 SIMULTANEOUS MICROSCOPY AND CAPACITANCE MEASUREMENTS REVEAL THAT MEMBRANE FUSION PRECEDES GRANULE SWELLING DURING EXOCYTOSIS IN BEIGE MOUSE MAST CELLS. J. Zimmerberg, DCRT, National Institutes of Health, Bethesda, MD; M. Curran, M. Brodwick, Biophysics and Physiology, UTMB, Galveston, TX; and F.S. Cohen, Physiology, Rush Medical College, Chicago, Il.

During exocytosis, secretory granules of mast cells show dramatic morphological changes. We have investigated whether the swelling of the secretory granule, the first of those morphological changes, precedes -- and therefore possibly drives -- secretion, or whether swelling occurs after fusion of the granule and cell membranes. Using the tight-seal whole-cell voltage clamp, we used cell membrane capacitance to detect the moment of continuity of granule and plasma membrane on the mast cells of Beige mice. We monitored membrane capacitance with a time-response of 2.9 milliseconds in wide bandwidth measurements. During degranulation of the large granules of this mast cell, we saw large transients in capacitance, and final increases in capacitance that we could correlate with the exocytosis of identifiable granules. The time course of the capacitance change was 17-100 msec. Simultaneously, we observed the same cells with high resolution video microscopy. In every experiment the capacitance increase preceded the granule swelling leading to exocytosis. We detected no other morphological changes before the capacitance rise. To rule out the possibility that a mechanical stress imparted by the internal pressure of a taut granule was driving fusion, we shrank vesicles with hyperosmotic solutions. Even with these flaccid granules, granule swelling occurred well after the capacitance rise. We conclude that vesicle swelling cannot be the driving force for membrane fusion in this system. Instead, granule swelling may be a sequellae of membrane fusion.

T-Pos328 FINAL STEPS IN EXOCYTOSIS IN MAST CELLS OF BEIGE MICE. L. Breckenridge & W. Almers, Physiology & Biophysics SJ-40, University of Washington, Seattle, Washington 98195.

We have exploited the abnormally large size of the secretory granules in the mast cells of the beige mouse (BM), mutants with Chediak-Higashi syndrome, to study three events in exocytosis, namely membrane fusion, vesicle swelling and loss of vesicular contents. Degranulation was induced by GTPγS in the patch pipette. The stepwise increase in cell membrane capacitance (C_m), thought to represent the fusion of single secretory vesicles with the cell membrane (Fernandez et al., 1984, Nature 312, 453), was ten times larger in cells from BM (222 $\pm$ 307 fF, (S.D.) n=418) than cells from normal mice (20.3 $\pm$ 8.1 fF, (S.D.) n=198). This would correspond to vesicles with diameters of 2.7μm and 0.8μm respectively. The fluorescent dye quinacrine, which concentrates in acidic spaces, selectively stained the vesicles and was used to monitor loss of vesicle contents. In the normal cell as C_m increased during exocytosis, the fluorescence diminished continuously, while BM cells demonstrated a stepwise decline coinciding with step increases in C_m. Some granules failed to stain suggesting that an intravesicular acidic pH is not required for exocytosis. Frequently intermittent increases in C_m preceded the steps but no dye loss was associated, suggesting the formation of an initial electrical connection between the vesicle interior and cell exterior that does not allow the escape of dye. Granule swelling, thought to be involved in membrane fusion, was also observed. The swelling of individual granules followed a mean of 440 $\pm$ 100ms after the C_m step caused by that granule. The step amplitude in BM was correlated with π x (initial granule diameter)^2. We conclude that as the C_m step preceeds granule swelling, the initial fusion process is not driven by membrane swelling. Supported by NIH AR 17803.

T-Pos329 INVESTIGATION OF THE FUSION PROCESS BETWEEN OLIGODENDROCYTES AND UNCOATED VESICLES.INSERTION OF THE MYELIN PROTEOLIPID APOPROTEIN (PLP).

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The time effect on the interacting process between oligodendrocytes and brain uncoated vesicles (UCV) loaded with PLP has been investigated.The protein tagged with fluorescein (PLP_f) was found to be rapidily associated with cells at 4 and 37°C.After 2.5 hours of incubation,the fluorescence intensity associated with oligodendrocytes decreased.After 3.5 hours it completely desappeared.By washing the mixture (cell+UCV) with KCl or triton X100,we showed that the cell labelling did not arise from UCV adsorption.The occurence of a fusion process was demonstrated by freeze-etching experiments and plasma membranes characterization.The fusion process was shown to be modulated by KCl and Ca^{++}.When we used BSA in place of PLP,cell labelling was significantly lower suggesting a fusogenic character for PLP.