

Th-AM-Min3-1 STUDIES OF EPITHELIAL TRANSPORT MECHANISMS BY EXTRA- AND INTRACELLULAR MICROELECTRODE TECHNIQUES. Luis Reuss, Washington University School of Medicine, St. Louis, MO. 63110.

Microelectrodes filled with aqueous electrolyte solutions or lipophylic membranes (containing either ion-exchangers or neutral ion carriers) have become standard experimental tools to measure cell membrane potentials and conductances and to estimate intracellular and extracellular ion activities in epithelia and other tissues. Advantages, disadvantages and novel applications of intracellular and extracellular microelectrode techniques are considered in reference to recent studies in amphibian gallbladder. Specific issues relating to the use of ion-sensitive microelectrodes include the following: a) measurements of K^+ activity, Cl^- activity and pH in the immediate (extracellular) vicinity of the apical cell membrane, to estimate transepithelial transport and apical membrane ion transference numbers, b) measurements of cell membrane potential with cation-sensitive microelectrodes which do not discriminate between sodium and potassium, in which case electrolyte leakage is prevented, c) conventional measurements of steady-state intracellular activities, d) estimates of net ion fluxes during modifications in the composition of the bathing solutions, and e) estimates of changes in cell volume, and apical membrane osmotic water permeability, by measurements of intracellular tetramethylammonium activity in cells pre-loaded with this cation. Supported by NIH Grant AM19580.

Th-AM-Min3-2 CHARACTERIZATION OF APICAL AND BASOLATERAL MEMBRANE CHANNELS IN THE MAMMALIAN COLON USING CURRENT FLUCTUATION ANALYSIS. N.K. Wills, Dept. of Physiology, Yale Univ. Sch. of Med., New Haven, CT 06510

The mammalian large intestine actively absorbs Na^+ and secretes K^+ . In studies of rabbit and human descending colons, current fluctuation analysis was used to assess the mechanisms of Na^+ and K^+ movement across the apical membrane. Na^+ entry across this membrane occurs via amiloride-blockable channels. The mean single channel Na^+ current was approximately 0.3-0.4 pA. The channel density was proportional to the Na^+ transport rate and was tentatively estimated as 2-6 channels per square micron. The method also revealed apical membrane K^+ channels in parallel with the Na^+ channels. These channels showed spontaneous conductance fluctuations and may play an important role in net potassium secretion by the colon. The basolateral membrane conductance in the rabbit colon is largely due to K^+ . Basolateral channels were studied by effectively removing the apical membrane resistance with nystatin. Unlike the apical membrane K^+ channels, basolateral channels were blocked by Ba^{2+} . Because current fluctuation analysis gives information concerning both channel current and density, this method is an important tool for investigating the regulation of Na^+ and K^+ transport systems. (Supported by N.I.H. grant #AM29962 to N.K. Wills.)

Th-AM-Min3-3 TRANSPORT-RELATED MEMBRANE AREA CHANGES IN TURTLE BLADDER MEASURED USING IMPEDANCE ANALYSIS TECHNIQUES. C. Clausen and T.E. Dixon. Dept. of Physiology, SUNY Stony Brook, NY, and Dept. of Medicine, Northport VA Medical Center, Northport, NY.

Epithelial transport processes are often regulated by altering exposed area of the different membranes. Transepithelial impedance techniques are capable of measuring membrane capacitances, which are proportional to membrane area ($1 \mu F \approx 1 \text{ cm}^2$). We are using these techniques to quantify membrane area changes associated with the regulation of H^+ transport in turtle bladder. A simple single-cell equivalent-circuit model incorporating a distributed lateral membrane impedance is sufficient to model accurately the measured impedance, but only when Na^+ transport is inhibited. Apical and basolateral capacitances (C_a and C_b) continuously decreased with time when ouabain and amiloride were used to inhibit Na^+ transport. We thought that slow cell volume changes, resulting from a compromised ability of the cells to volume regulate, could account for this decrease. By omitting ouabain and adding 2% albumen to the serosal solution, stable capacitance measurements were obtained. In tissues bathed under these conditions, removal of albumen caused an increase in C_b of 26% (mean of 5 bladders). In 5 bladders bathed in HCO_3^- -free Ringers, C_a was $3.82 \pm 0.87 \mu F/cm^2$, and H^+ transport was $8.1 \pm 1.2 \mu A/cm^2$. Addition of $50 \mu M$ serosal acetazolamide (AZ) reduced C_a to $3.48 \pm 0.82 \mu F/cm^2$, and reduced transport to $3.5 \pm 1.4 \mu A/cm^2$. Removal of AZ and addition of 24 mM HCO_3^- with 5% CO_2 increased C_a to $3.67 \pm 0.72 \mu F/cm^2$, and increased transport to $9.8 \pm 2.2 \mu A/cm^2$. Similar results were also found by varying transport by altering serosal $[HCO_3^-]$. Although these transport-related changes are small, they are consistent and similar to those predicted by morphometric analyses of fixed tissues.

Th-AM-Min3-4 SINGLE CHANNEL RECORDINGS FROM OCULAR EPITHELIA OBTAINED USING A PATCH VOLTAGE CLAMP. James L. Rae, Department of Physiology, Rush Medical College, Chicago, Illinois 60612.

By use of the technique of patch voltage clamping, it has been possible to resolve the currents through single ion channels in the apical membranes of the epithelium of the lens and the endothelium of the cornea of several species. The tissues require no enzymatic treatment to clean their surfaces and seals in the 10-300 G Ω range are routinely possible. The probability of obtaining gigaohm seals can be increased by choosing optimal tip geometries and by using specialty glasses such as Corning #7052 or #8161. For large seal resistances, the background noise is quite dependent on the electrode glass used with Corning #1723 and #7040 yielding particularly low noise. To date, more than 15 different ion channels (based on selectivity and/or single channel conductance) have been seen; 11 have been reasonably identified. They include a 440 pS-1.9nS channel non-selective for anions or cations, a 12 pS Na⁺ channel, a 4-7 pS Ca⁺⁺ channel and 3 non-selective cation channels with conductances of about 25, 50 and 100 pS. There is a diverse group of 5 different K⁺ channels with conductances of about 27, 40, 80, 130, and 230 pS determined with intracellular K⁺ concentrations on both sides of the channel. The 230 pS channel appears to be the "classical" Ca⁺⁺-activated K⁺ channel but the 130 pS channel also depends on internal Ca⁺⁺. With asymmetric K⁺ solutions on either side of the K⁺ channels, most of the channels rectify passing current more readily from the side on which the [K⁺] is highest. The channel density is high particularly in mammalian and avian species in which channel-free patches are rare. It is curious that to date, no Cl⁻ channels have been seen.

Th-AM-Min3-5 ELECTROPHYSIOLOGY OF EPITHELIAL PLASMA MEMBRANE VESICLES. Ernest M. Wright. Dept. Physiology, UCLA School of Medicine, Los Angeles, CA 90024.

Voltage-sensitive dyes have been used to study the ion permeability of renal brush borders. Membrane vesicles were prepared from the rabbit renal cortex by a standard Ca differential centrifugation procedure, and the electrical potential difference across the membranes of the vesicle suspension were measured using the voltage-sensitive cyanine dye 3,3'-dipropylthiadicarbocyanine iodide (diS-C₃-(5)) in a dual wavelength spectrophotometer. The absorbance signal was calibrated with K-diffusion potentials in the presence of the K-ionophore valinomycin and with Na-diffusion potentials in the presence of the Na-ionophore ETH 1097. Ion permeabilities were obtained from bi-ionic diffusion potentials and the constant field equation. At 22°C the permeabilities relative to Cl⁻ were: Br⁻(2.5) > NH₄⁺(1.5) > Li⁺(1.2) > Cl⁻(1.0) > K⁺ ~ Cs⁺ ~ Rb⁺(0.7) > Na⁺(0.3). Increasing the temperature to 38°C increased P_K⁺/P_{Cl}⁻ 4-fold. This increase was partially blocked (50-70%) with TEA but not TMA. The apparent inhibitor constant for TEA was approximately 10mM. These results demonstrate that renal brush border vesicles are perm-selective to ions, and that K may permeate via a temperature-sensitive, TEA blockable channel. Furthermore, the permeability data indicate that under physiological conditions, i.e. intra- and extra-cellular ion concentrations, the electrical potential across the membranes of these vesicles is about -30mV.

Th-AM-A1 RAPID PERFUSION OF EXCISED PATCHES - ACTIVATION AND DESENSITIZATION OF NICOTINIC RECEPTOR CHANNELS. James P. Dilger and Paul R. Adams. Department of Neurobiology and Behavior, State University of New York at Stony Brook, Stony Brook, NY 11794.

We have developed a technique for reversibly applying a known concentration change to an excised patch of membrane within 100 msec. We have used this method to apply agonists to outside-out patches of membrane from BC3H1 cells containing nicotinic receptor channels. When low concentrations of ACh are applied ($\lesssim 3 \mu\text{M}$) there is very little desensitization of the receptors within 200 msec. At higher concentrations of ACh, however, we detect the onset of a very fast desensitization process. For $30 \mu\text{M}$ ACh, this process occurs with a time constant of 200 msec; for $\geq 100 \mu\text{M}$ ACh, the time constant is 100 msec or less. Information on receptor activation has been obtained from examining the ensemble mean and variance of the current responses induced by repeated applications of known ACh concentrations. When no desensitization is present, the variance to mean ratio is independent of time. From binomial statistics, we expect the variance to mean ratio to be $i(1-p)$ where i is the single channel current and p is the probability of the channel being in the open state. For $3 \mu\text{M}$ ACh and -50 mV applied, $p \approx 0.1$. Using this probability and the ensemble mean current, we can calculate the number of activatable channels in the patch. We have obtained patches with as many as 200 channels. At higher concentrations of ACh, p decreases as the channels desensitize. The speed of the perfusion system limits our ability to calculate the value of p before the onset of desensitization. However, we estimate $p \approx 0.2$ for $10 \mu\text{M}$ ACh and $p \geq 0.4$ and 0.7 for 30 and $100 \mu\text{M}$ ACh respectively.

Th-AM-A2 ACTIVATION OF ACETYLCHOLINE RECEPTORS BY DIMETHYL-D-TUBOCURARINE. Sine, S.M. and J.H. Steinbach, Molecular Neurobiology Laboratory, The Salk Institute, La Jolla, CA.

We have examined activation of acetylcholine receptors (AChRs) by the classical antagonist, dimethyl-d-tubocurarine (dm-dTC), to gain further insight into the processes underlying channel opening and closing. AChRs on clonal BC3H-1 muscle cells were studied using high resolution single channel recording techniques. dm-dTC may be used to selectively occupy one binding site on the receptor oligomer because the two sites have widely separate affinities for dm-dTC ($K_1=0.3 \mu\text{M}$, $K_2=28 \mu\text{M}$; Sine and Taylor, *J. Biol. Chem.* **256**, 6692, 1981). Channel opening events were not detected in the presence of dm-dTC at concentrations that occupy only the high affinity binding site ($0.1-0.3 \mu\text{M}$). At $3 \mu\text{M}$ (fractional occupation: site 1 = 0.91; site 2 = 0.10) two classes of openings are observed, brief duration ($t_{\text{open}}=100 \mu\text{sec}$) and long duration ($t_{\text{open}}=10 \text{msec}$) in the ratio 1:1 (brief:long). Both duration classes have the same conductance. Long duration openings often appear in bursts of two or more openings separated by brief gaps. These brief gaps resemble closely the "nachsschlag" seen with all agonists and have a time constant of about $50 \mu\text{sec}$. At $30 \mu\text{M}$, (fractional occupation: site 1 = 0.99; site 2 = 0.52) brief and long duration openings are also seen, and the mean duration of long openings is 4-fold shorter. These observations suggest that binding of two antagonist molecules triggers both brief and long duration channel opening events. Since nachschlag appear so similar for agonists and dm-dTC they most likely result from coupling of the long open state to a second closed state common to all ligands that trigger channel opening. dm-dTC also blocks channels as evidenced by the reduction in open time seen at high concentrations. Supported by grants NS 13719, BNS-79-06013 and ONR contract N00014-79-C-0798, SNS is supported by a fellowship from the NIH. JHS was a Sloan Foundation Fellow.

Th-AM-A3 DIAZEPAM INCREASES GABA-ACTIVATED SINGLE CHANNEL BURST DURATION IN CULTURED MOUSE SPINAL NEURONS. Greg Redmann, Harold Lecar, and Jeffery Barker. Laboratories of Biophysics and Neurophysiology, NINCDS, National Institutes of Health, Bethesda MD 20205.

Single channel patch clamp recordings were made on cell-attached and excised membrane patches from neurons cultured for 2-6 weeks from the embryonic mouse spinal cord. Recordings were made in Hank's Basic Saline solution, which routinely contained TTX and 20mM TEA. Patch pipettes contained this solution plus $0.2-2.0 \mu\text{M}$ GABA, with or without $10 \mu\text{M}$ diazepam (DZM). In both cases, two kinetic populations of open and closed times occurred in most patches. Lifetime histograms were fit by double exponential functions to derive relative amplitude and time-constant values. The mean fast open time and mean slow open time were 2.8 ± 0.4 (SE) and 20 ± 2.4 ms in GABA patches, and were 2.8 ± 0.5 and 22 ± 5.3 ms in patches with GABA+DZM (N=5 in each case). Closed time distribution means were 3.8 ± 0.5 and 54 ± 24 ms for the GABA controls, and 3.0 ± 0.5 and 61 ± 16 for the patches with GABA+DZM. The amplitude ratio of the number of fast to slow events was 2.0 ± 0.2 for open times, and 1.3 ± 0.4 for the closed times in GABA, and 3.9 ± 1.1 and 5.1 ± 0.7 for GABA+DZM. The greatest effect of diazepam then, is to increase the relative number of fast closed events with respect to controls, which probably reflects increased re-openings, or bursts, of a liganded GABA receptor-channel complex. Calculated burst length, or the ratio of the summation of open and fast closed state times to the number of slow closed state times, was 26 ± 4 ms in GABA and 60 ± 7 ms in GABA+DZM. The single channel conductance for both fast and slow open states was the same, $28-30$ pS in symmetrical NaCl solutions, and was not affected by the addition of diazepam. (Supported by F32NS07044-01).

Th-AM-A4 THE MEASUREMENT OF THE RATE CONSTANTS FOR A COMPETITIVE ANTAGONIST OF THE NICOTINIC ACETYLCHOLINE RECEPTOR. M. E. Krouse, H. A. Lester, B. F. Erlanger* and N. H. Wassermann*, Division of Biology, California Institute of Technology, Pasadena, CA. 91125; *Dept. of Microbiology, Columbia University, College of Physicians and Surgeons, New York, N.Y. 10022.

We have used a photoisomerizable drug (2,2' Bis-Q) to investigate the interaction between a competitive antagonist and the nicotinic acetylcholine receptor of the *Electrophorus* electroplaque. The *cis*-isomer is more potent than the *trans*-isomer ($K_{cis} = 0.33 \mu M$, $K_{trans} = 1.0 \mu M$). Light is used to shift the mole fraction of *cis*-2BQ in solution near the membrane. Voltage-clamped cells are exposed to a solution containing a photostable agonist and 2BQ. Visible flashes increase the agonist-induced current; the relaxation has two exponential components. The faster component has the same characteristics as a subsequent voltage-jump relaxation, as both relaxations involve channel opening. UV flashes, which produce an increase in antagonist activity, cause a decrease in the number of open channels. This relaxation has a sigmoidal waveform. The delayed start arises because: (a) *cis*-2BQ does not bind to open channel receptors; and (b) some 2BQ molecules already bound to the receptor leave the binding site upon absorbing a photon allowing agonist molecules to bind and open new channels. When carbachol is the agonist this light-flash relaxation is at least 10 times slower than a subsequent voltage-jump relaxation. This slow relaxation is similar in time-course to the slower component of the visible flash relaxation. From the relation between [*cis*-2BQ] and the relaxation kinetics, the voltage- and agonist-independent binding rate constant of *cis*-2BQ is computed as $1.6 \times 10^8 M^{-1} s^{-1}$ ($20^\circ C$; $Q_{10} \sim 3$). This value typifies that estimated for agonists. [Supported by grant NS-11756].

Th-AM-A5 PATCH-CLAMP STUDIES OF ACh CHANNELS ACTIVATED BY PHOTOISOMERIZABLE AGONISTS. Lee D. Chabala, Alison M. Gurney, Henry A. Lester. Division of Biology, CalTech, Pasadena, CA. 91125.

We studied the reversibly bound and covalently bound ("tethered") photoisomerizable agonists, Bis-Q and QBr, at ACh receptors of cultured rat muscle ($15^\circ C$, $-100 mV$, $140 mM Cs^+/10 EGTA$ in the pipette). In whole-cell recordings, the *cis* isomers induce less than 5% the conductance of the *trans* isomers. The half-maximal concentration of *trans*-Bis-Q is greater than $2 \mu M$ (it is thus less than 1/10 as potent as at *Electrophorus* electroplaques); at higher concentrations, both Bis-Q isomers block channels, complicating dose-response studies. Light flashes from a xenon lamp modify the *cis/trans* ratio either of the Bis-Q molecules in solution near receptors or of the tethered QBr population. *Cis*-to-*trans* and *trans*-to-*cis* photoisomerizations increase or decrease the conductance, respectively; $\tau \sim 17$ (Bis-Q) and ~ 8 (QBr) msec. *Trans*-to-*cis* photoisomerizations of bound Bis-Q molecules produce transient decreases in the agonist-induced conductance ("phase 1"), revealing that the lifetime of the *cis*-Bis-Q channels once opened is $\sim 1/4$ that of the *trans*-Bis-Q channel.

In outside-out patches, single *trans*-Bis-Q ($100 nM$) channels show two lifetimes: ~ 1 and ~ 21 msec (relative frequencies $\sim 5:1$); tethered QBr channels show a dominant lifetime of ~ 5 msec. Channels are opened by repetitive flashes in the presence of $300 nM$ *cis*-Bis-Q. Summed flash episodes form a synthetic concentration-jump relaxation resembling the whole-cell data. The distribution of waiting times to the first opening has two components: an initial exponential ($\tau \sim 50$ msec) and a much slower phase (recovery from desensitization?). Supported by NS-11756, MDA (L.D.C.), Del E. Webb Foundation and Fulbright-Hays (A.M.G.).

Th-AM-A6 COVARIANCE ANALYSIS OF SINGLE CHANNEL CURRENTS FROM PURIFIED ACETYLCHOLINE RECEPTOR AND CHLORIDE CHANNELS IN PLANAR LIPID BILAYERS. P. Labarca†, J. Rice†, J. Lindstrom* and M. Montal†. University of California at San Diego† and The Salk Institute*, La Jolla, CA 92093.

Identification of the minimum number of ways in which open and closed states are entered and exited is a crucial step in defining the gating kinetics of multistate channels. We used certain covariance functions [1] to investigate the opening and closing kinetics of chloride channels and of purified acetylcholine receptor (AChR) channels in planar lipid bilayers. These covariance functions are conveniently computed from single channel current records and yield information on M, the minimum number of open entry states, open exit states, closed entry states and closed exit states. M gives a lower limit on the number of transition pathways between open and closed states. The chloride channel displays two open and two closed states. Covariance analysis of single channel currents shows that entrance into and exit from the open states occurs via a single pathway. This result and previous studies [2] indicate that the following scheme could account for the gating kinetics of the chloride channel: $C \rightleftharpoons C_1 \rightleftharpoons O_1 \rightleftharpoons O_2$ (C's and O's are closed and open states). The AChR displays two open states and several (>3) closed states [3]. Covariance analysis shows that $M \geq 2$ for the receptor channel and, therefore, entrance into and exit from open and closed states proceeds via at least two pathways. Similar conclusions were reached with a different analysis of AChR channels *in situ* [4]. Thus, these covariance functions provide a simple and general strategy to obtain information on channel kinetics. [1] Fredkin, et al., Proc. Neyman-Kiefer Mem. Symp., L. Le Cam, ed. (1983); [2] Miller, C., Phil. Trans. R. Soc. Lond., 299:401-411 (1982); [3] Labarca, et al., J. Neurosci. (in press); [4] Jackson, et al., Biophys. J., 42:109-114 (1983). Supported by MDA, NIH and DAMR.

Th-AM-A7 VOLTAGE DEPENDENCE OF 1-GLU INDUCED CURRENT IS DUE TO GATING BY Mg IONS. °Nowak, L.M., °Ascher, P., °Bregestovski, P., °Herbet, A. & °Prochiantz, A. °Neurobiologie, Ecole Normale Supérieure and °Neuropharmacologie, Collège de France, Paris, France. (Intr. by R.T. Kado)

N-methyl-D-aspartate (NMDA) receptors, a class of excitatory amino acid receptors in mammalian CNS (Watkins & Evans, 1981, *Ann.Rev.Pharmacol.* 21:165), trigger responses that have two unique properties: 1) the current-voltage (I-V) curve has a region of negative slope conductance, and 2) the response is reduced by low concentrations of extracellular magnesium ions (Mg^{++}). We have analyzed these responses by the patch clamp method using the whole cell and outside-out recording modes (Hamill et al., 1981, *Pflügers Arch.*, 391: 85) on cultured mesencephalon neurons from 13 day old fetal mice (Beaujouan et al., 1982, *Mol.Pharmacol.*, 22: 48). Recordings were made in Ringer solution containing varying amounts of $MgCl_2$. Patch pipettes contained (in mM): 140 CsCl, 4 NaCl, 5 K-EGTA/0.5 $CaCl_2$ (pCa 7.3) and 10 K-HEPES (pH 7.2). The agonists 1-glutamate (1-GLU) and NMDA were used.

In Mg^{++} -free Ringer the I-V relations for 1-GLU activated whole cell current and single channel currents were linear and the currents reversed polarity at 0mV. Reducing $[Cl^-]_o$ did not change the reversal potential or the single channel conductance (49 ± 8 pS; 20-25°C). Addition of Mg^{++} (10 μ M-1mM) reduced 1-GLU evoked currents at negative potentials but not at positive potentials. At the single channel level, Mg^{++} induced: 1) rapid, brief, closings that interrupted the open state, and 2) a reduction of the frequency of channel opening. These results are consistent with the hypothesis that 1-GLU activates cationic channels, which Mg^{++} blocks in a voltage dependent way.

Th-AM-A8 MODULATION OF POST-TETANIC POTENTIATION OF QUANTAL RELEASE OF TRANSMITTER AT THE FROG NEUROMUSCULAR JUNCTION. Stanley Mislner, Depts. of Medicine and Physiology and Biophysics, Washington Univ., Sch. of Med., St. Louis, MO. 63110

At many neuromuscular junctions repetitive stimulation of the motor nerve, under conditions where the quantal content (m) of the endplate is low, results in large increases in both m and F, the frequency of miniature endplate potentials, which develop and decay along parallel but complex time courses. Recently we, [Mislner and Hurlbut, *P.N.A.S.* 80: 315, 1983] reported (1) that a component of post-tetanic potentiation (PTP) of m and F, often lasting for minutes, can be observed in frog cutaneous pectoris muscles after several minutes of repetitive stimulation (at 5-20 Hz) in Ca^{2+} -free Ringer, if Ca^{2+} is restored after the tetanus and (2) that component of PTP is greater and more prolonged the longer the post-tetanic (K^+)_o. We suggested that much of PTP may be due to post-tetanic accumulation of Ca^{2+} _i, due to Ca^{2+} _o - Na^+ _i exchange. Na^+ _i was postulated to accumulate during the tetanus when Na^+ - K^+ pump activity might not keep pace with accelerated Na^+ entry. The following new data are consistent with, though not definitive for, Na^+ _i- Ca^{2+} _o exchange.

A. Divalent cation independence: (1) After stimulation in the absence of Ca^{2+} _o, F is steeply dependent on $[Ca^{2+}]_o$ restored post-tetanicly. (2) Sr^{2+} _o but not Mg^{2+} _o can replace Ca^{2+} in sustaining PTP of F. (3) Concentrations of Sr^{2+} _o and Ca^{2+} _o which give similar resting values of m and F yield similar degrees of PTP of m and F. **B. Effect of presumed modulation of Na^+ pump activity:** (1) Brief accumulation with 10⁻⁶ M acetylcholinesterase reversibly augments PTP of m and F prior to affecting resting transmitter release. (2) PTP is substantially augmented if K^+ _o is substituted with Li^+ , but only modestly augmented if K^+ _o is substituted with Rb^+ . (Supported by MDA).

Th-AM-A9 ON THE ONSET AND TERMINATION OF NEUROTRANSMITTER RELEASE. H. Parnas^x, J. Dudel^{xx} and I. Parnas^x. ^xNeurobiology, Hebrew University, Jerusalem Israel. ^{xx}Institute of Physiology, Technical University, Munich, Germany.

Transmitter release was studied by graded depolarization at the crayfish opener muscle. Two pulses were applied (in the presence of TTX). The first, the prepulse varied in amplitude and duration, the second, the test pulse was kept constant. Facilitation is defined here as the ratio of the test pulse response after the prepulse to that of the test pulse alone. The short term facilitation (F_c) and the duration of facilitation (T_F) both reflect the amount of Ca that entered at the prepulse. It was found that as the prepulse depolarization increased the quantal content of the prepulse increased while the F_c and T_F first increased but later decreased with strong prepulse depolarization. Thus a discrepancy between Ca entry and transmitter release at different depolarizations is demonstrated. When the terminal is loaded with Ca^{++} by repetitive stimulation, a small depolarization pulse that by itself released 0.008 quanta per pulse, if placed 17ms after the train showed a quantal content of 9, a facilitation of ~1600. Thus, the rapid termination of release after the train, could not result from rapid sequestration of Ca.

The time course of evoked release was determined directly by a histogram of synaptic delay following an action potential or a depolarizing pulse. Termination of release seems to occur via the repolarization-induced cessation (with a certain time constant) of processes that were activated during depolarization.

We found that the onset and termination of release are directly controlled by depolarization and are independent of changes of intracellular Ca concentration.

Th-AM-A10 INTERACTION OF CELL SURFACE ACETYLCHOLINESTERASE WITH THE CYTOSKELETAL FRAMEWORK OF EMBRYONIC MUSCLE CELLS IN CULTURE. Anthony F. Ross and Joav M. Prives (Intr. by Susan H. Gilbert). Department of Anatomical Sciences, State University of New York at Stony Brook, NY 11794.

To study the role of the cytoskeletal framework in the expression of cell surface acetylcholinesterase (AChE) during myogenesis in culture we have used a mild detergent extraction that removes lipids and soluble proteins and preserves internal structure, and we have measured the partitioning of AChE activity between the cytoskeletal framework and the soluble detergent-extracted phase. We have observed that a minor (~30%) proportion of total cellular AChE activity is retained on the detergent insoluble cytoskeletal framework in newly fused myotubes, and that this proportion increases with maturation. As AChE is distributed in the cytoplasm as well as on the cell surface, we have used pharmacological agents to distinguish cell surface from intracellular AChE. To selectively measure surface AChE activity, the irreversible inactivator diisopropyl fluorophosphate was used to block intracellular AChE activity while the surface enzyme was protected by the reversible inhibitor BW28451. To selectively measure intracellular AChE, the enzyme on the external surface of intact myotubes is inactivated by echothiophate iodide, an irreversible AChE inhibitor that does not penetrate the cell membrane. We have found that during myotube development the proportion of cell surface AChE retained on the cytoskeletal framework increases markedly. In contrast, only a minor proportion (~30%) of intracellular AChE is retained on the cytoskeletal framework at all stages of development. These results suggest the participation of the cytoskeletal framework in the developmental expression of cell surface AChE.

Th-AM-B1 TWO PROTEINS THAT SEVER F-ACTIN CAUSE AN INCREASE IN STEADY STATE NONFILAMENTOUS ACTIN CONCENTRATION. L. Wang, P. Silverman, and J.A. Spudich, Department of Structural Biology, Stanford University School of Medicine, Stanford, California 94305. (Intr. by L.M. Griffith)

Severin, a 40,000 dalton protein from *Dictyostelium*, and a newly purified 45,000 dalton protein isolated from unfertilized sea urchin eggs have been shown to sever F-actin in the presence of μM Ca^{2+} . Experiments using either a nonhydrolyzable ATP analogue or ADP reveal that the severing activity is not dependent on the hydrolysis of ATP. In addition, both sedimentation and fluorescence energy transfer experiments indicate a net disassembly of actin filaments and an increase in the steady state nonfilamentous actin concentration. The effect of severin and the 45,000 dalton protein on the rise of nonfilamentous actin is substoichiometric. For example, addition of the 45,000 dalton protein to F-actin (1 mg/ml) in a 1:100 ratio causes an increase in the nonfilamentous actin concentration from 30 $\mu\text{g/ml}$ to 300 $\mu\text{g/ml}$. Experiments with severin give similar results. The nonfilamentous actin (designated HSS-actin) generated by these proteins is capable of repolymerization. This was demonstrated by adding 10-fold G-actin to fluorescently labeled HSS-actin (0.3 mg/ml) and observing a depletion of fluorescent actin from the supernatant after high speed centrifugation. Energy transfer experiments also show that HSS-actin repolymerizes on its own but at a much slower rate than the same concentration of G-actin. We are currently investigating the mechanism for the increase in HSS-actin and are exploring the possibility that there is a chemical modification of the actin molecules. (NIH GM 25240 to JAS)

Th-AM-B2 ANALYSIS OF ACTIN POLYMERIZATION: FRAGMENTATION VS. OTHER MECHANISMS. Marilyn F. Bishop and F. A. Ferrone, Department of Physics, Drexel University, Philadelphia, PA 19104.

We have recently developed a perturbation method for analyzing nucleation-controlled polymerization that is augmented by a secondary pathway for polymer growth. [Bull. Am. Phys. Soc. 28, 402 (1983)] We have used this approach to analyze the data of Wegner and Savko [Biochemistry 21, 1909 (1982)], who followed actin polymerization in the presence of Mg, Ca, and K. They found that a mechanism that assumes fragmentation gave good agreement with their data for Mg and Ca, and was not required for the K data. In our analysis, the nature of the secondary pathway need not be assumed, but appears as a result. We find that, although fragmentation reproduces most of the systematics of the data, a mechanism of lateral growth onto existing polymers produces even better agreement for the Mg data. Our method also determines the size of the homogeneous nucleus independent of assumptions about the secondary mechanism. In agreement with Wegner and Savko, we find that the nucleus size depends on the salt; however, we obtain nucleus sizes that differ from theirs. For Mg we obtain 2.92 ± 0.11 (vs. 4), for Ca we obtain 4.66 ± 0.11 (vs. 4), and for K we obtain 3.54 ± 0.13 (vs. 3).

Th-AM-B3 PHALLOIDIN AFFECTS ACTIN ASSEMBLY AT THE NONPREFERRED END OF ACTIN FILAMENTS. L.M. Coluccio and L.G. Tilney (Intr. by C.F. Armstrong) Dept. of Biology, Univ. of Penn., Philadelphia, PA 19104.

Incubation of the isolated acrosomal bundles of *Limulus* sperm with skeletal muscle actin results in assembly of actin onto both ends of the bundles. These crosslinked bundles of actin filaments taper allowing one to distinguish directly the preferred end for actin assembly from the nonpreferred end; the preferred end is thinner. Incubation of the bundles with actin in the presence of phalloidin in a 1:1 molar ratio in 100 mM KCl, 1 mM MgCl_2 , and 0.5 mM ATP at pH 7.5 results in nearly identical association rate constants at the preferred end as in the absence of the drug (0.45 $\mu\text{m/min}\cdot\mu\text{M}$ vs. 0.46 $\mu\text{m/min}\cdot\mu\text{M}$). This is also true for the dissociation rate constants at this end (0.033 $\mu\text{m/min}$ vs. 0.045 $\mu\text{m/min}$; con. vs. exp.). Consequently, the critical concentrations at the preferred ends are very similar (0.07 μM vs. 0.10 μM ; con. vs. exp.). There is also no change in the rate constant of association at the nonpreferred end in the presence of phalloidin (0.044 $\mu\text{m/min}\cdot\mu\text{M}$ vs. 0.049 $\mu\text{m/min}\cdot\mu\text{M}$; con. vs. exp.); however, we find the dissociation rate constant at the nonpreferred end to be one-eighth that of the control (0.047 $\mu\text{m/min}$ vs. 0.006 $\mu\text{m/min}$). Moreover, we find the critical concentration at the nonpreferred end to be 1.07 μM whereas in the presence of phalloidin, it is reduced to 0.13 μM . Dilution-induced depolymerization at the nonpreferred end is considerably slower in the presence of phalloidin. Thus, phalloidin has no effect on the association or dissociation rate constant or the critical concentration at the preferred end, but has a marked effect on the critical concentration at the nonpreferred end, not by increasing the rate constant of association but as a consequence of decreasing the dissociation rate constant at that end. NIH grant 14474 to LGT and MDA Postdoc Fellowship to LMC.

Th-AM-B4 FITTING THE ACTIN MONOMER INTO THE ACTIN HELIX: A COMBINATION OF X-RAY DIFFRACTION AND ELECTRON MICROSCOPE DATA R.Mendelson***, W.Gebhard*, K.Holmes*, W.Kabsch*, D.Suck**, J.Couch***, E.Morris*** and E.O'Brien*** Max Planck Institute* and European Molecular Biology Laboratory**, Heidelberg, F.R.G. and Medical Research Council Cell Biophysics Unit, King's College, London, U.K.***

The structure of the actin:DNaseI complex has been determined to 0.6nm resolution (Suck *et al.*, 1981, P.N.A.S. 78, 4319) and that of DNaseI alone to 0.2nm (Suck, 1983, E.M.B.O. J., submitted). Subtraction of the DNaseI from the map of the complex yields the actin monomer at 0.6nm, showing it as kidney-shaped, being elongated and divided into two unequal domains. The longest dimension is about 6.7nm and the shortest about 3.0nm. The domains are joined by a narrow neck, in the region of which ATP appears to be bound. The N-terminus is in the small domain. An elongated shape divided roughly into two domains is indicated in three-dimensional reconstructions computed from electron micrographs of paracrystals of filaments of actin + tropomyosin (O'Brien *et al.*, 1983, in "Actin, its structure and function in muscle and non-muscle cells", Academic Press, Sydney). The reconstructions also show tropomyosin well resolved as a continuous column of density which can be subtracted to provide F-actin alone, at a resolution of about 3.5nm.

A systematic analysis by computer graphics of the possible ways of fitting the actin monomer into the F-actin reconstruction indicates four general orientations. In each case the long dimension of the monomer lies approximately normal to the helix axis. The large domain may be either at low or high radius and may point up or down. The criteria used to assess the models are: fit to the e.m. data, cross-sectional radius of gyration, extent of inter-subunit contacts, and low-angle X-ray data from oriented fibres. The most favoured solution has the large domain at high radius.

Th-AM-B5 MODIFICATION OF LYS-237 ON ACTIN BY 2, 4-PENTANEDIONE: EVIDENCE FOR SEVERAL FUNCTIONAL SITES FOR TROPOMYOSIN ON ACTIN.

SALEH C. EL. SALEH*, RICHARD THIERET⁺, PETER JOHNSON⁺ AND JAMES D. POTTER*. Dept. of Pharmacology and Cell Biophysics, Univ. of Cincinnati, College of Med., Cincinnati, OH 45267 and ⁺The Dept. of Chemistry and College of Osteopathic Med., Ohio Univ., Athens, Ohio 45701.

Modification of actin at Lys-237 with 2, 4-pentanedione reversibly blocks the protein's ability to bind tropomyosin (Tm) at high ionic strength in the absence of Ca²⁺ and Mg²⁺. In the presence or absence of Ca²⁺, the binding of Tm to this modified actin did not inhibit actin-activated HMM-Mg²⁺-ATPase activity. However, under the same conditions, Tm binding to unmodified actin caused a 55% inhibition of the ATPase activity. In the presence of Mg²⁺ and in the absence of Ca²⁺ the binding of Tm to control actin, in the presence of Tn (-Ca²⁺ + Mg),

greatly inhibited the ATPase activity (80%). However, under identical conditions, using modified actin, the ATPase activity was slightly activated (133%) rather than inhibited. When Ca²⁺ was added to the above systems the ATPase activation by modified actin in the presence of Tm and Tn was slightly enhanced (145%) as compared to 100% activation using control actin under equivalent conditions. These results can be explained in terms of the ability of the actin molecule to assume conformations that can provide several states for the Tm-Tn complex that affect actin-myosin interaction. These may include: a blocking, non-blocking (active) and activated states. Supported by grants from the Muscular Dystrophy Assoc (P J), the NIH (HL22619, 3A (J P) and by NIH training grant (HL 07382 to S.C.E.).
*Present Address: Pharmacology, U. of Miami, R-189, P.O. Box 016189, Miami, FL 33101.

Th-AM-B6 FLUID DYNAMICAL SIMULATION OF CONTRACTILE ACTIN NETWORKS, Micah Dembo, Theoretical Division, Los Alamos National Laboratory, Los Alamos, New Mexico 87545

If appropriate simplifying assumptions are made, then it is possible to derive a pair of equations for the density (A) and velocity (V) of a contractile actin network [see Dembo *et al.* (1984), in: *Cell Surface Phenomena: Concepts and Models* (eds. A. Perelson *et al.*) Marcel Dekker, NY]. Letting μ , C and D be the coefficients of viscosity, contractility and solvent drag respectively, then for one dimensional motion these equations take the form

$$\partial_t A = - \partial_x AV + R(A) \quad (1a)$$

$$\text{and} \quad 0 = \partial_x \mu A \partial_x V + CA \partial_x A - DAV \quad (1b)$$

where R(A) is a function giving the rate of chemical assembly (or disassembly) of the actin network.

Numerical simulations indicate that solutions of Eqs. (1a) and (1b) consist of a number of localized regions of high actin density towards which actin flows under the influence of contractile forces. The continued accumulation of actin at a localized contractile center is counterbalanced by the chemical reaction of network disassembly. In numerical simulations, contractile centers are seen to arise due to local instabilities, grow for a time and eventually annihilate by interacting with larger centers. Depending on the initial conditions, the boundary conditions and the parameter values, the interactions between contractile centers can give rise to steady, periodic and even chaotic behavior. At least on a qualitative level, the dynamics of contractile centers seem to provide a straightforward and unified account of many seemingly unrelated aspects of amoeboid motion.

Th-AM-B7 INHERENT FLEXIBILITY OF TROPOMYOSIN MOLECULES. George N. Phillips, Jr., Dept. of Physiology and Biophysics, Univ. of Illinois, Urbana, IL 61801.

Models for thin filament regulation invoke movements of tropomyosin relative to actin. But it now seems unlikely that tropomyosin moves in an "all-or-none" fashion from an off to an on state (Wegner and Walsh, *Biochemistry*, 1981). Instead, Ca^{++} sensitive aspects of troponin-tropomyosin interactions seem to shift the equilibrium distributions of tropomyosin strands on actin. It is therefore essential to establish the inherent flexibility of tropomyosin to see if such models are realistic in terms of mechanical properties of the molecule.

New three-dimensional X-ray crystallographic data have been collected from rabbit skeletal tropomyosin at two temperatures, 0° and 15°C. The results confirm preliminary two-dimensional studies (Phillips, Fillers, and Cohen, *Biophys. J.*, 1980) that indicated significant flexibility even at 0°, and extend those findings to allow estimates of the flexibility of two 105-125 Å long stretches of tropomyosin at physiological temperatures. In the crystal, the N-terminal segment shows temperature independent 5 Å RMS deviations from the equilibrium position, and the C-terminal segment shows deviations of 7 Å at 0°, 7.7 Å at 15° and estimated RMS deviations of 9 Å at 37°. These results can be interpreted to suggest that for the C-terminal half of the molecule, even if only one tropomyosin-actin contact were to be broken, that "unhitched" segment could spend up to 25% of its time 10 Å away from the equilibrium position. The deviations for the N-terminal half of the molecule would be about half as large, but still considerable.

GNP is an Established Investigator of the American Heart Association.

Th-AM-B8 FLUORESCENCE STUDIES OF THE CONFORMATION OF RABBIT SKELETAL TROPOMYOSIN BOUND TO F-ACTIN. Y. Ishii and S.S. Lehrer, Department of Muscle Research, Boston Biomedical Research Institute, Boston, Ma. 02114.

The fluorescence spectrum of rabbit skeletal tropomyosin labeled with pyrene maleimide (PTm) consists of an excimer (E, 485nm) and a monomer band (M, 385nm) (Betcher-Lange and Lehrer, 1978, *JBC*, 253, 3757). E originates from pyrenes at Cys190 of each chain that can interact only if the coiled-coils locally separate. Effects of increasing temperature, T, on E were interpreted as being due to a shift in equilibrium from a localized chain-closed state to a chain-open state in a pretransition, prior to the main transition where the chains completely dissociate (Graceffa and Lehrer, 1980, *JBC*, 255, 11296). Addition of excess F-actin (F) to PTm in 0.03M NaCl, 5mM MgCl₂, pH 7.5, in the pretransition T region, produced a decrease in the E/M ratio and the F+Tm spectra vs. T showed that the pretransition was shifted 10°-15° up, indicating that F stabilizes the closed state. To correlate these spectral changes with known perturbers of Tm stability, effects of ethanol (which stabilizes) and high pH (which destabilizes) on E and M were studied. Ethanol addition (10%) to PTm alone at pH 7.5 decreased E/M and shifted the pretransition 5°-10° up; increasing the pH to 8.5 increased E/M and shifted the pretransition 5° down. In all these studies the isomissive point (440nm) did not change indicating that these additions shifted the pretransition equilibria without directly affecting the fluorescence characteristics of the pyrene probe. For F+PTm, this suggests that the stabilizing effect of F is due to a shift in equilibrium to the closed state rather than a direct interaction with the Cys190 region of Tm. Thus, since F-actin stabilizes the closed state, resulting in less localized unfolding near physiological T, the flexibility of Tm is reduced when bound to F-actin. (Supported by NIH HL22461 and the MDA).

Th-AM-B9 ARCHITECTURE OF TITIN-CONTAINING CYTOSKELETAL MATRIX IN STRIATED MUSCLE -- MAPPING OF DISTINCT EPITOPES OF TITIN SPECIFIED BY MONOCLONAL ANTIBODIES, K. Wang and R. Ramirez-Mitchell, Clayton Foundation Biochemical Institute, Department of Chemistry, and Cell Research Institute, The University of Texas, Austin, Texas 78712.

Titin, a giant myofibrillar protein (~10⁶ M₀ subunit) abundant in striated muscles, has been proposed as a component of a set of cytoskeletal filaments which coexists with thick and thin filaments within the sarcomere (K. Wang in *Crossbridge Mechanisms in Muscle Contraction*, 1983, Plenum Press). Due to its size and antigenic complexity, sarcomeric localization data obtained with conventional polyclonal antibodies to titin have been difficult to interpret in detail (PNAS 76, 3698 (1979)). We have now prepared a library of monoclonal antibodies directed to four distinct epitopes of rabbit titin. The distribution of these epitopes along the length of isolated titin molecules is being studied by electron microscopy of shadowed titin-antibody complexes. In parallel experiments, these epitopes are being mapped in sarcomeres of various lengths by immunocytological techniques at both light and electron microscopy levels. It is likely that a detailed comparison of epitope distribution on the isolated molecule and in the sarcomere may provide structural data essential for a deeper understanding of the architecture and dynamics of the cytoskeletal matrix. (Supported by NIH AM20270 and The American Heart Association, Texas Affiliate, Inc.)

Th-AM-B10 CHARACTERIZATION OF CA BINDING SITES OF TROPONIN-C IN CHEMICALLY SKINNED CARDIAC MUSCLE FIBERS. Pan B.* and Solaro R.J. University of Cincinnati, College of Medicine, Cincinnati, OH 45267.

Chemically skinned muscle cells were prepared from porcine ventricular trabeculae by extraction in a lubrol/glycerol solution. Bound Ca was measured with a double-isotope technique. The troponin-C (TNC) content of the fibers was 0.4 nmol/mg fiber protein as determined by quantitative gel electrophoresis. At 0 mM Mg the fibers in rigor bound 2.4 nmol Ca/mg protein at pCa 5, which could be accounted for in terms of the divalent metal binding sites of TNC and myosin. At 2 mM Mg the Ca titration curve was shifted downwards with a bound Ca of 1.4 nmol/mg at pCa 5, i.e., slightly more than 3 mol Ca/mol TNC. A smaller downward shift was observed upon further increase of Mg to 10 mM. These findings are in agreement with biochemical evidence that there are two Ca binding sites per mole myosin, which have high affinity for Mg, and three Ca binding sites per mole TNC, at least two of which bind Mg with low affinity. The binding data were further analyzed using a nonlinear least-squares curve fitting program. The Ca binding parameters of the three sites of TNC were: $K_1=K_2=1.2 \times 10^7 M^{-1}$, $K_3=5.1 \times 10^5 M^{-1}$ at 2 mM Mg; $K_1=3.6 \times 10^6 M^{-1}$, $K_2=2.6 \times 10^6 M^{-1}$, $K_3=2.5 \times 10^5 M^{-1}$ at 10 mM Mg. Experiments were also conducted in which the bound Ca and force were measured simultaneously in the presence of ATP. An increase of Mg from 2 to 10 mM had an effect on Ca titration of TNC similar to that seen with fibers in rigor. This was accompanied by a 0.5 pCa unit shift of the pCa-tension relation to lower pCa's. The relation between Ca occupancy of TNC and force development showed a steep rise in slope at about 2 mol bound Ca/mol TNC and did not vary with Mg concentrations.

Th-AM-B11 BIOCHEMICAL CHARACTERIZATION OF META-VINCULIN, A 150KD PROTEIN IMMUNOLOGICALLY RELATED TO VINCULIN. Janet D'Angelo Siliciano and Susan W. Craig. Department of Physiological Chemistry, The Johns Hopkins School of Medicine, Baltimore, Md. 21205

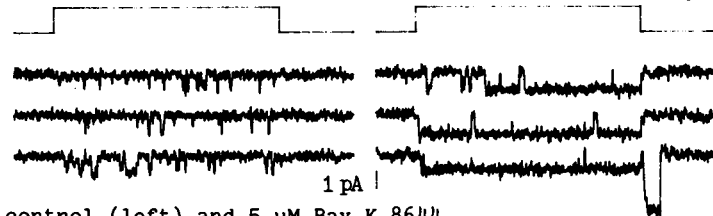
We have shown previously that in chicken gizzard smooth muscle there is a 150kd protein, designated meta-vinculin, that is antigenically related to the 130kd actin-binding protein vinculin. We have examined the structural basis for the immunological relatedness between these two proteins using both one-dimensional and two-dimensional peptide analysis. One-dimensional peptide analysis reveals that vinculin and meta-vinculin share chymotryptic peptides of identical electrophoretic mobility in SDS gels and that these shared peptides are cross-reactive with affinity purified anti-vinculin antibodies, indicating domains of homology between the two proteins. Two-dimensional tryptic, chymotryptic, and *S. aureus* V8 protease peptide maps of immunoprecipitated, ¹²⁵I-labelled vinculin and meta-vinculin reveal that: 1. tryptic peptide maps of vinculin and meta-vinculin are identical, 2. both *S. aureus* V8 protease and chymotryptic peptide maps show that meta-vinculin contains all the tyrosine containing peptides found in vinculin but in addition has several unique peptides. The results from the peptide mapping suggested there might be a precursor:product relationship between vinculin and meta-vinculin. To determine if one of the molecules is processed to the other with time, embryonic gizzard smooth cell cultures were pulsed with ³⁵S-methionine. The label was then chased with cold methionine for various lengths of time. The results of these experiments indicate that vinculin is not processed to yield meta-vinculin and that meta-vinculin is not proteolytically processed to vinculin. This information, together with the peptide map data suggests that vinculin and meta-vinculin are synthesized from separate mRNAs.

Th-AM-C1 DIHYDROPYRIDINE DERIVATIVES: VOLTAGE-DEPENDENT MODULATION OF CALCIUM CHANNEL CURRENT. M.C. Sanguinetti and R.S. Kass, Department of Physiology, University of Rochester Medical Center, Box 642, Rochester, New York 14642.

We have studied the influence of membrane potential on block and potentiation of Ca channel current in calf cardiac Purkinje fibers by dihydropyridine (DHP) compounds. In these experiments outward currents were reduced by tetrabutylammonium injection and membrane current was measured with a two microelectrode arrangement. We find that use-dependent block develops in the presence of nisoldipine (200 nM) when pulse frequency exceeds .5 Hz, and that block is more pronounced when pulses are applied from depolarized (>-40 mV) holding potentials. These effects of nisoldipine (pKa < 3.5), brought on by the neutral form of the drug, contrast with the actions of verapamil (pKa = 8.7) because they do not require repetitive activity to develop. Instead, the effects of voltage on nisoldipine block can be brought on by changing holding potential, resulting in a 20 mV hyperpolarizing shift of the apparent steady-state inactivation curve for Ca channel current. This result suggests an interaction between this dihydropyridine and the inactivated state of the Ca channel. The effects of voltage on block by nifedipine (pKa = 7.0) are intermediate to nisoldipine and verapamil. Potentiation of Ca channel current by the DHP partial agonist, Bay K 8644 (200 nM) is also voltage sensitive. In this case, enhancement of current is observed when pulses are applied from negative holding potentials. But when holding potentials are more positive than -40 mV, these effects are less pronounced, and even indicate block. These results show that membrane potential, and perhaps inactivation of the calcium channel, is pivotal in the regulation (block or enhancement) of calcium channel current by DHP derivatives.

Th-AM-C2 MODULATION OF SINGLE CALCIUM CHANNELS BY THE CALCIUM AGONIST BAY K 8644. P. Hess, J. B. Lansman and R. W. Tsien, Department of Physiology, Yale University, New Haven, CT 06510.

Calcium agonists are a new class of drugs that increase cardiac contractility even though they are 1,4-dihydropyridines like nifedipine and other Ca channel blockers. We studied effects of an exemplar Ca agonist, Bay K 8644, on Ca channels in single guinea pig and frog ventricular cells. At a test potential of +10 mV with 10 mM Ca or Ba outside, 1 μ M Bay K 8644 very rapidly produces a 3-4 fold increase of the peak inward Ca channel current. Half-maximal effects are obtained at 50-100 nM drug. The enhancement is greatest at weak depolarizations, and decreases progressively as the potential becomes more positive. In the presence of Bay K 8644 the peak current occurs earlier, the current during the pulse decays more rapidly and inward tail currents deactivate more slowly. Nonstationary fluctuation analysis suggests that the predominant mechanism of Bay K 8644 is to



control (left) and 5 μ M Bay K 8644
110 Ba in pipette
140 msec pulse from RP-40 to RP+110 mV

increase the probability for a channel to be open. This is confirmed in cell-attached patch recordings of single Ca channels: (1) the probability of opening is increased and there are fewer sweeps with no openings, (2) channel openings last longer on average, and the distribution of open times shows more than one exponential component, suggesting the presence of more than one conducting state and (3) closed times and latency-to-first opening are abbreviated.

Th-AM-C3 A NIFEDIPINE-DERIVATIVE (BAY k 8644) THAT INCREASES Ca CURRENTS IN MYOCARDIAL CELLS: A NOVEL POSITIVE INOTROPIC AGENT. C.J. Cohen and M. Chung, Miles Inst Preclin Pharm, New Haven, CT.

Bay k 8644 is a nifedipine-derivative with positive inotropic activity (Schramm et al., Nature 303:535, (1983)). The drug causes a dose-dependent increase in the action potential duration of calf ventricular muscle and Purkinje fibers. Effects on APD are eliminated by nisoldipine, which specifically blocks Ca channels. The interaction of Bay k 8644 with Ca channels in calf Purkinje fibers was studied using the 2 microelectrode voltage clamp technique. Current through Ca-activated channels was minimized by intracellular injection of Cs and replacement of extracellular Ca and K by Sr and Cs. BAY k 8644 increases I_{Sr} and alters the time- and voltage-dependence of channel opening. The greatest percent increase in ionic current occurs for weak depolarizations. For strong depolarizations, like those occurring during a cardiac action potential, I_{Sr} is most enhanced immediately after the voltage step. Bay k 8644 acts directly on Ca channels because increases in I_{Ca} mediated by changes in Ca_i do not occur when Sr is the charge carrier. The drug also differs from catecholamines, which do not alter the time course of I_{Ca} . Bay k 8644 increases a slow tail current (I_{tail}) following repolarization ($\tau_{off} \approx 0.3s$). I_{tail} is also increased by: 1) more positive test pulses over the voltage range that increases Ca channel opening; 2) longer test pulses in proportion to $\int I_{Sr} dt$. I_{tail} is not proportional to instantaneous g_{Ca} . Nisoldipine blocks I_{tail} and peak I_{Sr} with the same potency. I_{tail} is not a Sr-activated transient inward current because: 1) reduced Na_o changes the amplitude but not the time course of I_{tail} ; 2) changes in Mg_o have no effect; 3) the amplitude increases monotonically as the repolarization voltage is made more negative. Sr apparently slows the rate of deactivation of some Ca channels.

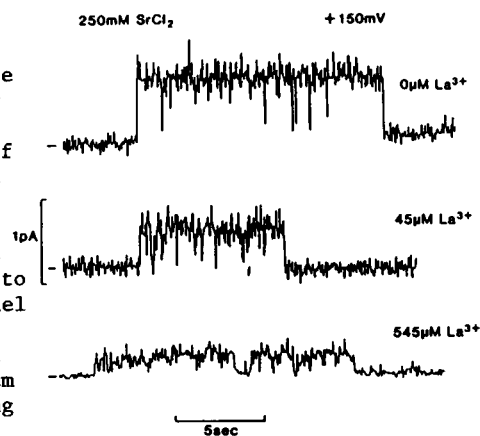
Th-AM-C4 TEMPERATURE AND PHARMACOLOGICAL REGULATION OF H³-NITRENDIPINE BINDING TO CANINE CARDIAC SARCOLEMMAS: EFFECTS OF BEPRIDIL AND DILTIAZEM. Joseph Balwierczak, Ingrid Grupp, Gunter Grupp, & Arnold Schwartz, Department of Pharmacology and Cell Biophysics, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267

Certain calcium channel blocking drugs (CCB) can regulate the binding of the dihydropyridine (DHP) class of the calcium antagonists, which includes nitrendipine (NTD) and nimodipine (NMD). It has been shown that d-cis diltiazem (DTZ) stereospecifically increases the binding of H³-NTD to membrane sites. Depending upon the tissue source and temperature, this effect is due to either a change in the affinity (Yamamura *et al.*, BBRC 108: 640, 1982) or a change in the maximum number of binding sites (B_{max}) (DePover *et al.*, BBRC 108: 110, 1982). At a temperature of 37°C, we have found that both DTZ and another CCB whose structure is unrelated to DTZ, bepridil (BEP), stimulates DHP binding to canine cardiac sarcolemma (SL) and that this effect is due to an increase in B_{max}. We attempted to relate the stimulatory effects of these two drugs to a pharmacological action in rat hearts perfused by the Langendorff procedure. DTZ and BEP significantly decrease the IC₅₀ of the negative inotropic action of NMD by about 30 and 10 fold, respectively. The kinetics of binding of H³-NTD to SL was measured at 37°C. The association rate constant (k₊₁) and dissociation rate constant (k₋₁) were found to be 0.44 min⁻¹ nM⁻¹ and 0.22 min⁻¹, respectively. The value for k₊₁ was also measured at 2°, 10°, 20°, and 30° C. A decrease in temperature, which must affect the kinetics of calcium channel gating and may affect the availability of binding sites, seems to cause an increase in the B_{max} of DHP binding. This increase is similar to that seen by DTZ and BEP. Supported by NIH Training Grant T32 HL 07382-07 and PO1 HL 22619-06.

Th-AM-C5 REDUCTION OF SINGLE CALCIUM CHANNEL CURRENTS BY LANTHANUM AND CADMIUM.

Mark T. Nelson, Depts Physiol. & Biophys., University of Maryland, Baltimore, MD 21201

Single calcium channels from rat brain membrane vesicles were incorporated into planar lipid bilayers (Nelson, 1983, Soc. Neurosci. Abstr. 9, 508). When the membrane vesicles were added to one ("cis") side of the bilayer, the channels usually oriented in the bilayer such that cis-positive voltages caused the channel to open. Lanthanum and cadmium, potent blockers of macroscopic calcium currents in many preparations, reduced the current through single calcium channels in a dose dependent manner (Figure 1). In the presence of 250 mM SrCl₂, 0.15 mM lanthanum or 9.4 mM cadmium in the cis side reduced the single channel current by 50%. In contrast, addition of cadmium (up to 20 mM) to the trans side had little effect on the single channel current. Voltage (+100 mV to +150 mV) did not affect the relationship between cadmium concentration in the cis-side and single channel conductance. These results suggest that cadmium or lanthanum can block macroscopic calcium currents by reducing the single channel conductance. (Supported by the American Heart Ass. & U.S. Army Res. Dev. Cont.).



Th-AM-C6 CHANGES IN CELL CAPACITANCE USED TO MEASURE EXOCYTOSIS ARE PREVENTED BY THE CALMODULIN INHIBITOR, TRIFLUOPERAZINE. David Clapham and Erwin Neher, Max-Planck-Institut für biophysikalische Chemie, D-3400 Göttingen, F.R.G.

Using patch clamp techniques, exocytosis was studied in single chromaffin cells maintained 1-5 days in culture. The effect of the calmodulin inhibitor, trifluoperazine (TFP), on vesicle fusion events was observed by measuring evoked increments in cell capacitance from whole cells (Neher & Marty 1982). The effect of TFP was also examined on Na, Ca, and ACh channels.

TFP in concentrations up to 10 μM had no effect on Na channel currents. TFP was a potent anticholinergic agent which slowly blocked or allosterically modified the ACh channel. TFP concentrations of 0.1 to 1 μM decreased net inward ACh currents in 0.5 or 20 μM ACh and was only slowly reversible. Recovery from desensitization was prolonged in direct proportion to [TFP]. Single ACh channel kinetics were analyzed. Whole cell calcium currents (I_{Ca}) were reduced in 10 μM TFP by ~50%.

Capacitance increments of 1-200 fF were elicited by voltage clamp steps from rest (-70 mV) to +10 mV in 1 mM [Ca]_o but were absent in "zero" [Ca]_o. The size of the steps were roughly dependent on I_{Ca}. No capacitance steps were observed in 10 μM TFP at levels of I_{Ca} that elicited large capacitance steps in controls. Thus TFP was shown to block exocytosis independent of its actions on Ca or ACh currents.

Neher and Marty (1982) PNAS USA 79, 6712-6716.

(Supported by Deutsche Forschungsgemeinschaft and a Fulbright award (DEC)).

Th-AM-C7 ³H-NIMODIPINE (NIM) AND ³H-NITRENDIPINE (NTD) BINDING TO RABBIT SKELETAL T-TUBULAR MEMBRANES: EFFECTS OF D-cis-DILTIAZEM (DTZ) AND ATTEMPTS TO SOLUBILIZE AND AFFINITY LABEL THE PUTATIVE CALCIUM CHANNEL. T.L. Kirley and A. Schwartz, Department of Pharmacology and Cell Biophysics, University of Cincinnati, Cincinnati, Ohio 45267.

High receptor density for dihydropyridine (DHP) Ca²⁺ antagonists NIM and NTD appears to be in skeletal muscle t-tubules - about 50 pmol/mg @ 10°C (Fosset *et al.*, *J. Biol. Chem.* 258, 6086, 1983). However, in our preparations, B_{max} varies from 5-15 pmole/mg. For NTD, K_D = 1.8 ± 1.0 nM @ 10°C and 4.04 ± 0.67 nM @ 37°C. Contrary to Fosset *et al.*, binding is stimulated by DTZ @ 10°C and 37°C, primarily due to an increase in B_{max} (1.3- and 1.6-fold @ 10° and 37°C, respectively). For NIM, K_D = 2.59 ± 0.13 nM and 3.52 ± 0.60 nM @ 10° and 37°C, respectively. DTZ increases B_{max} 1.5- and 1.9-fold @ 10° and 37°C. DTZ also stimulates by decreasing K_D @ 37°C (but not @ 10°C). The density of NIM and NTD binding sites is very similar.

Solubilization of DTZ-stimulable receptors was effected by sodium deoxycholate. Affinity labeling reveals a 40 Kd protein. Calsequestrin binds DHPs but is not the high affinity binding protein.

Supported by NIH Training Grant HL 07382-07 and P01 HL 22619-06.

Th-AM-C8 INHIBITION OF SECRETION FROM A PITUITARY CELL LINE BY DIHYDROPYRIDINES: EVIDENCE FOR A MEMBRANE POTENTIAL DEPENDENT BLOCKADE OF CA⁺⁺ CHANNELS. John J. Enyeart and Patricia M. Hinkle (Intr. by S.-S. Sheu). Department of Pharmacology, University of Rochester, Rochester, NY 14642.

In a variety of muscle cells, dihydropyridine (DHP) Ca⁺⁺ antagonists potently block Ca⁺⁺ flow through voltage-sensitive channels. Many endocrine cells possess Ca⁺⁺ channels of this kind which function in depolarization-stimulated secretion. We compared several DHPs with respect to their ability to inhibit agonist and depolarization-dependent secretion from a clonal line of pituitary cells (GH₄C₁). These cells respond to thyrotropin-releasing hormone (TRH) with large increases in the number of mainly Ca⁺⁺ dependent action potentials, and in the amount of prolactin secreted. Co⁺⁺ suppresses both of these responses. We found that the DHPs nifedipine, nisoldipine, and nimodipine at concentrations ranging from 1 to 1250 nM had little or no effect on basal or TRH-stimulated prolactin secretion. Depolarizing the cells with 20 or 50 mM KCl produced 3- to 5-fold increases in secretion. This response was completely inhibited by the DHPs at concentrations as low as 10 nM. We also found that the tritiated DHP, [³H]nitrendipine, bound specifically to GH₄C₁ membrane fragments at nanomolar concentrations. The [³H]nitrendipine was displaced by the DHPs at concentrations comparable to those that blocked KCl-induced secretion. These results indicate that the DHPs can function as potent Ca⁺⁺ antagonists in an endocrine cell line. They further suggest that the effectiveness of these agents in blocking Ca⁺⁺ currents depends strongly on the resting membrane potential, and increases dramatically at depolarized levels. This might account for the large disparity between the DHP concentrations required for physiological effects in whole cells, and the dissociation constants determined from radioligand binding to membrane fragments.

Th-AM-C9 EFFECT OF ENKEPHALINS ON A PROTOZOON'S LIGHT AVOIDANCE. J. K. Randolph, Physics Department, University of Tulsa, Tulsa, OK 74104 (Intr. by H. A. Pohl).

The effect of neurohormones on the light avoiding reaction of a protozoon (*Stentor Coeruleus*) was studied. The avoiding reaction itself, a stop in swimming followed by resumed swimming to the side, is known to involve an action potential with Ca²⁺, K⁺ and perhaps Cl⁻ currents¹. (The absorption of light is known to be followed by proton release from pigment granules².)

The probability of light avoidance was measured by watching protozoa with a 15x stereo-microscope. The dim levels of background light caused very few stop reactions. A strobe light was triggered when a protozoa was observed swimming smoothly and quickly toward the lamp. Responses were scored as positive even if the stop was not followed by swimming to the side. With these precautions ambiguous responses were only 2-6% and were excluded from the probability. Controls with unexposed protozoa from the same culture were always run within the hour. The role of Ca²⁺ in the reaction was first verified with the Ca²⁺ blocker nifedipine (J. K. Randolph, to be published). At micromolar concentrations light avoiding probability was reduced to values in the 20%-50% range compared to a control's 88%. Results with two neurohormones known to affect calcium transport were

		Probability	Control	σ
Met-enkephalin	2.0 μM	65%	96%	8.0%
Leu-enkephalin	1.0 μM	82%	87%	6.0%

(supported in part by the Research Office of The University of Tulsa).

1. D. C. Wood, *J. Comp. Physiol.* 146, 537-550 (1982).

2. P. S. Song, D. P. Hader, and K. L. Poff, *Arch. Microbiol.* 126, 181-186 (1980).

Th-AM-C10 CHARACTERIZATION OF CALCIUM CHANNELS FROM PARAMECIUM CILIA IN A PLANAR LIPID BILAYER.

B.E. Ehrlich, A. Finkelstein, M. Forte*, C. Kung#. Department of Physiology and Biophysics, Albert Einstein College of Medicine, Bronx, NY, *Department of Biology, Case Western Reserve University, Cleveland, OH, and #Laboratory of Molecular Biology, University of Wisconsin, Madison, WI.

Vesicles made from Paramecium cilia can be incorporated into a planar lipid bilayer. We chose Paramecium for these studies for four reasons: 1) Ca⁺⁺ currents in Paramecium and in a variety of vertebrate and invertebrate cells are similar, 2) Paramecium can be manipulated genetically and biochemically, 3) data from electrophysiological and behavioral studies can be used to compare with our results, and 4) it is possible to prepare large quantities of pure ciliary membranes, the only membrane in which Ca⁺⁺ channels exist in Paramecium. We have shown previously that Paramecium cilia contain two types of divalent cation channels (Ehrlich et al., Biophys. J. 41:293a, 1983). Although in both channels anions and monovalent cations are virtually impermeant, these channels differ in size, divalent ion selectivity, and voltage-dependence. The larger (30 pS) channel does not select among the divalent cations and is only weakly voltage-dependent. In the smaller (2 pS) channel, Ca⁺⁺ and Ba⁺⁺ can be current carriers, but Mg⁺⁺ is virtually impermeant. This channel is also strongly voltage-dependent: we see an e-fold change in membrane conductance for a 9 mV change in membrane potential. The voltage-dependent parameter may be the probability of channel opening since the mean open time does not vary significantly with voltage. From these results and studies using vesicles from mutants with altered Ca⁺⁺ currents, we suggest that the smaller channel is responsible for the Ca⁺⁺ current measured in intact Paramecium.

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Th-AM-D1 T-TUBULE MEDIATED CALCIUM RELEASE FROM SARCOPLASMIC RETICULUM IN VITRO OCCURS AT PHYSIOLOGICAL RATES. Noriaki Ikemoto, Bozena Antoniu and Do Han Kim. Dept. of Muscle Res., Boston Biomed. Res. Inst.; Dept. of Neurol., Harvard Med. School, Boston, MA 02114

A heavy microsomal fraction of rabbit skeletal muscle, which is enriched in the T-tubule/SR complexes, was incubated with 0.15M K.gluconate and then mixed with 0.15M choline Cl to produce membrane depolarization. The time course of depolarization induced Ca^{2+} release is biphasic as determined with a dual beam stopped flow spectrophotometer and with arsenazo III as Ca^{2+} indicator at 27°. In the initial phase of Ca^{2+} release, which takes place after lag phase of about 50 ms, 15-20 nmol Ca^{2+} per mg SR protein is released rapidly ($k_1=88-140\text{s}^{-1}$); and in the second phase 30-80 nmol Ca^{2+} per mg is released at a slower rate ($k_2=0.5-1.0\text{s}^{-1}$). Upon dissociation of the T-tubule from SR by a French press treatment, the rapid Ca^{2+} release as well as slow release were diminished. Re-association of the T-tubule/SR complex by incubation in 0.42M K.cacodylate completely restored the rapid Ca^{2+} release, while slow Ca^{2+} release was partially restored. These results suggest that the rapid Ca^{2+} release is triggered via depolarization of the T-tubule. The slow phase is probably induced by the Ca^{2+} released in the rapid phase. The rate constant of rapid Ca^{2+} release is on the same order as that expected for Ca^{2+} release *in vivo* (e.g. $t_{1/2}$ of the tension development in the electrically stimulated twitch muscles is in the range of 5-25 ms). T-tubule depolarization determined by the potential-sensitive dye NK2367 trapped within T-tubules occur in the initial 6 ms of the lag phase which precedes rapid Ca^{2+} release. It is proposed that the remaining 44ms of the lag phase represents the time required for the signal transmission from the T-tubule to SR. Supported by grants from NIH (AM16922) and MDA.

Th-AM-D2 INHIBITION OF THE T TUBULE + SARCOPLASMIC RETICULUM LINKAGE BY ANTI T TUBULE MONOCLONAL ANTIBODIES. Mario S. Roseblatt, Ralph W. Nelson and Noriaki Ikemoto. Dept. of Muscle Res., Boston Biomed. Res. Inst.; Dept. of Neurol., Harvard Med. School, Boston, MA 02114

We have performed several successful fusions using the isolated T-tubule membranes and one using SR membranes as immunogens, and obtained a total of 46 clones producing anti-T-tubule monoclonal antibodies (MoAbs) and 8 anti-SR MoAbs. Of those cell lines, at least five react preferentially with T-tubule antigens (lines 5/25.7, 5/49.7, 5/131.9, 5/137.1 and 5/140.8). In order to investigate whether any of the protein components of the T-tubule membrane are involved in the T-tubule/SR linkage, T-tubule and SR fractions isolated from rabbit skeletal muscles were incubated in 0.42 M K.cacodylate to re-associate the T-tubule/SR complex (Casswell et al, J. Biol. Chem., 254, 202, 1979), in the presence and absence of the T-tubule specific MoAbs described above. The degree of the T-tubule/SR re-association was determined by separating free T-tubules by centrifugation in a discontinuous sucrose density gradient. It was found that the first three of the MoAbs listed above inhibit the T-tubule/SR re-association. None of the anti-SR antibodies had any effect on re-association. Screening of antigenic components of the purified T-tubule for these MoAbs is in progress using the blotting technique. It appears that the 35k and 42k Dalton proteins of the T-tubule are the candidates for such components. These results suggest that some of the molecular components of the isolated T-tubule are involved in the linkage between the T-tubule and SR. Supported by grants from NIH (AM16922, HL27229) and MDA.

Th-AM-D3 TEMPERATURE-DEPENDENCE OF THE MOLECULAR DYNAMICS OF BOTH THE CALCIUM PUMP PROTEIN AND LIPID IN SARCOPLASMIC RETICULUM MEMBRANES. Thomas C. Squier, Diana J. Bigelow, and David D. Thomas, Dept. Biochem., Univ. of Minn. Med. School, Minneapolis, MN 55455.

In sarcoplasmic reticulum (SR) breaks (changes in slope) in the Arrhenius plot of the Ca-ATPase activity have been clearly demonstrated. A correlation of this functional T-dependence with the T-dependence of physical properties may provide clues about the mechanism of calcium transport. We have used conventional and ST-EPR to measure the rate of rotational mobility ($1/\tau_C$) of both the calcium pump protein and the lipid in native sarcoplasmic reticulum. Previous attempts to measure the rotational mobility of the calcium pump protein using ST-EPR were analyzed with lineshape parameters, which have proven unreliable at higher temperatures in SR due to interference from weakly immobilized probes. We have demonstrated that, unlike lineshape parameters, saturation and intensity parameters reject the contribution of weakly immobilized probes, and thus measure large-scale molecular motions unambiguously. Using these parameters we observe that the overall rotational mobility ($\log 1/\tau_C$) of the calcium pump protein varies in a linear manner with $1/T$, demonstrating no breaks in an Arrhenius plot. An abrupt change in oligomerization or a large conformational change would change the apparent rotational correlation time ($\log 1/\tau_C$) of the calcium pump protein in a discontinuous manner. This result is inconsistent with models in which an abrupt change in the oligomeric state or a large conformational change occurs with increasing temperature (Hoffman et al., 1979, PNAS 76: 5860, Kirino et al., 1980, J. Biochem. 88: 1837). Similar studies were undertaken on the lipid component using stearic acid spin labels in both SR and SR lipids.

Th-AM-D4 EFFECT OF GLYCEROL ON RABBIT CARDIAC SARCOPLASMIC RETICULUM. A.L. Jacobson, University of Calgary, University Biochemistry Group, Calgary, Alberta, Canada T2N 1N4.

Differential Scanning Calorimetry has been used to study transitions of SR in 0.125M NaCl at pH 7.5. Both heating and cooling scans have been studied. On first heating transitions centered at 20, 34, 47 and 61°C are observed. On cooling transitions at 7, 17, 25 and 32°C are apparent. On reheating only 1 transition is observed (22°C). On recooling hysteresis is marked and transitions are observed at 9, 17 and 28°C. In the presence of 50% v/v glycerol transitions at 15, 37, 60 and 71°C are observed on first heating and at 25°C on reheating. The hysteresis on recooling is not observed and transitions are centered at 9 and 18°C. The (Ca²⁺)-ATPase is specifically activated by glycerol. The (Mg²⁺)-ATPase is unaffected by glycerol. Glycerol specifically affects both the lipid and protein thermal transitions of cardiac SR. Glycerol appears to specifically affect a specific portion of the (Ca²⁺)-ATPase.

Th-AM-D5 IDENTIFICATION OF CANDIDATE COMPONENTS FOR THE CALCIUM RELEASE CHANNEL OF SARCOPLASMIC RETICULUM USING RUTHENIUM RED AS A PROBE. Do Han Kim and Noriaki Ikemoto. Dept. of Muscle Res., Boston Biomed. Res. Inst.; Dept. of Neurol., Harvard Med. School, Boston, MA 02114

Ruthenium red (RR) inhibits several types of Ca²⁺ release induced by a) increase in extravesicular [Ca²⁺] b) addition of caffeine or quercetin, and c) ionic substitution which produces membrane depolarization, with an apparent $k_d = 0.2 \mu\text{M}$. Since these different types of Ca²⁺ release are induced by different triggering mechanisms, the inhibitory effect of RR is presumably due to blocking of Ca²⁺ release channels. In an attempt to identify the molecular components responsible for the channel function, RR binding was determined by measurements of A₅₃₃ after separating bound and free RR by centrifugation. SR fractions which showed the highest Ca²⁺ release activity (cf. Kim, Ohnishi and Ikemoto, J. Biol. Chem. 258, 9662, 1983) had two classes of RR binding sites (high affinity, $k_d = 0.3 \mu\text{M}$, $n = 0.7 \text{ nmol mg}^{-1}$; low affinity, $k_d = 1 \mu\text{M}$, $n = 4 \text{ nmol mg}^{-1}$). RR binding to the high affinity class, not to the low affinity class, parallels inhibition of Ca²⁺ release functions. The high affinity class binding was not observed in the partially purified Ca²⁺ ATPase preparation nor in the purified T-tubule fraction. Specific RR binding roughly doubled in a sedimentable fraction of the SR membrane after centrifugation of the membranes partially solubilized with C₁₂E₈ (2.0 mg detergent/mg protein). Several proteins (M_r range: 20-50k, 200-300k) are enriched in this fraction, but the 100k dalton Ca²⁺ ATPase is virtually absent. These results suggest that the molecular components of SR other than the Ca²⁺ ATPase protein are responsible for the Ca²⁺ release channel functions. Supported by grants from NIH (AM16922) and MDA; D.H.K. is a postdoctoral fellow of MDA.

Th-AM-D6 EFFLUX of ⁴²K FROM SINGLE, SKINNED SKELETAL MUSCLE FIBERS. Philip M. Best, Brian Masters, Carla Abramcheck, Department of Physiology, University of Illinois, Urbana, IL 61801.

The efflux of ⁴²K from single, skinned (sarcolemma removed) muscle fibers was measured. Loading (250 mCi/mole K) and washout (no isotope) solutions contained 120 mM K⁺, 1 mM MgATP, 2 mM Mg²⁺, 10 mM EGTA, 16 mM MOPS (pH = 7.4 at 10°C) and 40 μM Quercetin. Mathematical fits to the efflux curves were generated using a non-linear least squares routine. Control experiments showed that multiple washouts obtained from the same fiber under identical conditions gave identical kinetics. Therefore, three washout curves were obtained from each fiber. The first acted as a control. During the second washout the fiber was exposed to 120 μM Decamethonium, an SR K channel blocker. The third washout followed a 30 min treatment with 0.5% Triton X-100 to destroy intracellular membranes. In all cases (n=5) the control and decamethonium washout curves were best described (chi-squared analysis) as the sum of two exponentials. Under control conditions the rate constant (k) of the fast components was $.35 \pm .01 \text{ sec}^{-1}$ while that of the slow component was $.026 \pm .008 \text{ sec}^{-1}$. The compartment of the fast component was about 90% of the total fiber volume while that for the slow component was about 6% of the fiber volume. Decamethonium treatment had no effect on the fast component but reduced the slow component by about half compared with the control value. After triton treatment the efflux data was best described by a single, fast exponential decay. Fibers loaded with ¹⁴C-sucrose also showed only a single, fast component of washout. Assuming the slow component of ⁴²K efflux under control conditions arises entirely from isotope crossing the SR membrane and that $V_{SR} = 0$, the permeability of the SR can be calculated to be approximately 10^{-7} cm/sec . Supported by MDA and NIH (AM 32062).

Th-AM-D7 STIMULATED ^{45}Ca EFFLUX IN SKINNED MUSCLE FIBERS: ATP DEPENDENCE OF Ca^{2+} -INSENSITIVE COMPONENT. Elizabeth W. Stephenson, Dept. of Physiology, UMDNJ-NJ Med. Sch., Newark, NJ 07103

Stimulation of ^{45}Ca efflux from skinned twitch fibers by depolarizing ionic gradients at constant ion product is Ca^{2+} dependent, but a small component is present in 5 mM EGTA (Biophys. J. 41:231a, 1983). The present studies show that ATP removal inhibits this Ca^{2+} -insensitive stimulation, and prevents its potentiation by quercetin (Proc. IUPS XV:156, 1983), a bioflavonoid thought to stabilize phosphorylation of the SR Ca pump. ^{45}Ca efflux and isometric force were measured on segments of frog semitendinosus fibers skinned by microdissection. The fibers were loaded with buffered ^{45}Ca , rinsed, and exposed to ATP-free (or control) solutions containing K methanesulfonate (KMes) and 0.1 mM EGTA for 90 s; in ATP-free solution, rigor force developed and stiffness increased. After increasing EGTA to 5 mM, fibers were stimulated by choline Cl replacement of KMes and efflux followed for 1 min; residual ^{45}Ca was extracted in Triton X-100 + 5 mM CaEGTA. In 5 mM ATP, stimulated fibers released 12% fiber ^{45}Ca compared to 6% in control fibers (KMes). In zero ATP_0 , control release increased slightly but not significantly, while stimulated release decreased significantly ($P < 0.01$); stimulation above control loss fell from 6.0% fiber ^{45}Ca ($P < 0.01$) in ATP to 1.75% ($P < 0.05$) in zero ATP_0 . In ATP, 100 μM quercetin increased stimulated release from 11% fiber ^{45}Ca to 23%, but in zero ATP_0 release was at control levels. The ability of the non-hydrolyzed analogue AMPPCP to support stimulation is under study. These results imply that activation of the channel mediating this Ca^{2+} -insensitive component of stimulated efflux requires phosphorylation or nucleotide binding. Supported by NIH Grant AM R01 30420.

Th-AM-D8 MEMBRANE CRYSTALS OF Ca^{2+} -ATPase IN SARCOPLASMIC RETICULUM OF FAST AND SLOW SKELETAL AND CARDIAC MUSCLES. Laszlo Dux and Anthony Martonosi, Dept. of Biochemistry, SUNY Upstate Medical Center, Syracuse, New York 13210.

Crystalline arrays of Ca^{2+} transport ATPase develop in sarcoplasmic reticulum (SR) membranes after treatment with Na_3VO_4 in a calcium free medium (Dux, L., and Martonosi, A., (1983), J. Biol. Chem. 258, 2599, 10111, 11896, 11903). Vanadate promotes crystallization by interaction with the Ca^{2+} -ATPase in the E_2 conformation. The proportion of vesicles containing Ca^{2+} -ATPase crystals in microsome preparations isolated from rat muscles of different fiber types (semimembranosus, levator ani, extensor digitorum longus, diaphragm, soleus, and heart) correlates with the Ca^{2+} -ATPase content and Ca^{2+} -modulated ATPase activity. This implies that the concentration of Ca^{2+} -ATPase in sarcoplasmic reticulum membranes of fast and slow skeletal or cardiac muscles differs only moderately, and the low Ca^{2+} transport activity of "sarcoplasmic reticulum" preparations isolated from red skeletal and cardiac muscles is largely due to the presence of large amount of non-SR membrane elements. This is in accord with the relatively small differences in the density of 85 Å intramembranous particles seen by freeze-etch electron microscopy in sarcoplasmic reticulum of red and white muscles. The dimensions of the Ca^{2+} -ATPase crystal lattice are similar in SR membranes of different fiber types; therefore if structural differences exist between "isoenzymes" of Ca^{2+} -ATPase these are not reflected in the crystal-lattice. (Supported by research grants from the NIH (AM 26545) and the Muscular Dystrophy Association.)

Th-AM-D9 HALOTHANE-INDUCED Ca^{2+} RELEASE FROM SARCOPLASMIC RETICULUM SUBFRACTIONS ISOLATED FROM RAT SKELETAL MUSCLE. Troy J. Beeler, Department of Biochemistry, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814.

Sarcoplasmic reticulum vesicles were separated into low-density, high-density and triad-enriched fractions. The low- and high-density sarcoplasmic reticulum vesicles were prepared from the microsomal fraction (10,000 g - 53,000 g cut) while the triad-enriched fraction was prepared from the crude mitochondrial fraction (1500 g - 10,000 g cut). The triads were separated from the mitochondrial and myofibril contaminants by centrifugation on Percoll and sucrose gradients. The high-density vesicles are believed to be derived from the terminal cisternae. The triad-enriched fraction contained terminal cisternae vesicles with the transverse tubule membrane still attached. Halothane at clinically significant concentrations (>0.5 mM) initiated Ca^{2+} release from the high-density and triad-enriched fractions but did not influence Ca^{2+} accumulation by the low-density sarcoplasmic reticulum vesicles which are believed to be derived from nonjunctional sarcoplasmic reticulum. The concentration of Halothane which gave half maximal Ca release was 60 μM . Halothane-induced Ca^{2+} release required ATP and was inhibited by Mg^{2+} (10 mM) and ruthenium red (5 μM). The data suggest that Halothane-induced Ca^{2+} release occurs at specific sites on the sarcoplasmic reticulum membrane and is not due to a generalized alteration of the membrane by Halothane. The site involved in Halothane-induced Ca release may also be involved in Ca^{2+} release during excitation-contraction coupling. Supported by NIH (GM-29300) and ONR.

Th-AM-E1 SEARCH FOR A CODE OF DNA SEQUENCE RECOGNITION BY PROTEINS. Allen T. Ansevin, Physics Department, University of Texas System Cancer Center, Houston, Texas 77030.

A catalog was prepared of permissible, double hydrogen bond contacts between amino acid side chains and base pairs exposed in the wide groove of B DNA. Observations made with a CPK, space-filling model were compiled for each of the 16 arrangements of base pairs taken two at a time. The amino acid side chains considered were only those capable of making at least two hydrogen bonds with properly disposed donors or acceptors. The major feature of the catalog was the high degree of degeneracy in H-bonding capacity, even for bidentate contacts. For instance, arginine appeared to have no value for specificity since contacts were sterically possible with every set except AA:TT and TT:AA. Glutamine and asparagine made centrally located contacts with all but TA:TA, TC:GA, CG:CG, and GC:CG. Good specificity was found only for the possible interaction of charged aspartic acid or glutamic acid with the set CG:CG. Although histidine appeared capable of recognizing most cytosines, an effective recognition of base sequences by proteins would require a simultaneous identification of two or more distinguishing features of base pair sets. This almost certainly would depend highly on steric hindrance (e.g., thymine methyl) and other positional features of DNA structure.

The observation of greatest single interest is that the C5 methylation of cytosine should interfere specifically with histidine binding. It is suggested that this provides the mechanism for an important control system of higher organisms as well as the inhibition of restriction enzymes, often seen in prokaryotes.

Th-AM-E2 CHICK BRAIN MICROTUBULE PROTEINS INTERACT SPECIFICALLY WITH SATELLITE DNAs OF CHICKEN, MOUSE AND *D. MELANOGASTER*. K.A. Marx, T. Denial and T. Keller, Department of Chemistry, Dartmouth College, Hanover, NH 03755.

Chick brain microtubule protein interacts with ³⁵S labelled tracer DNA from higher organisms as measured by an equilibrium nitrocellulose filter binding assay. A highly ionic strength dependent interaction between the MAP protein fraction and DNA has been demonstrated in this system. Increasing concentrations of competitor *E. Coli* DNA successfully compete away all but 1-3% of ³⁵S chicken, mouse or *D. melanogaster* tracer DNAs in the microtubule binding system. When challenged by homologous unlabelled competitor DNA, all filter binding was abolished. In general, heterologous higher organism competitor DNAs showed effects intermediate between *E. Coli* and the homologous DNA competitor. However, the best competitor in all cases was chicken DNA. This result suggests that the interaction under study occurs *in vivo* and that MAPs and their high affinity DNA sequences may have co-evolved.

The high affinity mouse, chicken and *D. melan.* ³⁵S tracer DNA sequences bound in *E. Coli* competitor excess were isolated from nitrocellulose filters. Reassociation kinetic analysis showed all three fractions to be highly enriched in repetitive DNA sequences. Satellite DNAs were found in CsCl gradients to comprise a major fraction of the ³⁵S tracer DNAs isolated from all three organisms.

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Th-AM-E3 UNFOLDING OF MONONUCLEOSOMES BY INTERCALATING LIGANDS, Kenneth S. Schmitz and Mei Lu, Department of Chemistry, University of Missouri-Kansas City, Kansas City, Missouri 64110

Chromatin exists in a repeat unit structure, where the repeat unit consists of DNA tightly wrapped about the surface of an octameric histone complex. We have used optical absorbance and sedimentation methods to examine the effect of proflavin on the conformation of mononucleosome units having 147 base pairs of DNA (core particle) and 192 base pairs of DNA (nucleosome). The results of these studies suggest a two-stage unfolding process, as summarized below; for particles with "excess DNA".

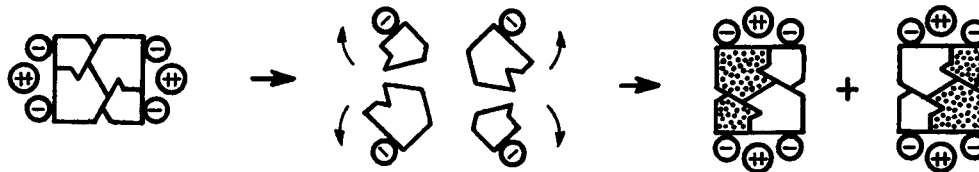
CONFORMATION	tight, compact	"end DNA" absorbs proflavin	completely unfolded structure
LIGAND/PHOSPHATE	0	< 0.02	> 0.02 (core) > 0.04 (nucleosome)
S _{20,w}	10.5-11.0	~ 9.5 (nucleosome)	6.0-7.0

Th-AM-E4 CHROMATIN ORGANIZATION OF A MOUSE INTERSPERSED REPEATED DNA SEQUENCE. Nancy Lynn Rosenberg and Douglas L. Vizard, M. D. Anderson Hospital & Tumor Institute, 6723 Bertner Avenue, Houston, Texas 77030.

The major interspersed DNA repeat of the mouse genome, MiF-1, is characterized by a sequence that is segmentally heterogeneous, but, nevertheless is distinguished by a highly conserved length 6 Kbp. Cytological studies have shown that the MiF-1 family is late replicating compared to the total DNA (Vizard and Rosenberg). To explain this finding experiments were performed to determine if MiF-1 was associated with a specific chromatin structure. Using a combination of DNP (deoxy-ribonucleoprotein) electrophoresis and hybridization techniques we have shown MiF-1 to be associated with a higher order nucleoprotein structure.

Th-AM-E5 A CHECKING MECHANISM FOR REPLICATION FIDELITY R.C. Hopkins, Univ. of Houston-Clear Lake Houston, TX 77058

The probability of occurrence of base tautomers suggests that base substitutions should occur in DNA once per 10^4 - 10^5 base pairs replicated, yet in eukaryotes the rate is about 10^6 - 10^7 times lower. A checking mechanism which explains this extreme fidelity comes from a proposal that four-stranded DNA may occur in nature (R.C. Hopkins, *Comments Mol. & Cell. Biophys.*, in press) as a consequence of an alternative structure for DNA [*Science* 211, 289 (1981); *J. Theor. Biol.* 101, 327 (1983)]. Shown below is a specifically paired tetrad from four-stranded DNA, with divalent cations in the minor grooves. During replication, the strands are unpaired (e.g., by enzymatic sequestering of cations and repulsion of the anionic chains). Each strand is replicated, and two daughter tetraplexes are formed. Each new tetraplex has had 3 opportunities for checking: during replication of each of its two parent strands and during the final pairing of the homologous base pairs. Thus, the high overall fidelity in vivo (3 steps) is consistent with one-step error rates of one in 10^3 - 10^4 for replication and repair enzymes in vitro. Supported by Robert A. Welch Foundation Grant E-889.

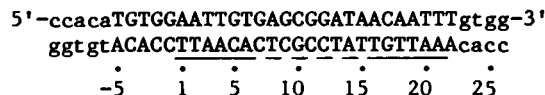


Th-AM-E6 PICOSECOND FLUORESCENCE DECAY TIME MEASUREMENTS OF NUCLEIC ACIDS AT ROOM TEMPERATURE IN NEUTRAL AQUEOUS SOLUTION. S. Georghiou*, Thomas N. Nordlund†, and A. M. Saim*,* Biophysics and Chemical Physics Lab., Department of Physics, University of Tennessee, Knoxville, TN 37996-1200 and †Departments of Radiation Biology-Biophysics and Physics, University of Rochester, Rochester, NY 14642. (Intr. by W. S. Riggsby).

Understanding the nature of the excited states of nucleic acids is a prerequisite for elucidating the mechanisms of action of UV radiation on cells. We report room temperature picosecond fluorescence decay time measurements for calf thymus DNA methylated at position N-7 of G and for its corresponding fluorophore 7-methyl-GMP as well as for nonmodified DNA. The samples were excited with single, 30 ps, 265 nm laser pulses from a frequency-quadrupled Nd:YAG laser with a repetition rate of 1 Hz. Fluorescence was detected with a streak camera-optical multichannel analyzer system that has a time jitter ≈ 2 ps. For methylated DNA, the major component has a decay time of 20 ps. A minor component has a decay time of 80 ps and makes a contribution of 3-20% depending on the transmission characteristics of the emission filters employed. For 7-methyl-GMP, the decay profile is single exponential with a decay time of 200 ps. There is more uncertainty in the measurements for DNA due to its very low fluorescence quantum yield and the very fast decay. Nevertheless, we detected a major component that has a decay time of 8 ± 5 ps. The absence of a component of negative amplitude in the decay profiles points against excited-state complex formation. The implications for transfer of excitation energy along the helix will be discussed. Supported in part by American Cancer Society grant IN-89H, NSF grants PCM-83-02601 and PCM-80-18488, and the Sponsors of the Laser Fusion Feasibility Project at the Laboratory for Laser Energetics of the University of Rochester.

Th-AM-E7 LAC OPERATOR: CORRELATION OF DYNAMIC HETEROGENEITY WITH FUNCTION. S. Cheung, K. Arndt, and P. Lu, Department of Chemistry, University of Pennsylvania, Philadelphia, PA 19104.

The imino protons of the double helical DNA sequence containing the lactose operon operator DNA has been examined by NMR. The intensities of the imino proton resonances as a function of increasing temperature indicates that a 36 base pair DNA fragment containing the operator not only melts sequentially from the ends, but also from a region inside the lac operator, i.e. at position 6 in the sequence:



In order to examine this phenomenon in detail, we have synthesized two sections of the above sequence, from -5 through 12 and from 11 through 27. Saturation recovery experiments were done to measure the T_1 's of the imino protons of both of these fragments as a function of temperature. At the higher temperatures where proton exchange dominates the relaxation rate, it is clear that the melting effects seen at position 6 are reflected in the higher exchange rate at the GC neighbors, 5 and 7. This same 3 base pair region, when altered, results in operator constitutive mutations. This phenomenon of a GTG/CAC sequence where the TA imino proton exchanges more rapidly has been seen by Kearns and coworkers (Biochemistry 20, 3756-3764, 1981).

This GTG/CAC triplet occurs in a number of DNA sites that specifically interact with proteins in prokaryotic and eukaryotic systems. (Supported by grants from the NIH)

Th-AM-E8 DNase I FOOTPRINTING ANALYSIS AS A MEANS OF STUDYING DRUG-DNA INTERACTIONS. M. J. Lane, D. J. Vestal, E. L. Fish, J. N. Vournakis and J. C. Dabrowiak. Departments of Biology and Chemistry, Syracuse University, Syracuse, New York 13210.

A powerful method for determining the sequence specificity of DNA binding ligands is footprinting analysis (1-3). Location of the specific binding sequences associated with a drug-DNA contact depends on the ability of a DNA bound ligand to protect regions of DNA from digest by DNase I or other endonucleases.

We have completed preliminary investigations on the scope and limitations of DNase I footprinting by studying eight equilibrium binding ligands having a diversity of molecular size and binding kinetics. Our data, though limited, indicated that these parameters do not seriously affect detection of specific binding sequences. We have also completed analysis of both strands of a 142 bp. Hind III/Hae III restriction fragment with regard to its ability to bind actinomycin D and netropsin. Both ligands bind to several sequences on this fragment and the protected regions are staggered by several base pairs when data from both strands are compared. Actinomycin D is also able to enhance DNase I cleavage away from its principle interaction site. The potential sources of the asymmetric protection patterns and the enhancements will be discussed. Quantitative approaches to analyzing footprinting autoradiograms using microdensitometry will also be presented and discussed. Supported by N.I.H. Grant GM 31895.

1. VanDyke, M. W. Hertzberg, R. P. and Dervan, P. R. (1982) PNAS, 79, 5470.

2. Lane, M. J. Dabrowiak, J. C. and Vournakis, J. N. (1983) PNAS, 80, 3260.

3. Dabrowiak, J. C. (1983) Life Sci., 32, 2915.

Th-AM-E9 AUTOMATION OF DNA SEQUENCING. Lloyd M. Smith, Michael W. Hunkapiller*, Tim Hunkapiller, Steve Schultz, Suzanna Horvath, Leroy Hood. California Institute of Technology, Pasadena, CA, and Applied Biosystems, Inc., Foster City, CA.

Current nucleic acid sequencing techniques are rapid, reliable, and widely used. However, it still requires a very substantial amount of time and money to sequence a long stretch of DNA (greater than 1000 base pairs). The work involved is difficult enough to require a highly trained person, yet repetitive and tedious by nature. Therefore it would be of great benefit to develop a means of automating the process, both to enable more rapid and inexpensive sequencing, and to free highly-trained personnel for more creative work.

DNA sequencing normally utilizes radiolabeled DNA molecules which are electrophoresed on high resolution polyacrylamide gels and detected by autoradiography. We have developed chemistry for the attachment of colored (and fluorescent) dyes to the oligonucleotide primer used in dideoxy sequencing and are developing instrumentation for the detection of these molecules during electrophoresis through the gel. This will enable us to automate the DNA sequencing process. The chemistry for synthesis of the labeled DNA molecules will be described, as well as some aspects of the instrumentation for automated sequencing. In addition, some of the numerous other possible applications of labeled oligonucleotides will be discussed.

Th-AM-E10 COMPUTER GRAPHICS AND MOVING PICTURES IN THE ANALYSIS OF MOLECULAR DYNAMICS SIMULATIONS. S.C. Harvey, F.L. Suddath, and M. Prabhakaran, Department of Biochemistry, University of Alabama in Birmingham, Birmingham, AL 35294.

The standard molecular dynamics film consists of pictures of successive molecular structures generated by the simulation; with a typical time step of 1-10 fs ($1 \text{ fs} = 10^{-15} \text{ s}$) between the images chosen for projection, 1 ps (10^{-12} s) of simulation is seen over a real time period ranging from a few seconds to a minute. We are experimenting with alternative representations of the data to uncover molecular motions which might otherwise go unnoticed. Examples of these representations are: (1) The speed of the usual film is appropriate to show the fluctuations in bond lengths, bond angles, and torsional angles (dihedrals), but it does not reveal collective motions which occur on much longer time scales. If we change the time step of the movie to 100 fs, for instance, motions with periods in the range 1-10 ps become evident. (2) It is not necessary to represent atoms or bonds at all. If we calculate the displacement of each atom from its mean position (averaged over the whole simulation) and represent atomic displacements by vectors, the picture is a collection of arrows whose collective motions reveal certain dynamic features. (3) Double helical portions of nucleic acids can be represented by idealized representations of helices that show the fluctuations in twist, bend, and length. (4) Various combinations of these ideas can be used. The method has been applied to investigate the motions of phenylalanine transfer RNA. (Supported by grants from the National Science Foundation (PCM-8118827) and from the University of Alabama in Birmingham.)

Th-AM-E11 THE DYNAMIC BEHAVIOR OF tRNA^{Phe}: A MOLECULAR DYNAMICS APPROACH. M. Prabhakaran^a, Stephen C. Harvey^a, and J. Andrew McCammon^b, (a) Department of Biochemistry, University of Alabama in Birmingham, Birmingham, AL 35294 and (b) Department of Chemistry, University of Houston, Houston, TX 77004

The intramolecular motions of tRNA^{Phe} have been simulated over a period of 30 ps using the molecular dynamics algorithm with the normal empirical energy functions. The trajectory has been studied by the usual analysis of time series, correlation functions, and moments of displacement, and we have also developed new methods using computer-generated moving pictures for the study of collective modes of motion (S.C. Harvey, F.L. Suddath and M. Prabhakaran; the abstract for that work appears elsewhere in this volume). We report here on our recent progress in the data analysis, which has covered the following features: (1) the spatial anisotropy of the atomic displacements; (2) the stability and fluctuations in the hydrogen bonding; (3) the motions of the backbone torsional angles and sugar puckers; (4) the collective motions of the double helical portions of the molecule; and (5) a comparison of backbone motions and base stacking patterns in the nonhelical regions with those of the helical regions. (Supported by grants from the National Science Foundation.)