Th-AM-Sym II-1

CALCIUM AND PHOSPHOLIPID REGULATION OF ACTIN CYTOSKELETON. Fu-Xin Yu, Koji Onoda, and Helen L. Yin, Department of Physiology, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75235-9040.

Receptor-mediated stimulation induces marked actin polymerization and cytoskeletal reorganization in a variety of cells. The activities of at least two potent vertebrate actin modulating proteins are activated by micromolar Ca^{2+} and inhibited by polyphosphoinositides. These include gelsolin, an actin filament capping and severing protein, and gCap39, a recently identified gelsolin-like actin filament capping protein with no severing function. Gelsolin and gCap39 coexist in a variety of mammalian cells, and may have unique as well as complementary roles in the regulation of the actin cytoskeleton in response to agonist stimulation. Immunofluorescence studies show that while both gelsolin and gCap39 are enriched in the subplasmalemmal cortical cytoplasm, they have distinct distribution. Structure-function analysis by limited proteolysis as well as expression of truncated gCap39 define the domains essential for actin filament end capping. Furthermore, analysis of gelsolin-gCap39 chimeras establish that the lack of severing by gCap39 in spite of a 49% sequence identity with gelsolin is due to its inability to bind to the side of actin filament.

Th-AM-Sym II-2

MOLECULAR ANALYSIS OF AMOEBOID CHEMOTAXIS: THE CORTICAL EXPANSION MODEL. John S. Condeelis, Dept. of Anatomy, Albert Einstein College of Medicine, Bronx, NY 10461.

Th-AM-Sym II-3

ACTIN-MEMBRANE INTERACTIONS AT SITES OF CELL-SUBSTRATUM ADHESION. Mary C. Beckerle, Maria E. Bertagnolli, Aaron W. Crawford and Raymond K. Yeh. Dept. of Biology, University of Utah, Salt Lake City, UT 84112.

Cell adhesion and migration depends on the ability of cells to establish transmembrane connections between extracellular ligands and the cytoskeleton at sites of membrane-substratum association. We have recently identified and characterized a novel 82 kDa protein found at sites of actin-membrane interaction. The 82 kDa protein is a low abundance cytoplasmic component of adherens junctions such as the focal contacts of fibroblasts and the dense plaques of smooth muscle cells. It is an elongated, monomeric phosphoprotein that exists as a family of isoforms having isoelectric points ranging from 6.4 to 7.2. We are currently examining the possibility that the 82 kDa protein interacts with actin or other known components of adherens junctions.

In order to approach the question of how cell-substratum adhesion is modulated in vivo, we have focused our attention on platelets, cells that are specialized for regulated adhesion. Platelets exhibit high levels of adherens junction components. One of these proteins, talin, undergoes an activation-dependent change in its subcellular distribution, moving from the cytoplasm to the plasma membrane as the platelets develop the capacity to adhere. Since platelets are anucleate cells having little active translational machinery, talin's subcellular distribution must be controlled at the post-translational level. Talin can be modified by proteolytic cleavage and phosphorylation and we are exploring the possibility that such modifications occur during the platelet activation response. Nucleated cells, on the other hand, may also be able to regulate their adhesiveness by controlling the level of expression of adherens junction components. Indeed, we have found that chicken embryo fibroblasts exposed to an adhesive substratum exhibit significantly higher levels of mRNA encoding two adherens junction components, vinculin and talin, than do cells exposed to a non-adhesive substratum. Thus, cells may utilize a variety of strategies to regulate substratum-adhesion.

Th-AM-Sym II-4

MEMBRANE GLYCOPROTEIN MOVEMENTS ASSOCIATED WITH CELLULAR MOTILITY AND SIGNALING. M. P. Sheetz, Department of Cell Biology, Duke University Medical School, Durham, N.C. 27710.

We have been using submicron latex and gold particles to visualize the movements of membrane glycoproteins on cells in order to understand the mechanism of cell movement and its control. A major finding of those studies was that the rearward migration of material on the surface of motile cells was coupled with the attachment of material to the cytoskeleton and even in the most rapidly migrating cells there was no directed flow of bulk membrane (Kucik et al., J. Cell Biol. 111:1617 (1990) & Sheetz et al., Nature 340:284(1989)). In studies designed to validate the method of Single Particle Tracking (SPT) and to define its limitations we have shown that particles as large as 0.2 microns will diffuse at the same rate as the dimeric form of the lectin, con A (succinimidyl con A), when they are attached to keratocyte membranes (Kucik, Elson, and Sheetz, in preparation).

It is the leading edge of the lamella region in cells which first contacts other cells and extracellular matrix molecules and is expected, therefore, to be the primary site for control of motility. In neuronal growth cones and migrating keratocytes there is evidence that the leading edge of the lamella is differentiated both through the concentration of specific glycoproteins in that region (Sheetz et al., Cell 61:231 (1990)) and attachment of particles to the cytoskeleton occurs preferentially there (Kucik et al., J. Cell Biol. in press). In the latter study we have used a laser optical trap to move particles to specific regions of the lamella and find that particles become attached to the cytoskeleton when held at the leading edge. Attachments to the cytoskeleton are generally weak initially and at early times can be broken by the force of the laser trap (7 piconewtons). This opens up the possibility of measuring directly the strength of glycoprotein attachments to the cytoskeleton. We feel that this new technology opens up many ways of understanding the physical changes in glycoproteins which accompany cell migration and signalling phenomena.

Th-AM-F1

SEPARATION OF CALCIUM- AND VOLTAGE-MEDIATED PROCESSES IN THE INACTIVATION OF CARDIAC CALCIUM CURRENT.
P.-F. Méry, P. Lechêne, R. C. Levi & R. Fischmeister, INSERM U-241, Université Paris-Sud, F-91405 Orsay.

Whole-cell experiments on cardiac cells have conclusively demonstrated that the L-type Ca current (I_{Ca}) inactivates upon a depolarization of the membrane potential by a mechanism that depends to some extent on the influx of Ca ions. An important finding supporting this thesis was that in the absence of Ca ions, e.g. when Ba or Na ions are carrying the current, I_{Ca} inactivates with a much slower time course. To investigate whether this slower inactivation reveals an intrinsic voltage-dependent mechanism associated with Ca channels, we examined the inactivation of I_{Ca} in zero Ca (nominal) solution, i.e. when no net current flows through Ca channels. Experiments were performed in frog ventricular myocytes using the whole-cell patch-clamp technique and a fast perfusion device. The mouths of four capillary tubings (400 μm inner diameter) were glued together horizontally and attached to a voice coil from a commercially available loud speaker. Servo control of the position of the coil allowed the mouth of any of the capillaries to be positioned near the cell within 5-6 ms. Different solutions were flowing out of the capillaries at variable velocities. At a flow rate of ≈5 cm/s complete exchange of the external environment of the cell was achieved within 20-30 ms, as evidenced by the change in background K current at -100 mV upon a sudden variation in extracellular K concentration. For I_{Ca} measurements, Cs ions were used instead of K, and the cells were held at -80 mV holding potential and depolarized every 8 sec to various potentials during 400 ms. Three capillaries were used in each experiment, allowing current to be measured either in physiological Ca concentration (I_{Ca}), in zero Ca ($I_{Ca,0}$) or in 0Ca + 5 mM EDTA, when Na ions flow through Ca channels ($I_{Ca,Na}$). Inactivation of $I_{Ca,0}$ was investigated by exposing the cell to 0Ca 50 ms prior to a depolarization and by measuring the peak I_{Ca} or $I_{Ca,Na}$ that developed when either Ca ions or EDTA were suddenly re-applied to the cell at increasing times (0 to 350 ms) following the beginning of the depolarizing pulse. In the absence of net current flow through Ca channels, $I_{Ca,0}$ inactivated with a similar time course as $I_{Ca,Na}$ and about three times slower (at 0 mV) than I_{Ca}. These results suggest that a current-independent mechanism, which may represent an intrinsic voltage-dependent property of Ca channels, participates in the inactivation of I_{Ca}. At physiological Ca concentration, however, this process plays a lesser role in the inactivation of I_{Ca} as compared to the influx of Ca ions through the channels.

(Supported by an exchange program INSERM/CNR and a grant from Bayer-Pharma.)

Th-AM-F2

ABSENCE OF A DIRECT, MEMBRANE-DELIMITED PATHWAY IN THE ß-ADRENERGIC MODULATION OF CALCIUM CURRENT IN FROG VENTRICULAR MYOCYTES.
P.-F. Méry, G. Szabo* & R. Fischmeister (Intro. by R. Ventura-Clapier), INSERM U-241, Université Paris-Sud, F-91405 Orsay, & *Department of Physiology, University of Virginia, Charlottesville, VA 22908.

A direct activation of L-type Ca channels by the stimulatory G protein (G_s) has been demonstrated recently to participate in the stimulation by isoprenaline (Iso) of the whole-cell Ca current (I_{Ca}) in guinea pig cardiac myocytes (Yatani & Brown, *Science* 245:71-74, 1989). Here, we present the lack of evidence for such a pathway in frog ventricle. Experiments were performed in frog ventricular myocytes using the whole-cell patch-clamp technique, an internal perfusion system, and the fast extracellular perfusion device described in Méry et al. (*Biophys. Soc. Abstr.*, 1991). For a frequent monitoring of I_{Ca}, the cells were depolarized every 2 sec to 0 mV during 200 ms from -100 mV holding potential. Experiments were performed at 20-24°C. The effects of rapid (< 30 ms) applications of maximal concentrations of Iso (2-10 μM) were compared with similar applications of forskolin (Fo, 10 μM) or the dihydropyridine BayK (-) 8644 (BK, 1 μM). I_{Ca} was unchanged at the first depolarization that followed by 50 ms to 2 sec the application of Iso. A small (20-50%) increase in I_{Ca} was detectable only at the second or third depolarization, i.e. I_{Ca} increased with a delay (δ) of 2-4 sec following the application of the agonist. I_{Ca} then rose abruptly with a half-time (t_{on}) of 12-20 sec whether Iso was still present in the solution surrounding the cell or not. When the cell was exposed to Iso for only short periods (350 ms to 10 sec), I_{Ca} increased during 25 to 40 sec to a level 150% to 1000% over control and then relaxed monoexponentially back to control level with a time constant (t_{off}) of 25-35 sec. When Fo was applied under similar conditions, all time parameters (δ, t_{on}, t_{off}) were increased by >50 %. However, similar applications of BK induced a much faster increase in I_{Ca}, with a delay of 150 ms and a t_{on} of ≈2 sec. This demonstrated that a fast stimulatory action on Ca channels was not prevented by the experimental approach used. These results also demonstrate that a fast action of G_s on Ca channels is essentially absent in frog ventricular cells. The difference in the time course of Iso and Fo stimulation of I_{Ca} might suggest, however, that a direct activation of Ca channels by G_s takes place in the stimulatory effect of Iso on a slower time scale than in guinea pig heart. However, in cells that were internally dialysed with the protein kinase A inhibitor (PKI_{15-22}, 16 μM) neither Iso nor Fo were able to produce a stimulation of I_{Ca}, even during a sustained application of the drugs. Therefore, it is concluded that ß-adrenergic stimulation of I_{Ca} in frog ventricle is exclusively due to activation of cAMP-dependent pathways. *(Supported by grants from Bayer-Pharma [France] to RF, MRT [France] and NIH #HL37127 to GS.)*

Th-AM-F3

CAN cAMP-DEPENDENT PHOSPHORYLATION EXPLAIN THE EFFECTS OF β-ADRENERGIC AGONISTS ON CALCIUM CURRENT AND CONTRACTION IN HEART? *H. Criss Hartzell & Daniel Bucknitz. Emory University School of Medicine. Atlanta, GA.*

Calcium current (I_{Ca}) plays a key role in initiating and maintaining contraction in heart. Force of contraction correlates closely with I_{Ca} amplitude. It has been suggested that I_{Ca} is regulated both by cAMP-dependent phosphorylation and direct G-protein binding. However, in frog ventricle, we have found no evidence for a direct regulation of I_{Ca} by G-protein, because the effect of isoproterenol (ISO) on I_{Ca} was completely blocked by internal perfusion with 8 μM PKI or 1 mM Rp-cAMP-S. It has also been suggested that phosphorylation is too slow to explain beat-to-beat regulation by sympathetic nerve stimulation. We have examined this question by comparing at 24°C the time courses of changes in (1) heart contractility upon sympathetic stimulation, (2) I_{Ca} upon rapid exposure to ISO, and (3) I_{Ca} upon photolysis of caged cAMP. Isolated frog hearts were perfused in a retrograde direction and the vagosympathetic trunk stimulated by a suction electrode in the presence of concentrations of atropine to block completely parasympathetic effects. Force of contraction increased upon nerve stimulation after a ~6 sec lag and beat frequency increased after a ~4 sec lag. Effects of parasympathetic stimulation in the absence of atropine and presence of propranolol were measurable within less than 2 sec. In isolated myocytes, I_{Ca} measured by patch clamp in Cs-containing solutions increased monotonically after a 3 sec lag upon exposure to isoproterenol. The time required to equilibrate solutions, calibrated by measuring the change in current in response to shifting from Cs to K solution, was less than 100 msec. An effect of ACh on K conductance in atrial cells, which is mediated directly by G-protein, could be measured in ~100 msec. Rapid release of cAMP from caged cAMP in single myocytes produced an increase in I_{Ca} within 0.5 sec upon photolysis of caged cAMP. These results suggest that in frog ventricle, direct G-protein modulation of I_{Ca} is not detectable and that cAMP-dependent phosphorylation is rapid enough to explain changes in contractility in response to sympathetic stimulation. In rat heart at 37°C, the increase in contractility in response to sympathetic nerve stimulation and the increase in I_{Ca} in response to rapid isoproterenol exposure were much more rapid and could be measured within 1.5 sec. The increase in I_{Ca} in response to isoproterenol was monotonic after the ~1.5 sec lag. Experiments are underway to determine whether any component of this response is mediated directly by G-proteins.

Supported by the NIH and Georgia Heart Association.

Th-AM-F4

CA CHANNEL KINETICS DURING THE SPONTANEOUS HEART BEAT IN EMBRYONIC CHICK VENTRICLE CELLS. Louis J DeFelice, Yuan-mou Liu, Stefania Risso, Michele Mazzanti, Anatomy and Cell Biology, Emory University, Atlanta GA 30322

To understand Ca conductance in the beating heart, we have used two electrodes to record patch current and whole-cell voltage simultaneously. Cells were maintained in normal physiological saline at room temperature. The whole-cell electrode contained an intracellular-like solution, while the cell-attached patch electrode contained various test solutions. In this configuration cells beat spontaneously for minutes. With 20 mM Na or 20 Ba in the patch electrode (Ca less than 10-7 M), L-type Ca channels are usually open throughout the plateau and early repolarization phases of the action potential. These long openings are interrupted by brief closings that become more frequent as the cell repolarizes. With 20 Ca in the electrode channel kinetics consisted of short openings during the early plateau phase. Long openings also occurred in 20 Ca; however, they were extremely rare. They were more frequent in 10 Ca; however, in either 10 or 20 Ca solutions long openings were less common with several channels in the patch. Single-channel events were unresolved with normal saline in the patch pipette (1.5 Ca, 120 Na); nevertheless, we occasionally observed long openings reminiscent of Na or Ba kinetics. The conductance of long openings in 1.5 Ca was 22 pS, suggesting that the carrier was not Ca but Na. We interpret these observations by a model of Ca channels that includes a voltage-dependent inactivation state and a current-dependent blocked state. The transition to the blocked state depends on the total patch current and therefore on the number of channels in the patch. Ca current during normal beating is therefore an admixture of kinetics that depends on channel density. NIH HL-27385 supports this work.

Th-AM-F5

BLOCK OF CARDIAC CALCIUM CHANNELS BY FENDILINE IN GUINEA PIG VENTRICULAR CELLS. W. Schreibmayer*, O. Tripathi# and H.A. Tritthart*. (Intro. by L. Hymel)
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In order to test the hypothesis that diphenylalkylamines act directly as organic calcium channel blockers, the modulation of the L-type calcium current (I_{Ca}) of guinea pig ventricular myocytes by fendiline was investigated. The results show that fendiline blocks I_{Ca} with an IC_{50} of 17.0 ± 2.4 μM. The kinetics of I_{Ca} is modulated by the interaction with fendiline in such a way that inactivation is significantly faster and the availability curve is shifted to more negative membrane potentials. In the presence of 30 μM fendiline (I_{Ca} partially blocked) the agonistic 1,4-dihydropyridine compound (4R,4S)-Bay K 8644 at a concentration of 1 μM led to a further increase in block instead of the expected increase in I_{Ca} (4R,4S)-Bay K 8644 did not exert a similar blocking behavior in combination with either verapamil, diltiazem or nifedipine, but instead the expected agonistic behavior was observed in all cases. Thus we conclude that diphenylalkylamines most likely act on a site of the voltage-sensitive calcium channel that is physically different from the sites for 1,4-dihydropyridines, phenylalkylamines and benzothiazepines.

This work was supported by the Austrian Research Fund (S 4504B).

Th-AM-F6

CARDIAC Na^+ and Ca^{++} CHANNEL GATING CURRENTS. Ira R. Josephson and Nick Sperelakis. Department of Physiology & Biophysics, University of Cincinnati, College of Medicine, Cincinnati, OH 45267-0576.

Non-linear charge movement (gating current) associated with the opening of Na^+ channels and Ca^{++} channels was recorded from enzymatically-isolated single ventricular myocytes cultured from embryonic chick hearts, using the whole-cell patch clamp method. The myocytes were exposed to the appropriate intracellular and extracellular solutions designed to block Na^+, Ca^{++} and K^+ ionic currents. The linear components of the capacity and leakage currents during test voltage steps were eliminated by adding scaled hyperpolarizing control step currents. Upon depolarization from negative holding potentials the non-linear charge movement displayed two kinetic components: (a) an early rapidly-decaying component (< 500 μs in duration) had a threshold of -70 mV and saturated above 0 mV. The ON charge was partially immobilized during brief (< 10 msec) voltage steps, or by the application of less-negative holding potentials. (b) a second slower-rising and longer-duration (1 - 5 msec) component was activated at test potentials positive to -60 mV and showed saturation above +20 mV. The second component was partially immobilized by 5 sec holding potentials over a more positive voltage range than for the early component. The voltage dependence of both activation and inactivation of the Na^+ and Ca^{++} ionic currents was determined for cases in which these currents were not blocked. There was a strong correlation between the voltage dependence of activation and inactivation of the Na^+ and Ca^{++} ionic currents and the activation and immobilization of the fast and slow components of charge movement. This evidence suggests that the two components of charge movement are associated with the voltage-sensitive gating steps that precede Na^+ and Ca^{++} channel opening and current flow. Supported by NIH HL31942.

Th-AM-F7

ENDOTHELIN INCREASES CYTOSOLIC Ca^{++} AND ENHANCES CARDIAC MYOCYTE Ca^{++} CURRENT BY A G-PROTEIN-DEPENDENT MECHANISM.

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Endothelin (ET) is a vasoactive peptide released from endothelial cells which has potent cardiac inotropic effects. The effect of ET on intracellular Ca^{++} transients was studied in single cardiac myocytes using the fluorescent Ca^{++} indicator Indo-1. In addition, the effect of ET on the voltage-dependent Ca^{++} current (I_{Ca}) in single myocytes was examined using the whole cell voltage clamp technique. ET (20 nM) consistently raised the diastolic signal level of the Ca^{++} transient and also increased the net amplitude of the transient by 24% (N=7). Using a standard pipette solution, extracellular application of ET (20 nM) not only failed to increase the peak I_{Ca}, it in fact caused a small reversible decline in the maximum current (903 ± 109 pA without ET, 727 ± 95 pA with ET (N=14, P<0.05)). However, if GTP (100 μM) was added to the dialyzing pipette solution, extracellular application of ET (20 nM or 0.2 nM) caused a large, reproducible increase in peak I_{Ca} (871 ± 85 pA without ET, 1230 ± 110 pA with 20 nM ET (N=10, P<0.05)). Myocyte pre-incubation with pertussis toxin (PTX) (500 ng/ml) failed to block the ET effect (N=4). GDPßS is a GDP analogue which binds to G proteins blocking their normal GTP-activated intracellular cycling. With the addition of both GDPßS (0.5-5.0 nM) and GTP (100 μM) to the pipette solution, the ET effect on peak I_{Ca} was blocked (1062 ± 86 pA without ET, 1170 ± 134 pA with ET (N=11, P>0.05)). These results indicate that ET enhances I_{Ca} through a G-protein-mediated pathway, but the G-protein involved is PTX-insensitive. By modulating the voltage-dependent Ca^{++} current, endothelin may be an endogenous regulator of cardiac inotropic state.

Th-AM-F8

POLYCLONAL ANTIBODY TO CARDIAC RYANODINE RECEPTOR PHOSPHORYLATION SITE IDENTIFIES THE GUINEA PIG BRAIN RYANODINE RECEPTOR.
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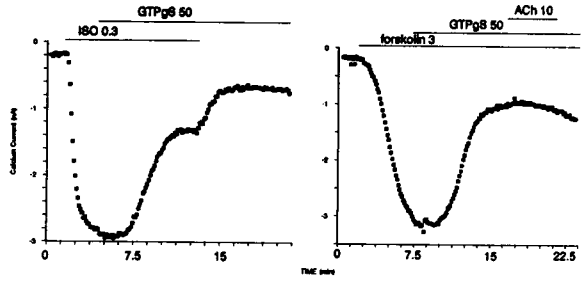
The cardiac ryanodine receptor has been shown to be identical to the sarcoplasmic reticulum Ca^{2+} release channel, and Northern blot analysis suggests that the cardiac isoform of the receptor is expressed in brain (Otsu et al., J. Biol. Chem. 265:13472, 1990). In this study, we prepared a polyclonal antibody to the sequence of the cardiac ryanodine receptor (residues 2805-2519), which contains the unique phosphorylation site for multifunctional Ca^{2+}/calmodulin-dependent protein kinase (CaM K). This antibody was used to identify the brain ryanodine receptor, and does not cross-react with the skeletal muscle receptor. More than 80% of solubilized [^{3}H]ryanodine binding sites from guinea pig brain membranes were specifically immunoprecipitated with the cardiac phosphorylation-site specific antibody. The immunoprecipitated brain ryanodine receptor had the same mobility on SDS-PAGE as the cardiac ryanodine receptor. Like the cardiac ryanodine receptor, the brain ryanodine receptor was shown to be an excellent substrate for CaM K. These results confirm that the cardiac isoform of the ryanodine receptor is expressed in brain, and furthermore show that the unique cardiac phosphorylation site is conserved. Since phosphorylation of the cardiac isoform of the ryanodine receptor increases Ca^{2+} channel activity, our results suggest that phosphorylation of the brain ryanodine receptor may provide a mechanism for increasing intracellular Ca^{2+} concentration during membrane depolarization and/or neurotransmitter release.

Th-AM-F9

Neurotransmitter regulation of I_{Ca} in frog ventricular myocytes is inhibited by GTPγS. T.D. Parsons and H.C. Hartzell. (Intro. by R.L. DeHaan). Dept. of Anatomy & Cell Biology, Emory University School of Med, Atlanta, GA 30322.

We have previously reported that internal perfusion of voltage-clamped cardiomyocytes with GppNHp, a hydrolysis-resistant analog of GTP, resulted in a near complete and irreversible inhibition of calcium current (I_{Ca}) stimulated by either isoproterenol (ISO) or forskolin (FORSK). To examine further the role of G proteins in the regulation of cardiac I_{Ca} by neurotransmitters, we have internally perfused cardiomyocytes with GTPγS, another hydrolysis-resistant congener of GTP. ISO-I_{Ca} (0.3 μM) was inhibited ~45% following internal perfusion with 50 μM GTPγS (n=7). The I_{Ca} was reduced another ~50% following washout of ISO (n=3), leaving, in the absence of hormone, an I_{Ca} that was ~3.5-times greater than basal I_{Ca}. This "persistently activated" I_{Ca} was resistant to inhibition by acetylcholine (10 μM) (ACh) (n=3). Furthermore, FORSK-I_{Ca} (3 μM) was reduced ~55% following internal perfusion with 50 μM GTPγS (n=13). This I_{Ca} was also insensitive to subsequent inhibition by ACh (n=5). Curiously, in many cells, prolonged internal perfusion with GTPγS resulted in partial reversal of the inhibition of FORSK-I_{Ca} (n=6). These results suggest that GTPγS stimulates inhibitory G proteins, as does GppNHp, but is more effective at activating stimulatory G proteins in the frog ventricular myocyte.



Th-AM-G1

SITE-SPECIFIC MUTAGENESIS OF HYDROPHOBIC RESIDUES IN THE CYTOPLASMIC SIDE OF THE PROTON TRANSPORT PATHWAY IN BACTERIORHODOPSIN S.Subramaniam, D.A.Greenhalgh K.J.Rothschild* and H.G.Khorana, MIT, Cambridge, MA 02139, and *Boston University, MA 02215. To investigate the mechanism of proton uptake in bacteriorhodopsin (bR), we have attempted to systematically alter the molecular volumes and hydrophobicities of some of the amino acids that line the cytoplasmic side of the proton transport pathway. Replacement of Leu 93 by Ala, Thr or Val result in proteins that have dark-adapted absorption maxima at 532, 532 and 547 nm, and light-adapted absorption maxima at 532, 531 and 554 nm, respectively. The Leu 93→Ala mutant shows a 5-fold decrease in the rate of chromophore formation compared to wild type bR when regenerated with 13-cis retinal, although the corresponding rates with all-trans retinal are similar. In contrast to wild type bR, the λ_{max} of the chromophore formed during regeneration of the Leu 93→Ala mutant is identical with both 13-cis and all-trans retinal. The kinetics of M formation in the Leu 93→Ala mutant are similar to that of wild type bR, but a long-lived "N"-like intermediate is observed in the photocycle. The above observations indicate an important role for Leu 93 in determining the rate of thermal cis-trans isomerization in bR. In contrast to the Leu 93→Ala mutant, the Phe 219→Ala mutant displays dark- and light-adapted spectra with maxima at 550 and 560 nm respectively, identical to that observed for wild type bR. Under saturating yellow illumination, all the above mutants show steady state proton translocation at levels comparable to that of wild type bR. The double mutant Leu 93→Asp/Asp 96→Ala displays a broad chromophore with an absorption maximum at 532 nm and is active in steady state proton pumping. This shows that the proton uptake defect in the Asp 96→Ala mutant can be overcome by displacing the aspartic acid residue one turn of an α-helix closer to the Schiff base. (Supported by grants from the NIH, ONR and the Damon Runyon-Walter Winchell Cancer Fund.)

Th-AM-G2

TIME-RESOLVED X-RAY DIFFRACTION STUDY OF STRUCTURAL CHANGES ASSOCIATED WITH THE PHOTOCYCLE OF BACTERIORHODOPSIN

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The time course of structural changes accompanying the transition from the M_{412} intermediate to the BR_{568} ground state in the photocycle of bacteriorhodopsin (BR) was studied at room temperature with a time resolution of 15 ms using synchrotron radiation X-ray diffraction. The M_{412} decay rate was slowed down by employing mutated BR Asp96Asn in purple membranes at two different pH-values. The observed light-induced intensity changes of in-plane X-ray reflections were fully reversible. For the mutated BR at neutral pH the kinetics of the structural alterations ($\tau_{1/2}$ = 125 ms) were very similar to those of the optical changes characterizing the M_{412} decay, whereas at pH 9.6 the structural relaxation ($\tau_{1/2}$ = 3 s) slightly lagged behind the absorbance changes at 410 nm. The overall X-ray intensity change between the M_{412} intermediate and the ground state was about 9% for the different samples investigated and is associated with electron density changes close to helix G, B and E. Similar changes ($\tau_{1/2}$ = 1.3-3.6 s), which also confirm earlier neutron scattering results on the BR_{568} and M_{412} intermediates trapped at -180°C (Dencher et al., PNAS 86, 7876, 1989) were observed with wild type BR retarded by 2M guanidine hydrochloride (pH 9.4). The results prove that the tertiary structure of BR changes during the photocycle and that the changes recover during the N_{550} to BR_{568} transition.

Th-AM-G3

ULTRAVIOLET RESONANCE RAMAN SPECTROSCOPY OF BACTERIORHODOPSIN: EVIDENCE AGAINST TYROSINATE IN THE PHOTOCYCLE. J. B. Ames, S. R. Bolton, and R. A. Mathies, Department of Chemistry, Univ. of Calif., Berkeley, CA 94720

We have recently shown that time-resolved ultraviolet resonance Raman (UVRR) spectroscopy is a powerful technique for probing protein structure and structural changes in bacteriorhodopsin (*Photochem. Photobiol. 52*, 605, 1990). FTIR and UV absorption studies have proposed that light-adapted BR_{568} contains an ionized tyrosine (tyr-185) which protonates upon light absorption and when the protein relaxes to dark-adapted BR_{DA}. To probe the protonation state of the tyrosine residues in BR, we have obtained UVRR spectra of BR_{568} and BR_{DA} with 230, 240 and 253 nm excitation. The 253 nm spectra of BR_{568} exhibit no prominent line at 1600 cm^{-1} which might be attributed to tyrosinate. Similarly, no changes attributable to tyrosinate Raman scattering were observed in $BR_{568} \rightarrow BR_{DA}$ difference spectra with 240 nm excitation, showing that there is no change of tyrosine protonation state in this transition. To examine whether we have sufficient sensitivity to detect the change of one residue, 240 nm spectra of purple membrane samples containing one equivalent of tyrosine were recorded at pH 7 and 11. The difference spectrum exhibits the expected scattering from the ν_{8a} mode of tyrosinate at 1600 cm^{-1} with an intensity that could easily have been observed in our experiments. Finally, with 240 nm excitation the weak scattering at 1600 cm^{-1} does not support the presence of a tyrosinate in BR_{568} because the intensity is exactly that expected for the ν_{8b} mode of the 11 tyrosines in BR. These results suggest that tyrosinate does not play a role in the proton-pumping mechanism of bacteriorhodopsin (Ames et al., *J. Am. Chem. Soc.*, in press).

Th-AM-G4

Picosecond Time-Resolved Resonance Raman Spectroscopy of Bacteriorhodopsin's J, K, and KL Intermediates. Stephen J. Doig, Philip J. Reid and Richard A. Mathies, Chem. Dept., Univ. of Calif., Berkeley, CA 94720

Picosecond resonance Raman spectroscopy has been used to obtain structural information on the primary photointermediates of bacteriorhodopsin. A synchronously pumped dye laser was amplified at 50 Hz to produce a probe pulse at 599 nm while a second, spectrally distinct, pump pulse at 550 nm was generated by amplification of a 10 nm slice of a continuum produced from the probe pulse. This apparatus was used to record a series of photoproduct spectra for time delays from zero ps to 13 nanoseconds with 3 ps time resolution. At 0 ps the hydrogen-out-of-plane (HOOP) intensity is large and the fingerprint region consists of a broad series of lines centered at 1180 cm^{-1}. By 3 ps, the relative HOOP intensity drops to its lowest value and the fingerprint collapses to a single strong mode at 1189 cm^{-1}, characteristic of a 13-*cis* chromophore. These results argue that J contains a highly twisted chromophore which conformationally relaxes upon conversion to K and that isomerization to 13-*cis* is complete within 3 ps. Between 3 and 40 ps there is a resurgence in HOOP intensity which remains large and constant thereafter. A plot of the photoproduct ethylenic frequency versus time also shows a shift in approximately 70 ps from 1518 cm^{-1} to 1521 cm^{-1}. The HOOP intensity increase and the ethylenic frequency shift are assigned to a conversion from K to a more twisted KL species with a time constant between 15 and 120 ps.

Th-AM-G5

FTIR of Bacteriorhodopsin Isotopically Labelled Lysine

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We have been investigating the nature of the coupling of retinal/lysine vibrations in the retinylidene lysine chromophore of bacteriorhodopsin. FTIR spectra have been obtained from various purple membrane samples with isotopically-enriched lysine. Selective ^{2}H enrichment has been investigated with substitutions that include deuterium on the lysine alpha carbon, the intermediate carbon atoms of the side chain and the ε-lysine carbon. ^{11}N and ^{13}C substitutions have also been studied and the data have defined those modes that have lysine N-C and C-C character. In addition, modes that have been previously assigned to the retinal have been shown to include a significant lysine component, while other observed vibtrations in the FTIR spectrum appear to be lysine normal modes with retinal contributions. In general, the data highlights the critical role that lysine plays in the normal modes of the bacteriorhodopsin chromophore. Elucidating the lysine/retinal couplings will allow for deeper understanding of the light-induced conformational and configurational changes in the retinal-lysine complex.

Th-AM-G6

PHOTOACTIVATION OF SENSORY RHODOPSIN I (SR-I): SOME DIFFERENCES FROM BACTERIORHODOPSIN AND SIMILARITIES TO RHODOPSIN. B. Yan[1,2], R. Johnson[2], E.N. Spudich[1], K. Nakanishi[2], & J.L. Spudich[1]*. [1]A. Einstein College of Medicine, Bronx, NY; [2]Columbia University, New York, NY.

The retinal binding sites of SR-I apoprotein (SOP-I) and bacterioopsin (BOP) are compared by incorporating retinal isomers and retinal analogs. Unlike BOP, SOP-I does not form a retinylidene pigment with 13-*cis* retinal, as assessed by absorption measurements and SDS-PAGE isolation of SOP-I after [^{3}H]13-*cis* retinal addition and $CNBH_3^-$ reduction. Also unlike BOP (Gartner et al. Biochemistry 22:2637, 1983), SOP-I does not thermally isomerize all-*trans* 13-desmethyl retinal to 13-*cis* and the corresponding analog SR-I pigment does not exhibit photochemical reactivity (1 msec resolution). This indicates that photoactivation of SR-I requires a specific steric interaction between the protein and the 13-methyl group of the retinal. This requirement differs from that of BR but resembles that of rhodopsin, in which steric interaction between the protein and the retinal 9-methyl group is crucial for photoactivation (Ganter et al. Biochemistry 28:5954, 1989).

An additional analogy to rhodopsin, namely that the meta-II_{380}-like intermediate S_{373} is the physiologically active conformation in the SR-I single photon cycle, is supported by further analog studies. The lifetime of S_{373} was modulated by incorporating retinal analogs *in vitro* and *in vivo*. Photocycles by SR-I analog pigments exhibit the same reaction scheme and similar formation rates, but different decay rates, of S_{373}-like species as monitored by flash spectroscopy in membrane vesicle suspensions. Attractant receptor signaling efficiencies determined by computerized cell tracking are proportional to the lifetimes of the S_{373}-like intermediates of the receptor. This indicates that S_{373}, the repellent receptor form of SR-I, is an attractant signaling state of the molecule.

Th-AM-G7

ENERGETICS OF LUMIRHODOPSIN AS PROBED BY PHOTO-ACOUSTIC CALORIMETRY - Kevin S. Peters, Department of Chemistry and Biochemistry, University of Colorado, Boulder 80309-0215

The enthalpy and volume changes for the conversion of rhodopsin and isorhodopsin to lumirhodopsin have been investigated by time-resolved photoacoustic calorimetry which measures the dynamics of enthalpy and volume changes for photoinitiated reactions on the nanosecond-microsecond time scale. In the detergent dodecyl-β-D-maltoside, the conversion of rhodopsin to lumirhodopsin is endothermic by 3.9 ± 5.9 kcal/mole and is accompanied by an increase of 29.1 ± 0.8 ml/mole at ambient temperatures. The lumirhodopsins produced from the rhodopsin and isorhodopsin are energetically equivalent. These values are compared with a previous low temperature matrix photocalorimetry study (Cooper, 1981) where the enthalpy increase for the formation of lumirhodopsin from rhodopsin is 26.3 ± 4.2 kcal/mole. The apparent discrepancy in these two values will be discussed.

Cooper, A. (1981) *FEBS Let. 123*, 324-326.

Th-AM-G8

PHOTOSENSITIVITY OF SALAMANDER VISUAL PIGMENTS. C. L. Makino, W. R. Taylor and D. A. Baylor. Dept. Neurobiology, Stanford Medical School, Stanford, CA 94305

The efficiency with which light activates a visual pigment is specified by the photosensitivity, P, defined as the product of the absorbance coefficient and the quantum efficiency of bleaching. The photosensitivity of rhodopsin has been studied extensively; for the A1 chromophore, Dartnall found $P = 10.5 \times 10^{-17}$ $cm^2 \cdot molecule^{-1}$, and for A2, 7.4×10^{-17} cm^2. What is the photosensitivity of cone pigments? How does photosensitivity vary with pigment type and the nature of the chromophore? We determined P for the pigments of salamander photoreceptors by whole cell patch recording. The early receptor current that accompanies photoexcitation was used to follow bleaching. Salamander red- and blue-sensitive cone pigments had very similar P's, in spite of the 175 nm separation in λ_{max}. P for the cone pigments was similar to that of the rod pigment. The mean P for all three pigments was $(7.3 \pm 1.2) \times 10^{-17}$ cm^2 (mean ± SD, n = 33), expressed as the value expected for a molecule in solution. Replacement of the native A2 chromophore with retinal A1 increased P to $(9.6 \pm 0.7) \times 10^{-17}$ cm^2 (n = 4). We conclude that the photosensitivity of cone pigments is similar to that of rod pigments and independent of pigment λ_{max}. Photosensitivity does depend, however, on the nature of the chromophore, A1 or A2. (Supported by NIH F32 EY06195 and EY05750).

Th-AM-H1

MEMBRANE POTENTIAL TRIGGERS POLIPHOSPHOINOSITIDE CHANGES IN A HUMAN SKELETAL MUSCLE CELL LINE.

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A permanent cell line from human skeletal muscle, was developed in our laboratory. The cells divide continuously with a doubling time of 24 hrs., a plating efficiency of more than 20% and reach a saturation density of 42000/cm². When placed in conditions (low serum, insulin and transferrin, DMSO) that induce fusion, up to 14% of the nuclei are found into multinucleated, tubule-like structures. Cells exhibit markers of differentiated muscle that include positive inmunostaining for myoglobin, muscle type myosin, desmin, and MM type creatine kinase. The presence of several ion channels was evident from patch clamp recordings and from binding of specific channel blockers.

Cells were incubated for variable periods of time in the presence of ^{3}H-inositol; label incorporation to phosphoinositides was measured by TLC after solvent extraction and the different labeled inositol phosphates were separated in the aqueous phase by ion exchange chromatography. The label saturates within 24 hours and remains stable after 48 hrs. In the presence of 60 mM LiCl in the incubation media, 82-90% of the label in the organic phase was recovered as phosphatidylinositol; 2-6% as phosphatidylinositol monophosphate (PIP) and 2-4% as phosphatidylinositol bisphosphate (PIP_2). Another phosphoinositide, also present in these cells was labelled with ^{3}H-glucosamine. Incubation of cells with 47-95 mM KCl for periods of time as short as 45 sec., induced an increase of 100 to 200% of the label in PIP_2 and minor increases in PIP.

In the aqueous phase, inositol mono, bis, tris and tetrakis phosphates are detected in similar and easily measurable amounts; variations in the label of inositol polyphosphates with high potassium were also seen.

This cell line appears as a good model system to further study the effect of membrane potential on phosphoinositide metabolism in skeletal muscle.

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Th-AM-H2

DANTROLENE SODIUM BLOCKS THE INOSITOL (1,4,5) TRISPHOSPHATE EFFECTS ON $[Ca^{2+}]_i$ IN SKELETAL MUSCLE. J.R. López, IVIC, Caracas, Venezuela.

The role of inositol 1,4,5 trisphosphate ($InsP_3$) in excitation contraction coupling in skeletal muscle is still controversial. We reported that microinjection of $InsP_3$ at submicromolar concentration induced increments in the intracellular free calcium concentration ($[Ca^{2+}]_i$) in intact skeletal muscle (Biophys J. 57:169a, 1990). Now, we have measured $[Ca^{2+}]_i$ in quiescent intact fibers isolated from tibialis anterior muscle of a tropical amphibian Leptodactylus insularis microinjected with $InsP_3$ (0.5 uM) before and after the muscle bundles were incubated in dantrolene (10^{-6} M). Small bundles of intact fibers were mounted horizontally with both ends fixed between two stainless steel forceps attached to two micromanipulators (striation spacing 2.4 um). The $[Ca^{2+}]_i$ was measured with calcium selective microelectrodes at 22°C. The $[Ca^{2+}]_i$ before the $InsP_3$ microinjection was 0.12 ± 0.01 uM (M ± SEM, n=16) and 0.52 ± 0.02 uM (n=12) after the microinjection of $InsP_3$ (0.5 uM). The incubation of muscle fibers in dantrolene (10^{-6} M) for 10 minutes reduced the $[Ca^{2+}]_i$ to a mean value of 0.06 ± 0.01 uM (n=16). The microinjection of $InsP_3$ in these fibers did not induce any increase in $[Ca^{2+}]_i$: 0.07 ± 0.02 uM (n=12). These results suggest that the transient elevation in the $[Ca^{2+}]_i$ induced by $InsP_3$ was due to a release of Ca^{2+} from an intracellular store, most probably the sarcoplasmic reticulum (SR), since dantrolene inhibits the calcium release from the SR, and not by activation of the dihydropiridine calcium channels (Supported by Angelini Pharmaceuticals and NSF).

Th-AM-H3

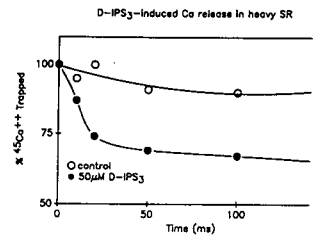
TEE EFFECTS OF INOSITOL (1,4,5) TRISPHOSPHATE ON $[Ca^{2+}]_i$ IN SKELETAL MUSCLE IS LENGTH DEPENDENT. J.R. López, IVIC, Caracas, Venezuela.

We have previously reported that microinjection of Inositol 1,4,5, trisphosphate ($InsP_3$) into intact skeletal muscle fiber induced transient enhacements of $[Ca^{2+}]_i$ which were dose deperdent (Biophys J. 57:169a, 1990). We have extended the previous study by exploring the effect of $InsP_3$ on $[Ca^{2+}]_i$ as a function of muscle length. Small bundles of intact fibers were isolated from the tibialis anterior muscle of a tropical amphibian Leptodactylus insularis and mounted horizontally in an experimental chamber with both ends fixed between two stainless steel forceps attached to two micromanipulators which allowed us to adjust the striation spacing (2.4 to 3.6 um) using the laser diffraction technique. $[Ca^{2+}]_i$ was measured using calcium selective microelectrodes. Streching the muscle fibers from 2.4 um to 2.6, 2.8, 3.0, 3.2, 3.4 and 3.6 um induced increment in $[Ca^{2+}]_i$ from an initial values of 0.12 ± 0.01 uM (M ± SEM, n=18) at 2.4 um, to 0.20 ± 0.03 uM (n=12), 0.29 ± 0.04 uM (n=14), 0.35 ± 0.02 uM (n=13), 0.48 ± 0.04 uM (n=12), 0.56 ± 0.05 uM (n=14) and 0.76 ± 0.11 uM (n=16) respectively. Pressure injection of $InsP_3$ (0.5 uM) induced transient increments in $[Ca^{2+}]_i$ which were length dependent. After the microinjection of $InsP_3$ the $[Ca^{2+}]_i$ was 0.43 ± 0.03 uM (n=6) at 2.4 um; 0.78 ± 0.15 uM (n=6) at 2.6 um; 1.15 ± 0.26 uM (n=6) at 2.8 um; 1.40 ± 0.22 uM (n=8) at 3.0 um; 1.95 ± 0.36 uM (n=7) at 3.2 um; 2.35 ± 0.41 uM (n=6) at 3.4 um and 3.50 ± 0.43 uM (n=8) at 3.6 um. These findings confirm previous results about the influence of muscle length on resting $[Ca^{2+}]_i$. In addition, it shows that the effects of $InsP_3$ on $[Ca^{2+}]_i$ is dependent on the muscle length. (Supported by grants from Angelini Pharmaceuticals and NSF).

Th-AM-H4

FAST RELEASE OF CALCIUM MEDIATED BY INOSITOL 1,4,5-TRISPHOSPHATE RECEPTOR CHANNELS IN THE SARCOPLASMIC RETICULUM OF SKELETAL MUSCLE. Carmen Valdivia, Donna Vaughan, Barry V.L. Potter* and Roberto Coronado. Department of Physiology, University of Wisconsin, Madison, WI 53706 and *University of Leicester, UK.

In this study we resolved the kinetics of IP_3-induced $^{45}Ca^{2+}$ release from the heavy SR of rabbit skeletal muscle. Measurements were done in a rapid filtration apparatus and release was stimulated with the non-hydrolyzable analog, DL-myo-inositol 1,4,5-trisphosphorothioate (IPS_3) used previously in triads (Valdivia et al, 57,1233, 1990). $^{45}Ca^{2+}$ was loaded into heavy SR vesicles passively, by equilibration in 5 mM $^{45}Ca^{2+}$, 100 mM KCl, 20 mM NaPipes pH 7.0, or actively, in the presence of 1 mM ATP, 2 mM $MgCl_2$, 150 mM KCl, 50 mM Mes-Tris pH 7.5. In both cases we found that 50 μM IPS_3 was capable of releasing ~30% of the loaded $^{45}Ca^{2+}$ in times ranging from 30 ms to 50 ms. Higher concentrations did not enhanced the rate nor the amount of $^{45}Ca^{2+}$ released. 2 μM IPS_3 induced release of 10% of the stored $^{45}Ca^{2+}$ following a delay of 100 ms. Control release mas measured in 2 mM free Mg^{2+} to effectively block ryanodine receptors. The presence of IP_3-sensitive channels in heavy SR was investigated in planar bilayers. In complete absence of ryanodine receptor activity we recorded a 30 pS channel that only opened in the presence of IPS_3. These results suggested that IP_3-sensitive channels present in heavy SR of skeletal muscle are different from ryanodine receptor channels. The $^{45}Ca^{2+}$ flux data suggests, much to our surprise, that IP_3 sensitive channels have adequate speed to be activated during excitation contraction coupling. Supported by NIH postdoctoral fellowship to C.V.



Th-AM-H5

INHIBITION OF Ca^{2+} TRANSIENTS ELICITED BY FLASH PHOTOLYSIS OF DM-NITROPHEN DURING THE PHYSIOLOGICAL RELEASE OF Ca^{2+} IN SKELETAL MUSCLE FIBERS.

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Calcium transients elicited either by electrical stimulation or flash photolysis of caged-Ca compounds, were recorded from frog single skeletal muscle fibers using the procedures described by DiFranco et al. (these proceedings). At 8°C, fluorescence transients induced by action potential stimulation (AP-transients) displayed triadic delays of 4 ms, reached peak values within 15 to 20 ms, and slowly decayed to resting values (200 to 400 ms). Flash photolysis Ca^{2+} transients (FP-transients) elicited by UV laser pulses on DM-Nitrophen-loaded muscle fibers, did not have delays and had faster exponential rising phases, reaching peak values within 5 to 6 ms after the flash. Interestingly, FP-transients triggered at different points during the time course of an AP-transient did not display constant amplitudes. They were maximal in resting fibers, were nearly abolished at the peak of the AP-transient, and gradually recovered their original amplitude along the decaying phase of the AP-transient. However, the time course of recovery from inhibition of the FP-transient was briefer than the AP-transient itself.

These observations are difficult to reconcile with the classical interpretation that Ca^{2+} ions released from the SR bind to myoplasmic buffers of constant capacity. Instead, they can be best explained assuming that a very high capacity, low affinity Ca^{2+} buffer compartment is made transiently accesible to myoplasmic Ca^{2+} ions during the AP-transient. We tentatively propose that the opening of SR Ca^{2+} channels creates a low resistance pathway which virtually couples the intra-SR Ca^{2+} binding proteins with the myoplasm. (Supp. by NIH AR-25201 and UCLA School of Medicine BRSG).

Th-AM-H6

DEPLETION OF CALCIUM FROM T TUBULES AND JUNCTIONAL SARCOPLASMIC RETICULUM DURING A SINGLE HEART BEAT

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Rabbit papillary muscles were mounted on a freezing head, where they were superperfused and stimulated while force was recorded isometrically. The perfusion solution contained $CoEDTA^-$ as an extracellular marker, and the stimulation was a continuous train of paired-pulses at 0.3 Hz. Stimulation and recording continued until the instant when each muscle was frozen by being "slammed" into a LN_2 cooled copper block. There were no changes in temperature (28°C) or force before contact with the block.

One group of muscles (*mid-rise*) was frozen at the instant when developed force rose to 50% of maximum (average 150 ms after stimulation). Another group (*end diastole*) was frozen at rest (10 ms before the next stimulus would have been given). Ultrathin sections were cut from the frozen muscles at -120°C, freeze-dried, and analyzed by electron probe x-ray microanalysis (EPMA).

Analysis of the freeze-dried fluid in the lumina of the T tubules indicated Ca depletion: The ratio of Ca/Co was 0.82 ±0.05 (n=24) at end diastole, and dropped to 0.59 ±0.05 (n=23) when developed force reached 50% of maximum ($P \leq 0.01$). Since the Co signal quantified the extracellular marker, this result indicates a 28% drop of luminal total [Ca]. However, if 28% of the resting T tubule Ca content entered the cells, it would be insufficient *by itself* to account for more than a small fraction of developed force.

Analysis of the junctional SR at end diastole yielded the highest [Ca] values yet reported for jSR in cardiac muscle, 22.1 ±2.6 mmol/kg dry wt (n=38); this decreased to 11.7 ±1.5 mmol/kg dry wt at mid-rise (n=37). The ratio of jSR to luminal Co x-ray intensities was used to correct jSR [Ca] for stray x-rays arising from the adjacent T tubular lumen. When this was done, the corrected values for JSR [Ca] were 11.8 ±1.6 and 4.0 ±1.0 mmol/kg dry wt at end diastole and mid-rise, respectively ($P \leq 0.001$). This 66% drop in jSR Ca is similar to that observed in rabbit papillary muscles when SR Ca was depleted by prolonged rest (Walsh & Tormey, Biophys. J. 55:485a), and is compatible with the hypotheses that (a) the SR releases most of its Ca on each beat, and (b) the amount released is sufficient for force development.
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Th-AM-H7

EFFECT OF TURNING OFF Ca^{2+}-CURRENT ON RELEASE OF CALCIUM FROM SARCOPLASMIC RETICULUM (SR).

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The effect on SR Ca^{2+}-release of stopping L-type Ca^{2+}-current was examined, in order to study the possible involvement of Ca^{2+}-induced release of Ca^{2+} in E-C coupling in cardiac muscle. In one protocol (#1), Ca^{2+}-current activated by depolarization to +10 mV was stopped nearly instantaneously by further depolarizing to +100 mV at various times after the initial depolarization. In another protocol (#2), Ca^{2+}-current was stopped by repolarizing to -45 mV at various times. The first method is different from the second in that it (#1) does not produce "tails" of Ca^{2+} current and that repolarization is avoided, which could turn off SR Ca^{2+}-release through a "charge-coupled release mechanism" (as in skeletal muscle). Previous work has shown that under the conditions of these experiments the rapid upstroke of the $[Ca^{2+}]_i$-transient is produced mainly by Ca^{2+} released from SR. In cells exposed to isoproterenol (10 μM), Ca^{2+}-current at +10 mV peaked in 5 msec (21°C), and the rise-time (from time of depolarization to 90% of peak) of the $[Ca^{2+}]_i$-transient was 20 msec. The delay in the $[Ca^{2+}]_i$-transient, (which may arise from slow fura-2 kinetics) was 5 msec. Stopping the Ca^{2+}-current by further depolarization (#1) during the first 5 msec (e.g. at 3 msec) reduced the rate of rise of the $[Ca^{2+}]_i$-transient. Stopping the Ca^{2+}-current at 5 msec or later had little effect on the rate of rise, or on the peak $[Ca^{2+}]_i$ reached (although the time course of the $[Ca^{2+}]_i$-transient was different after the peak). Stopping the Ca^{2+}-current by repolarizing (#2) at 5 msec or earlier also reduced SR Ca^{2+}-release. From these results, we suggest that the reduction of SR Ca^{2+}-release is related to the stopping of Ca^{2+}-current rather than to a change in membrane potential. In agreement with previous work, the results are not compatible with charge-coupled release mechanisms of E-C coupling, but are not clearly inconsistent with Ca^{2+}-induced release of Ca^{2+}.

Th-AM-H8

RYANODINE HAS NO EFFECT ON Ca^{2+}-CURRENT IN CARDIAC CELLS IN WHICH $[Ca^{2+}]_i$ IS BUFFERED.

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The inactivation of L-type calcium current in mammalian ventricular muscle is slowed in the presence of ryanodine. Two explanations have been advanced. Mitchell and her colleagues (1984, Br. J. Pharm. 81: 13-15) suggested that the inactivation is slowed because ryanodine reduces the availability of Ca^{2+} for inactivation of Ca^{2+}-current (Ca^{2+}-dependent inactivation). Cohen and Lederer (1988, J. Physiol. 406: 115-146) proposed that the effect is due to a mechanical interaction of the ryanodine receptor with the L-type Ca^{2+}-channel. We examined the effect of ryanodine in single voltage-clamped guinea-pig ventricular myocytes. We used fura-2 (pentapotassium salt) to confirm that the ryanodine we used was capable of abolishing SR Ca^{2+}-release during the period in which it was present. We perfused the cells with EGTA, at 10 mM, to block changes in $[Ca^{2+}]_i$. In the absence of internal EGTA, Ca^{2+}-currents displayed bi-exponential inactivation and Ca^{2+}-dependent inactivation (steady-state inactivation curves turned up at positive potentials). Inactivation was slowed by ryanodine at 10 uM. In cells perfused internally with EGTA, ryanodine had no effects. In particular, steady-state inactivation curves were not shifted to the right, as found by Cohen and Lederer (1988). We conclude that, in guinea-pig ventricular myocytes, the effects of ryanodine on Ca^{2+}-current do not provide the basis to postulate a physical connection between L-type Ca^{2+}-channel and ryanodine receptor (SR Ca^{2+}-release channel).

Th-AM-I1

ALTERATIONS IN TRANSIENT OUTWARD CURRENT PROLONG ACTION POTENTIAL DURATION IN AGED RAT MYOCARDIUM?

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Action potential (AP) duration is prolonged with aging in intact cardiac muscle. To determine if these age-related electrical alterations result from changes in myocyte properties rather than in vivo extracellular factors, AP's were studied in myocytes isolated from young, middle-aged and senescent rat hearts. AP's were recorded using suction pipettes with a current clamp technique. There were significant increases of AP duration at 25%, 50% and 75% repolarization points from young to old [(percentage increase from young to old) APD_{25}: 114.8% APD_{50}: 126.6% APD_{75}: 92.2%]. Mean amplitude increased by 4.4%. Previous studies have shown that age-associated changes in the L-type calcium current contribute less than 10% to these AP duration alterations. Thus, the effects of 3,4-DAP, a blocker of the transient outward current (I_{TO}), on AP duration were measured because I_{TO} helps determine AP duration in the rat heart. The effect of 3,4-DAP on APD_{50} was smaller (12% increase; n=10) in senescent myocytes compared to myocytes from young animals (31% increase; n=14; $.01<p<=.05$). These results suggest that alterations in I_{TO} may play a key role in age-related AP prolongation in rat myocytes.

Th-AM-I2

ON THE MECHANISM BY WHICH DOXORUBICIN ABOLISHES THE OSCILLATORY POTENTIAL. Q.Y. Liu and M. Vassalle (Intro. by P.M. Gootman), Department of Physiology, S.U.N.Y., Health Science Center. Brooklyn, N.Y. 11203.

The mechanism by which doxorubicin abolishes the oscillatory potential (V_{os}) induced by calcium overload was tested in isolated guinea pig ventricular myocytes. Overdrive in the presence of norepinephrine (NE, 0.1 μM) induced V_{os} superimposed on a depolarization of the resting potential which slowly subsided after overdrive (V_{ex}): doxorubicin (10-50 μM) abolished V_{os} but it exaggerated V_{ex}. Since doxorubicin blocks adrenergic receptors, in another approach Ca-overload was induced by overdrive in the presence of high $[Ca]_o$ (5.4-8.1 mM): doxorubicin had the same effects. Trains of voltage clamp steps were used to induce I_{os} and I_{ex} (the currents underlying V_{os} and V_{ex}, respectively): doxorubicin abolished I_{os} but not I_{ex}. Doxorubicin does not decrease the slow inward current and therefore the abolition of V_{os} is not due to such mechanism. The fact that doxorubicin abolishes V_{os} but increases V_{ex} suggests that it may act by inhibiting the uptake of Ca into the sarcoplasmic reticulum (SR). Indeed, caffeine (5 mM) (which blocks the uptake of Ca into the SR) abolishes V_{os} and increases V_{ex} and the underlying currents: in its presence, doxorubicin does not abolish either V_{ex} or I_{ex}. Ni (2 mM) (which has been reported to block the Na-Ca exchange) abolishes V_{os} and I_{os} but (in contrast to doxorubicin) also decreases or abolishes V_{ex} and I_{ex}. It is concluded that doxorubicin abolishes V_{os} and I_{os} but not by inhibiting the Na-Ca exchange. Instead, doxorubicin may act by suppressing the uptake of Ca into the SR by decreasing high energy phosphates and depressing the metabolism-dependent Ca uptake. (Supported by NIH grant HL 27038)

Th-AM-I3

BEPRIDIL-NITRENDIPINE INTERACTION IN CARDIAC MUSCLE: EVIDENCE FOR A MECHANISM WHICH INVOLVES CHANGES IN CALCIUM CHANNEL GATING.

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Dihydropyridine (DHP) calcium channel antagonists bind preferentially to the inactivated state of the L-type channel in heart. On the other hand, drugs with calcium antagonistic properties and a cationic amphiphilic structure can change gating properties of the channel, delaying recovery from inactivation. It is therefore conceivable that they favor the binding of DHPs, and thus possibly promote DHP action. Indeed, bepridil has been reported to increase the negative inotropic potency of the DHP nitrendipine. Therefore, we tested our hypothesis by studying the interaction of these two calcium channel blockers.

In guinea pig left atria, the negative inotropic potency of nitrendipine was enhanced by one order of magnitude when bepridil was present in a subthreshold concentration of 3 μM. This effect was abolished when the stimulation rate was lowered (from 2.5 to 1 Hz), and it was restored when the temperature was decreased (from 32 to 23°C). These bepridil effects on DHP potency were closely mimicked by slight depolarization (induced by elevating the $[K^+]_o$ from 2.7 to 5.4 mM).

In guinea pig myocytes, whole-cell calcium currents were measured using the patch-clamp technique. Bepridil (3 μM) prolonged the time course of recovery from inactivation. Again, this behavior resembled the influence of a mild depolarization (by reducing the holding potential from -90 to -75 mV). Furthermore, the steady state block of calcium current under continuous stimulation (1 Hz) reached 50% with bepridil (3 μM) plus nitrendipine (1 μM). Each of the compounds was without any effect when given alone in these concentrations.

In conclusion, mutual influences between different drugs acting on the same calcium channel seem to exist, which can lead to a marked functional interaction.

Th-AM-I4

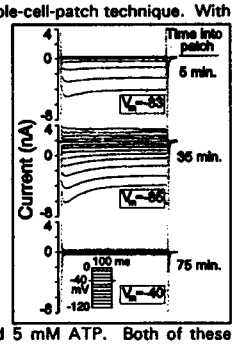
ADAPTATION OF THE CARDIAC NA/K PUMP CURRENT TO CHRONIC POTASSIUM DEFICIENCY. Hiroshi Matsuura, Michael J. Shattock and Jeremy P.T. Ward*. Cardiovascular Research and *Department of Medicine, St. Thomas' Hospital, London, U.K.

Potassium (K)-deficient diets induce a decrease in K concentration in plasma and skeletal muscle but not in cardiac muscle. It has been suggested that the heart is protected during K depletion by some modification to the sarcolemmal Na/K pump. Following chronic K depletion in the rabbit, plasma K falls to 1.5 mM and the affinity of Na-K ATPase for $[K]_o$ increases (half-maximal activation (K_m) by K falls from 1.04 to 0.62 mM) (Cameron & Ward *J Physiol* 1981:316, 8P). To investigate further the adaptation of the Na/K pump to K deficiency, we used whole-cell voltage clamp techniques to measure directly the $[Na]_i$ and $[K]_o$-dependence of Na/K pump current in ventricular myocytes isolated from control and K-depleted rabbits. Rabbits were fed either a normal or K-deficient diet for four weeks and ventricular cells were isolated by an enzymatic dissociation procedure. Perfusate and pipette solutions were designed to minimize all other components of membrane current. Under these conditions, currents measured in 0 $[K]_o$ or in the presence of 0.1 mM ouabain were identical. Na/K pump current was identified by subtracting the current recorded in the absence of $[K]_o$ from that measured in higher $[K]_o$. Maximally stimulated Na/K pump current density was similar in both groups (1.3-1.5 μA/cm²). The effect of varying $[K]_o$ between 0 and 10 mM on pump current was studied in cells equilibrated with 30 mM $[Na]_i$. Analysis of pump current against $[K]_o$ showed a significant left-shift in myocytes from K-depleted animals, such that the K_m was reduced from 1.24±0.10 to 0.77±0.19 mM. This observation is consistent with the biochemical evidence previously reported and would increase Na/K pump current by 14 % at the plasma K concentrations in the K depleted animals (1.5 mM). The effect of varying $[Na]_i$ (5 to 50 mM) on Na/K pump current was examined in cells superfused with 5 mM $[K]_o$. K_m was reduced from a control value of 18.8±2.1 to 14.1±1.2 mM in K-depleted cells. This would result in a 17% increase in Na/K pump current at 10 mM $[Na]_i$. We conclude that chronic K deficiency may induce a modification to the $[K]_o$ and $[Na]_i$-sensitivity of the Na/K pump of rabbit heart.

Th-AM-I5

INTRACELLULAR PHOSPHATE CAN MODULATE POTASSIUM CHANNEL CONDUCTANCE IN CAT CARDIOCYTES TE Schackow, PL Barrington, RE Ten Eick. Northwestern University, Chicago, IL 60611

It has been suggested that the intracellular ATP concentration ($[ATP]_i$) is important in regulating the activity of both the ATP-sensitive K channel ($I_{K,ATP}$) and the inwardly rectifying K channel (I_{K1}) but the role of the intracellular inorganic phosphate concentration ($[P]_i$) is presently unknown. To assess the role of $[P]_i$ in the control of these two K channels, we recorded action potentials and whole-cell ionic currents from adult cat cardiac ventricular myocytes using a conventional whole-cell-patch technique. With a control pipette (intracellular) solution containing 5 mM ATP and 1 mM phosphate, I_{K1} remained stable over long (> 1 hour) periods of time and $I_{K,ATP}$ was not observed. When the intracellular solution contained 0 mM ATP and 0 mM phosphate, a large $I_{K,ATP}$ developed within 20 minutes and the action potential became spike-like (APD < 50 ms); the resting potential however remained unchanged near E_K. Subsequent to this, $I_{K,ATP}$ began to diminish ("run down") and I_{K1} also began to decrease substantially. To determine whether these changes were the result of low $[ATP]_i$ or low $[P]_i$, we used an intracellular solution containing either (a) 1 mM phosphate and 0 mM ATP, or (b) 0 mM phosphate and 5 mM ATP. Both of these solutions prevented the development of $I_{K,ATP}$ and preserved the I_{K1} conductance, indicating that intracellular phosphate might be an acceptable substitute for ATP to maintain K channel function in the whole-cell configuration. We next tested the hypothesis that a phosphorylation event might be at least partially responsible for modulating K channel activity. With an intracellular solution containing 1 mM phosphate, 0 mM ATP, and 22.5 μM PKI_{6-24} (an inhibitor of the C-subunit of protein kinase A), $I_{K,ATP}$ developed rapidly while the resting potential remained stable. Subsequent to this, $I_{K,ATP}$ "ran down", the conductance of I_{K1} decreased markedly, and the resting potential depolarized (*see figure*). We conclude from these studies that (a) both ATP and phosphate can either directly or indirectly regulate K channel activity; (b) at least one phosphorylation event appears to be involved in the maintenance of I_{K1} conductance; and (c) inhibition of phosphorylation may have complex effects leading both to the development and "rundown" of $I_{K,ATP}$. TE Schackow is a Howard Hughes Medical Institute Predoctoral Fellow.



Th-AM-I6

EFFECTS OF OXIDANT STRESS ON CALCIUM INWARD CURRENT AND SODIUM PUMP CURRENT IN ISOLATED VENTRICULAR MYOCYTES. Michael J. Shattock, Hiroshi Matsuura and David J. Hearse. The Rayne Institute, St Thomas' Hospital, London, U.K.

Free radicals and consequent oxidant stress have been implicated as mediators of cellular injury, calcium (Ca) overload and arrhythmias during ischemia and reperfusion in the heart. We have investigated the cellular basis of Ca overload induced by oxidant stress (generated by photoactivation of rose bengal) using whole-cell voltage-clamped isolated rabbit ventricular myocytes. The possibility that oxidant stress enhances Ca inward current (i_{Ca}) was investigated in myocytes held at -40 mV and exposed to rose bengal (50 nM) for 5 min. Oxidant stress, however, depressed peak i_{Ca} from 1.59±0.09 (n=15) to 1.29±0.1 (n=11) nA (P<0.05) and similarly depressed the I-V relationship at all potentials studied. The slow phase of inactivation was shortened by oxidant stress (τ_{slow} = 52.9±6.1 (15) to 37.1±1.7 (11) msec; mean±sem (n), P<0.05) and cells showed evidence of Ca overload (transient inward currents, oscillatory contractions and blebbing). In a second series of experiments, intracellular sodium (Na) was reduced to zero by dialysing with Na-free pipette solutions and extracellular Na was replaced with tetraethylammonium (under these conditions, i_{Ca} can be recorded from a holding potential of -80 mV). In the absence of a Na gradient, i_{Ca} was unaffected by oxidant stress and cells showed little evidence of Ca overload. These results suggest that: (i) oxidant stress does *not* directly affect the Ca channel, (ii) the reduction of i_{Ca} in cells with a normal Na gradient may be due to Ca_i-induced inactivation of i_{Ca} and (iii) a normal Na gradient is necessary for oxidant stress-induced cellular Ca overload. This latter conclusion led us to investigate the influence of oxidant stress on Na pump function. Na pump current (i_p) was measured using whole-cell voltage clamp with pipette and Tyrode solutions formulated to inhibit voltage-gated channels and Na/Ca exchange. At all voltages, oxidant stress inhibited i_p in a time-dependent manner but did *not* affect passive membrane permeability. Thus, we conclude that oxidant stress-induced cellular Ca overload does not occur via non-specific membrane disruption or via increased i_{Ca} and may involve Na pump inhibition, a rise in Na_i and disturbance of Na/Ca exchange.

Th-AM-I7

TRANSIENT OUTWARD CURRENT (I_{to}) IN NORMAL AND HYPERTROPHIED CAT RIGHT VENTRICULAR MYOCYTES Ke Zhang, RD Harvey, PL Barrington, TE Schackow, AL Bassett, RE Ten Eick. Northwestern University, Chicago, IL 60611

The ventricular action potential (AP) is prolonged by cardiac hypertrophy, suggesting that membrane current flowing during repolarization and perhaps the properties of channels involved with the repolarizing currents may be altered by hypertrophy. Therefore, the kinetics of I_{to} were studied in single right ventricular myocytes, isolated from normal cats and cats with right ventricular hypertrophy (RVH), using the whole-cell-patch clamp technique. The incidence of I_{to} was higher in RVH (93%) than in normal myocytes (NM) (67%). The amplitude of I_{to} normalized to the cellular membrane capacitance was increased in RVH in the voltage range from 0 to +80 mV ($P < 0.01$). The mean steady state inactivation curve for I_{to} was slightly shifted to more negative potentials in RVH ($P > 0.05$). In contrast, the I_{to} activation curve in RVH was significantly shifted in the hyperpolarized direction ($P < 0.05$). As a result in RVH, the activation and inactivation curves overlap each other between -20 and 0 mV. No overlap is seen with NM. This suggests that an I_{to} "window current" might exist in RVH, but not in NM. The time course of I_{to} inactivation was fit by a single exponential in both RVH and NM; there were no significant differences between RVH and NM in the time constants of I_{to} inactivation or in the recovery of I_{to} from inactivation. Because 4-aminopyridine, an I_{to} selective blocker, can increase the AP plateau voltage and shorten AP duration, it is not unreasonable to speculate that changes in I_{to} from RVH might contribute to the changes of the plateau height and AP configuration seen during RVH.

Th-AM-I8

MYOCARDIAL ENERGETICS DURING VENTRICULAR FIBRILLATION

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Ventricular fibrillation (VF) is a lethal arrhythmia characterized by altered myocardial energetics. To elucidate the mechanism of the deterioration in energy metabolism during VF, the energy flux in myocardium was evaluated in isolated, perfused ferret hearts using phosphorus NMR spectroscopy (P-NMR). VF was induced either by perfusion with digitalis (strophanthidin, 30 μM) or by electrical stimulation. The flux in the forward reaction from phosphocreatine (PCr) to ATP, catalyzed by creatine kinase, was measured using the magnetization transfer method. During digitalis-induced VF, energy-related phosphates showed changes similar to those during hypoxia/ischemia: myocardial inorganic phosphate concentration ([Pi]) increased (1.0 ± 0.2 μmole/g wet wt in control [mean ± SE, N=8], 10.2 ± 1.2 during VF, P<0.01), and [PCr] decreased (12.4 ± 0.7 μmole/g wet wt in control, 4.3 ± 0.4 during VF, P<0.01). The energy flux during digitalis-induced VF (1.64 ± 0.16 μmole/g wet wt/sec) was significantly smaller than control (4.21 ± 0.74, P<0.01). On the other hand, VF induced by electrical stimulation showed no significant changes in [Pi] (0.6 ± 0.3 in control, 2.3 ± 1.4 during VF, N=4, P>0.20), [PCr] (11.2 ± 1.1 vs. 10.5 ± 1.2, P>0.50), or in the energy flux (3.35 ± 0.77 vs. 2.81 ± 0.49, P>0.65). These results indicate that the deterioration in energy metabolism during VF induced by digitalis was mainly due to a decreased production of energy, whereas energy supply remained unimpaired during VF induced by electrical stimulation. Intracellular calcium overload is expected to be more severe during VF induced by digitalis than during VF induced by electrical stimulation; severe Ca overload may compromise the capacity for energy generation by mitochondria during VF induced by digitalis. Thus, differences in cellular calcium loading may explain the difference in energetics in the two types of VF.

Th-AM-I9

EFFECT OF INTRACELLULAR SODIUM AND EXTRACELLULAR OSMOLARITY ON RECOVERY OF HEART CELLS FROM ACIDOSIS.

David Whalley, Paul Hemsworth, Helge Rasmussen (Intro. by P.H Barry). Royal North Shore Hospital, Sydney, Australia.

Increasing intracellular Na^+ activity (a^i_{Na}) inhibits H^+ extrusion via Na^+-H^+ exchange while a hyperosmolar milieu stimulates extrusion. Since a^i_{Na} changes in an isosmolar milieu the relationship between a^i_{Na}, osmolarity and Na^+-H^+ exchange is of interest. We measured, using ion sensitive microelectrode techniques, intracellular pH (pH_i) and a^i_{Na} of guinea pig papillary muscles in HEPES-Tyrode's solution. After a 20 mM NH_4Cl prepulse pH_i decreased by 0.3 units. Maximal pH_i recovery rate was 0.041 $\pm$ 0.006 units/min (Mean $\pm$ SE, n=6) in 5.6 mM K^+ isosmolar solution. Recovery increased to 0.064 $\pm$ 0.009 units/min ($p < 0.01$) in solution made hyperosmolar with 100 mM sucrose. In K^+-free isosmolar solution pH_i recovery after an NH_4Cl prepulse was 0.031 $\pm$ 0.003 units/min while a^i_{Na} increased to 20.1 $\pm$ 3.2 mM. In hyperosmolar K^+-free solution pH_i recovery was increased to 0.055 $\pm$ 0.006 units/min ($p < 0.01$) while a^i_{Na} was elevated to 32.7 $\pm$ 3.0 mM. Recovery rate of pH_i was reduced from 0.047 $\pm$ 0.005 units/min in isosmolar solution to 0.026 $\pm$ 0.006 units/min ($p < 0.01$) in solution with an osmolarity 0.75 of normal (constant Na_o) while at the time of maximal recovery a^i_{Na} was 9.7 $\pm$ 0.6 mM and 7.3 $\pm$ 0.9 mM respectively. In summary, 1) Hyperosmolarity enhances pH_i recovery even when a^i_{Na} is markedly elevated. 2) Hypoosmolarity inhibits pH_i recovery despite a decrease in a^i_{Na}. Conclusion: The effect of osmolarity and probably cell volume on Na^+-H^+ exchange overrides the effect of the transmembrane Na^+ gradient.

Th-AM-J1

INACTIVATION OF SARCOPLASMIC RETICULUM Ca ATPase BY NIPI *+**J.-J. Lacapere, G.C.R. Ellis-Davies, *N.M. Green** and **J.H. Kaplan.** *National Institute for Medical Research, Mill Hill, London NW7 1AA, U.K., Department of Physiology, University of Pennsylvania, Philadelphia,PA 19104-6085 and +CNRS, URA 1290, Paris

Incubation of SR vesicles with NIPI, N-(2-nitro-4-isothiocyanophenyl)-imidazole, a new lysine-reactive probe for P-type ATPases (Ellis-Davies and Kaplan, *J. Biol.Chem.*, in press) results in inactivation of Ca ATPase activity. The inactivation kinetics are similar in the presence of Ca^{2+} or EGTA and the inactivation is prevented by the presence of ADP. The use of [^{3}H]NIPI shows that (i) in SDS PAGE covalent [^{3}H]NIPI incorporation occurs exclusively in the Ca ATPase protein and (ii) almost complete inactivation is obtained with 1 molecule of [^{3}H]NIPI bound per enzyme molecule. The modified enzyme has unaltered Ca^{2+} binding properties (as shown by intrinsic fluoresence measurements) but has lost the capacity to bind [^{14}C]ADP. There is also inhibition (about 50%) of the usual phosphorylation level obtained with [^{32}P]P_i. These results suggest that modification of a single ATP-protectable lysine residue on the SR Ca ATPase results in a loss of ATP binding and consequent inactivation. Tryptic digestion and peptide isolation by HPLC is underway to identify the essential lysine residue.

Th-AM-J2

CALCIUM TRANSPORTING ATPase: CALCIUM IONS OCCLUDED ON THE PHOSPHOENZYME MIX BEFORE DISSOCIATING.
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Graduate Department of Biochemistry
Brandeis University, Waltham, MA 02254.

Several investigators have shown that two Ca^{2+} ions bind sequentially to kinetically distinct "inner" and "outer" high affinity sites on the cytoplasmic side of nonphosphorylated CaATPase of sarcoplasmic reticulum (SR). Phosphorylation of the enzyme by ATP results in occlusion of the two Ca^{2+} ions and is followed by dissociation of the ions into the lumen of the SR. The rate at which the occluded Ca^{2+} ions become insensitive to ADP (25 °C, pH = 7.0, 100 mM KCl) as they are internalized into empty or passively loaded (20 mM Ca^{2+}) native intact SR vesicles was measured by quenching with 7 mM ADP/10 mM EGTA. It was shown by selectively labeling the Ca^{2+} ions bound to the free enzyme that both the "inner" and "outer" Ca^{2+} ions become insensitive to the ADP quench and are internalized with the same rate constant of 26-34 s^{-1}; there was no lag for either ion when the lumenal [Ca^{2+}] was 20 mM. The observed rate constants were found to vary from 18-50 s^{-1}, however, with different enzyme preparations.

A single Ca^{2+} ion inhibits the hydrolysis of E-P by reversing the reaction E-P·Ca·Ca ⇌ E-P·Ca + Ca_{in} [Khananshvili et al.. (1990) FEBS Lett. 260, 83], which is consistent with sequential dissociation of Ca^{2+} from E-P·Ca_2. However, 20 mM lumenal [Ca^{2+}] does not affect the rate of loss of ADP-sensitivity of either the "inner" or "outer" Ca^{2+} ion. Therefore, the order of binding of the two Ca^{2+} ions to the free enzyme is lost following phosphorylation by ATP; the two ions mix rapidly (> 10^3 s^{-1}) when bound to the phosphorylated enzyme before they dissociate. This conclusion differs from a previous report [Khananshvili & Jencks (1988) Biochemistry 27, 2943]. Mixing may occur because phosphorylation causes a conformational change that weakens the interaction of carboxylate and other ligating groups with the two ions in E-P·Ca_2 (low affinity) compared with E·Ca_2 (high affinity).

Th-AM-J3

CHANGES IN ION ACTIVATION OF THE Na,K-ATPase IN ALTERNATING CURRENTS. Martin Blank and Lily Soo, Dept. of Physiology and Cellular Biophysics, Columbia University, 630 West 168th Street, New York, NY 10032

Low frequency alternating currents (AC) through cell suspensions lead to ion accumulation by cells via the Na,K-ATPase (Serpersu and Tsong, J. Biol. Chem. 259:7155,1984). We have shown changes in ATP splitting by the enzyme when AC flows through Na,K-ATPase vesicle suspensions (Blank and Soo, Bioelectrochem. Bioenerg.22:313,1989). The AC signals decrease ATP splitting by the normal enzyme, with the maximum effect at about 100 Hz, and increase the enzyme activity, when the activity is lowered. Both inhibition and activation by AC can be explained by variations in ion activation of the enzyme brought about by an apparent increased binding of Na and K ions (Blank and Soo, Bioelectrochem. Bioenerg. 24:1990 in press). The frequency dependence appears to arise from the kinetics of reactions at membrane surfaces (Blank, J.Electrochem. Soc. 134:343, 1112, 1987). Our estimated threshold for the effect of AC on ATP splitting is about 20μV/cm for 15 minute exposures, which is comparable to the induced electric fields in experiments reporting changes in protein synthesis. These studies suggest a mechanism by which induced currents from low frequency electromagnetic signals affect cellular processes without penetrating the cell membrane.
(We thank the ONR and EPRI for their support.)

Th-AM-J4

PARADOXICAL EFFECTS OF HYDROSTATIC PRESSURE ON (Na,K)-ATPase: EVIDENCE THAT INTERMEDIATES WITH OCCLUDED CATIONS HAVE DECREASED VOLUME. **P.A. George Fortes, with the technical assistance of E. Almendarez. Department of Biology, University of California San Diego, La Jolla, CA 92093-0116.**

We have studied the effect of pressure on ATP hydrolysis by purified (Na,K)-ATPase from dog kidney at pH 7.5 and 25 C as a function of ATP, Na, and K concentration. Pressure (1-800 bar) inhibited ATPase activity in the presence of 125 mM Na + 20 mM K; there was no difference in the responses to pressure with either 25 uM or 3 mM ATP. With 125 mM Na, and no K, increasing pressure stimulated the ATPase activity (~300% at 400 bar). The stimulation by pressure was larger at lower Na concentration (with 25 mM Na 400-500% stimulation at 400-800 bar), and was smaller at higher Na concentration: with 0.4 M Na 20-50% stimulation at 200-400 bar followed by inhibition at higher pressure. Oligomycin inhibited the stimulation by pressure. The effects of high pressure on Na-ATPase activity resemble the effect of increasing Na concentration at 1 bar. The simplest explanation of these results is that intermediates of the ATPase reaction that contain occluded (or bound) cations are favored by high pressure because they occupy a smaller volume. In the presence of Na and K, deocclusion of K would be inhibited by pressure, thus inhibiting turnover. In the presence of Na alone high pressure would favor Na binding and stabilize EP(Na_2), which hydrolyzes faster than EP, and, at higher Na, high pressure would stabilize EP(Na_3), which has a low turnover rate and interacts with oligomycin. **(Supported by ONR N-00014-88-K-0324).**

Th-AM-J5

CONFORMATIONAL ANALYSIS OF THE H,K-ATPASE. E.C. Rabon, G. Sachs and S.J.D. Karlish*; UCLA & VAMC 90073 and *Weizmann Institute of Science, Rehovoth, Israel 76100

Following a recent demonstration that H,K-ATPase can actively transport Na^+ at a low rate (Polvani, C, Sachs, G & Blostein, R. (1989) *J.Biol.Chem.* 264, 17854-17859), we have looked for and found effects of Na^+ ions on the conformational state of gastric H,K-ATPase labelled with fluorescein isothiocyanate (FITC). Na^+ ions reverse the K^+-induced quench of the fluorescein fluorescence and somewhat enhance fluorescence in the absence of K^+ ions. Equilibrium titrations of the cation effects show that Na^+ and K^+ ions are strictly competitive with apparent dissociation constants of $K_{Na}^* = 62$ mM (n=2) and $K_K^* = 6.6$ mM (n=2). The observations demonstrate that Na^+ ions bind to and stabilize the high fluorescence E_1 form of the protein while K^+ ions stabilize the low fluorescence E_2 form. Elevation of pH from 6.4 to 8.0 increased the apparent affinity of the Na^+ ions from approximately 62 mM to 10.2 mM, consistent with competition between H^+ and Na^+.

The action of Na^+ to stabilize the E_1 form was used to measure the rate of the E_2K-- E_1Na transition with a stopped-flow fluorimeter. The rate at pH 6.4 and 20 C is 18.1 sec^{-1}. In addition the rate of the reverse conformational transition E_1K - - E_2K has been measured at several K^+ concentrations. From the hyperbolic dependence on K^+ concentration a maximal rate of 211±32 sec^{-1} and intrinsic K dissociation constant on E_1 of 64.6 ± 3.3 mM have been estimated. The kinetic and equilibrium data are self-consistent and support the proposed action of Na^+ and K^+ ions.

Compared to Na,K-ATPase, the H,K-ATPase exhibits a lower affinity for Na^+ on E_1 and a much faster rate of the E_2K -- E_1Na transition, but a similar affinity for K^+ ions on E_1 and rate of the transition E_1K -- E_2K. The significance of the increased rate of transition E_2K to E_1K in the H,K-ATPase is that K^+ occlusion, well characterized in the Na,K-ATPase, is not easily detected in the H,K-ATPase due to the rapid dissociation of K^+ from the ATPase. It is also evident that this rapid transition does not serve to minimize the movement of K^+ "uncoupled" from phosphate transfer. It is possible that the high rate of K^+/K^+ exchange of this enzyme may indicate that coupling between K^+ transport and ATP hydrolysis is not tight. [Supported by NIH 34286 & VA]

Th-AM-J6

DO HYDROGEN BONDED WATER MOLECULES CONDUCT PROTONS WITHIN THE MEMBRANE DOMAIN OF PROTON-DEPENDENT ATP SYNTHASES? D.W. Deamer and M. Akeson, Department of Zoology, U.C. Davis, 95616.

Water in the form of hydrogen bonded chains has been proposed as a proton transport mechanism in the Fo subunits of coupling membranes (Williams, 1985; Sebald and Hoppe, 1984; Schulten and Schulten, 1985; Deamer and Nichols, 1989). This concept is consistent with several observations: 1) A single strand of H-bonded waters within the gramicidin channel can transport protons at a rate equivalent to the proton flux required for ATP synthesis (Akeson and Deamer, 1990). 2) Interfaces between α-helices of synthetic peptides can conduct protons with high selectivity (Lear et al., 1988) perhaps along water strands similar to those spanning the gramicidin channel. 3) The interfaces between *c* subunits and the *a* subunit of Fo are plausible candidates for a site at which H bonded water might conduct protons to an active site. 4) Sodium ions as well as protons can carry charge through the Fo subunit of a halophilic bacterium, *P. modestum*, thereby driving ATP synthesis (Laubinger and Dimroth, 1989). In this special case, membrane waters could either transport protons by a H-bonded chain mechanism or partially hydrate Na^+ and thus facilitate its diffusion as is true in gramicidin. We will present an integrated model for charge transport by Fo which incorporates the above observations. Supported by ONR Contract N00014-85-0231.

Th-AM-J7

PROBING PROTEIN DYNAMICS IN A YEAST H^+-ATPASE. S.L. Harris[1], SongQing Na[1], J.E. Haber[1], D. Seto-Young[2], B.C. Monk[2] and D.S. Perlin[2]. [1]Dept. of Biology, Brandeis University, Waltham, MA and [2]Dept. of Biochemistry, The Public Health Research Institute, New York, NY.

A genetic approach has been used to probe protein dynamics in the plasma membrane H^+-ATPase from *Saccharomyces cerevisiae*. The starting point for this study was the finding that a Ser368-->Phe mutation near the site of phosphorylation (Asp378) caused a general depolarization of steady-state membrane potential (hygromycin B-resistance) and a sensitivity to acid pH (<pH 4.0) in whole yeast cells. The biochemical properties of the mutant enzyme revealed an acid-sensitive Vmax component and a pronounced insensitivity to inhibition by vanadate. These distinct cellular and biochemical properties facilitated a detailed revertant analysis to identify protein structure domains that interact directly or indirectly with the localized catalytic region defined by the Phe368 mutation. Partial revertants were selected that maintained a depolarized membrane potential but were no longer sensitive to acid pH. Revertant enzymes were identified which either changed the primary site (Phe368) mutation or left the Phe368 mutation intact and altered a secondary site amino acid. Second site mutations were found within the catalytic domain and putative transmembrane segments 1,2,3 & 7. Of 13 such second site mutations, Cys148->Ser was found 4 times and Val289->Phe was found 3 times. The revertant enzymes all have a stable Vmax in the acid pH range but display vanadate sensitivities ranging between the insensitive Phe368 mutant and the fully-sensitive wild type enzyme. A similar analysis was carried out for another pH sensitive mutation that changed Ala135->Val. This mutation is expected to lie within transmembrane segement 1 and second site mutations restoring full activity were found in transmembrane segments 2 and 3. A structural model emphasizing transmembrane segments 1 & 2 has been used to explain how mutations within the bilayer may influence a cytoplasmically-located catalytic region.

Th-AM-J8

POSSIBLE INVOLVEMENT OF A VACUOLAR TYPE H^+-ATPASE IN THE MAINTENANCE OF A HIGH INTRACELLULAR pH IN HUMAN TUMOR CELLS. Raul Martínez-Zaguilán, Gloria M. Martínez, and Robert J. Gillies Dept. Biochem. U. Ariz., Tucson, AZ 85724.

Insertion of a yeast H^+-ATPase in the plasma membrane of normal NIH-3T3 cells renders them tumorigenic[1]. These cells maintain a higher pH^{in} than their non-tumorigenic counterparts under physiological conditions[2]. These observations raise the possibility that H^+-ATPases are present in naturally occurring tumor cells. In the present study, we screened 9 different normal and transformed primary human cell lines for the presence of plasmalemmal H^+-ATPase activity using specific inhibitors of vacuolar-type (bafilomycin) and H^+/K^+ (SCH-28080) H^+ ATPases. Our results indicate that most tumorigenic cells maintain a higher pH^{in}, a higher rate of H^+ extrusion, and a higher rate of glycolysis, compared to non-tumorigenic cells. Bafilomycin induces a significant decrease in pH^{in} in melanoma, leiomyosarcoma, mesothelioma and breast carcinoma cells, but not in other tumor or normal cells. In contrast, SCH28080 did not affect pH^{in} in any of these cells. Simultaneous measurement of pH in cytoplasmic and endosomal-lysosomal compartments indicates that the effect of bafilomycin on pH^{in} is due to inhibition of H^+-ATPase at the plasma membrane and not at the vacuole. These results suggest that a vacuolar type H^+-ATPase may be expressed in the plasma membrane of some tumor cells. (R01 GM43046-01) [1]Perona and Serrano, Nature 334: 438, 1988; [2] Gillies et.al., PNAS 87: 1990 (In press).

Th-AM-J9

MULTINUCLEAR MAGNETIC RESONANCE STUDIES OF HIGH DENSITY MAMMALIAN CELLS. RJ Gillies, PG Scherer and J-P Galons; Depts. Biochem., Physiol. and Radiol.; Univ. ARIZONA HSC; Tucson, AZ 85724.

NMR analyses of cultured cells is an important extension of this powerful technique. However, NMR signals are inherently weak, necessitating extremely high cell density cell cultures, which are difficult to stabilize. Over the past few years, we have developed systems to grow and maintain normal and tumorigenic mammalian cells to densities approaching those in tissues. These systems are hollow fiber bioreactor circuits wherein the custom, 25mm bioreactors are perfused continuously with media whose composition is maintained through constant bleed-and-feed. Medium temperature and gas composition are finely regulated. ^{31}P spectra of these systems can be obtained in as little as 30 seconds for periods exceeding months. We have used these systems to investigate bioenergetic and metabolic differences between static and proliferating normal and tumor cells. We have used ^{31}P spectroscopy to observe intracellular pH, Mg^{++}, phospholipid metabolism, and ATPase kinetics via saturation transfer. These data indicate that there are differences in phosphoryl choline and ATP metabolism between growing and non-growing cultures, and that there are reversible effects of pH on glycerol phosphoryl diester metabolism. We have also used ^{13}C spectroscopy to follow the metabolism of labeled glucose. These spectra allow observation of intermediates, which can be used to determine control strengths in the glycolytic pathway. In addition, ^{1}H and ^{2}H NMR have been used to monitor water relaxation rates (T_2, T_1) during culture growth. (supported by R24 RR05625-02)

Th-AM-K1

TWO CLASSES OF CAP BINDING SITES

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Introduction: Thirty-one CAP binding sites, previously confirmed by footprinting or deletion experiments, revealed the consensus aAa-TGTGA-tctaga-TCACA-tTt. The subset of these sites with 8 bp spacers instead of 6 bp produced the consensus AAa-tGTGa-taaatttta-tCACa-TTt [Barber A. and Zhurkin V., *J. Biomol. Struct. Dyn.* 8(2), 1990]. We proposed that there is a correlation between the length of the spacer and its sequence.

Methods: To investigate the significance of the eight base pair (bp) spacer, we used electrophoresis mobility shift assay to test how well synthetic DNA sequences with 8 bp spacers bind CAP.

Results: We found that sequences with an eight bp spacer were capable of binding CAP. In accord with other authors we also found that the sequences most similar to the consensus sequence bound CAP best. The abilities of the synthetic DNA binding sites to bind CAP were in the following order:

TGTGA - GTTAGC - TCACA	Strongest binding
TGTGA - GTTAGC - TCACT (*lac*)	2nd
TGTGA - TGGCGCCA - TCACA	3rd
TGTGA - GTTAGC - TCTGA	4th
TGTGA - TAAATTTA - TCACA	5th
TGTGA - TTCTAGAA - TCACA	6th
TGTGA - TAAATTTA - TCTGA	7th
TGTGA - TAAATTTA - CTTGA	No binding

Conclusion: Thus, we see two classes of CAP binding DNA sequences: (1) those with a conventional six bp spacer between GTG and CAC boxes and (2) those with an eight bp spacer. This is the first time that CAP binding sites with an 8 bp spacer have been shown to bind CAP *in vitro*.

Th-AM-K2

LACTOSE OPERON REGULATION BY PROTEIN DISPLACEMENT AND BINDING COOPERATIVITY

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The lac repressor and catabolite gene activator protein (CAP) regulate the activity of the E. coli lactose operon. Because these proteins occupy overlapping DNA sequences, binding competition resulting in the displacement of one or both proteins is expected. Using coupled DNaseI footprinting and electrophoresis mobility shift assays, we find that the expected displacements do occur, but that the resulting complexes are 100-1000-fold more stable than expected. This result suggests that strong CAP-repressor interactions compensate for the loss of specific protein-DNA contacts in these complexes. Such interactions may play a role in the control of the lac operon. If they do, it is one that is not predicted by current regulatory models.

Th-AM-K3

DNA BENDING INDUCED BY CRO PROTEIN BINDING AS REVEALED BY GEL ELECTROPHORESIS

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Cro is one of a number of proteins that participate in regulation of transcription of lambda bacteriophage. Recent crystallographic diffraction and NMR studies of the DNA-Cro complex can help to elucidate the structure of the Cro protein in the complex, but little is known about protein induced changing of DNA conformation under solution conditions. Gel electrophoresis of DNA and protein-DNA complexes has been an important tool for studies of protein induced DNA bending. We report studies which explore the interaction of Cro protein and its primary site of action, the 17 bp lambda phage O_R3 sequence. Oligonucleotides 21 bp in length containing the O_R3 recognition site were synthetized and ligated both in the presence and absence of excess Cro. Circularization frequences were measured by 2D gel electrophoresis experiments on both types of samples. It is shown that in the presence of Cro protein, circular DNA molecules are formed with significant frequency. No circular molecules are observed in the DNA samples ligated in the absence of Cro. The experiments clearly demonstrate that DNA bending is induced by Cro protein to this operator site. The value of the DNA bending angle is estimated from these data.

Th-AM-K4

ROTATIONAL DIFFUSION OF NUCLEOSOME CORE PARTICLES - SHAPE CHANGES AT LOW IONIC STRENGTH

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We have used the decay of the fluorescence anisotropy of ethidium intercalated into the DNA of nucleosome core particles to monitor the rotational diffusion of the particles. At 10 mM ionic strength, where the core particles should have a "native" conformation, we find a rotational correlation time of 164 ns, which is consistent with hydrodynamic calculations based on the expected size and shape of the hydrated particle. Since the rotational correlation time is very sensitive to changes in shape, we can use it to observe changes in the shape of the core particles as a function of ionic strength. At low ionic strength (<1 mM), where the particle undergoes the "low salt transition" as measured by steady-state tyrosine fluorescence anisotropy, we observe very little change in shape until the ionic strength is decreased below ~0.2 mM. At the minimum ionic strength obtained (~0.01 mM) we measure a rotational correlation time of 290 ns, indicating that the particle has opened up to a more extended form. This opening is not a reversible transition in that the particle does not return to the closed form until the ionic strength is increased above 10 mM. At higher ionic strengths (≥600 mM) we also see an apparent increase in the rotational correlation time, but the overall features of the anisotropy decay are completely different from those at very low ionic strength. Here we observe changes which indicate an apparent increase in the DNA flexibility as the core particle begins to dissociate.

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Th-AM-K5

T4 REPLICATION ACCESSORY PROTEINS FORM A COMPLEX VISIBLE BY ELECTRON MICROSCOPY WITH NICKED BUT NOT INTACT DNA

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Replication of the bacteriophage T4 genome is accomplished by a core DNA polymerase, the product of T4 gene 43, along with a set of accessory proteins (products of genes 44,45 and 62), which are required to achieve a viable synthesis rate, processivity and fidelity of replication. This set of proteins, along with the T4 g32p, is a minimal requirement for *in vitro* template-directed DNA synthesis with properties comparable to the *in vivo* phenomenon. We have previously reported the observation, by cryo-electron microscopy, of elongated structures ("hash marks") formed on double-stranded phage DNA, by the accessory proteins in the presence of g32p. We have also established the requirement for ATP hydrolysis (catalyzed by the DNA-stimulated g44p ATPase) for the observation of these structures.

We have examined the DNA cofactor requirements for the formation of these structures, and find that nicks in the DNA are necessary. The complexes do not form on intact, supercoiled plasmids, nor on linear fragments; in contrast, plasmids specifically nicked by the fd g2p initiator protein are suitable substrates for formation of the complexes, as are linear fragments containing the nicks. The g44p ATPase activity, which is thought to drive the assembly of the accessory proteins complex, has a parallel requirement for nicks in the DNA : activity is low in the presence of supercoiled DNA, but stimulated by nicks, and even futher by gaps in the double-stranded DNA. Though only one nick is present in each DNA molecule, multiple, often clustered, hash marks are usually visible along strands of DNA. This observation suggests that the structures form at nicks, perhaps with some cooperativity, but may then migrate along the DNA.

Th-AM-K6

Idling Turnover Kinetics and Fidelity of *E. coli* RNA Polymerase

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We have been investigating the idling turnover kinetics of *E. coli* RNA polymerase stalled at a specific site on a DNA template. These experiments were performed on two DNA templates, pAR1707 (gift of Michael Chamberlin) and pDE13, which contain the T7A1 and λP_R promoters, respectively.

pAR1707: +1 +21
T7A1 promoter—AUCGAGAGGGACACGGCGAAUAGCCAU—
pDE13: +1 +25
λP_R promoter—AUGUAGUAAGGAGGUUGUAUGGAACAAC—

As can be seen from the transcript sequences shown above, stable stalled elongation complexes can be made by adding ApU and omitting UTP for T7A1 transcripts (A_{20} complexes), and by omitting CTP for λP_R transcripts (A_{24} complexes). The idling turnover rate is measured at these stalled sites by monitoring the production of ^{32}P-pyrophosphate ($^{32}PP_i$) from γ-[^{32}P]-ATP or γ-[^{32}P]-GTP. The turnover rates are measured as a function of $^{32}PP_i$ concentration as well as NTP concentrations. The rate of NTP turnover in these idling turnover experiments is 2000 to 10,000 times slower than the steady-state synthesis rate of ~30/sec. In addition, the observed rates appear to be a result of misincorporation and excision as opposed to correct incorporation and excision. The results will be discussed in terms a kinetic mechanism for RNA polymerase fidelity.

Th-AM-K7

DOES E. COLI RNA POLYMERASE PREVENT TRANSCRIPTIONAL ERRORS AT THE SITE OF BOND FORMATION?

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The fidelity of transcription is higher than predicted from the greater stability of Watson-Crick base pairs compared to other nucleotide base pairs. We are asking whether E. Coli RNA polymerase helps to prevent transcriptional errors at the point of internucleotide bond formation. To answer this question we use the system that we have developed for determining the geometry of interaction of NTP substrates at the active site of the enzyme. The two metals, Zn and Mg, at this site are used as probes; they are replaced by paramagnetic Mn, distances from metal to points on the substrates are measured by NMR using the paramagnetic effect on the relaxation of substrate nuclei, and metal-metal distances are determined by the Leigh method using EPR. We have found that the enzyme is flexible, as shown, for example, by changes in the distance between the metals bound to the enzyme. The addition of ATP or 3'dATP to the enzyme increases this distance. The addition of the complementary poly(dT) to enzyme bound to 3'dATP then decreases the distance, while addition of partly non-complementary poly(dAdT) to enzyme bound to ATP further increases the distance. Similar results are obtained in analogous systems using G and C bases instead of A and T. The 3'deoxynucleotides are used with complementary systems to prevent RNA synthesis from occurring while relaxation measurements are carried out. The results are compatible with the ability of the enzyme to assume two conformations, one to place bases complementary to DNA bases into an optimal configuration for bond formation, and the other to place non-complementary bases into a suboptimal configuration.

Th-AM-K8

GAMMA-RAY FOOTPRINTING OF EcoRI AT ELEVATED PRESSURE

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Irradiation of aqueous solutions with gamma-rays from the radioactive decay of ^{60}Co generates hydroxyl radicals which can be used for high-resolution footprinting of protein-DNA interactions. The production of hydroxyl radicals in this manner can be employed under a number of experimental conditions not amenable to conventional footprinting protocols. The effect of pressure ($\leq$ 200 MPa) on the the noncovalent interaction between EcoRI and its 6-base pair recognition sequence is being studied using gamma-rays. Qualitatively, as the pressure is increased the size of the DNA binding region becomes smaller, i.e. fewer base pairs are covered by the enzyme. This is in agreement with the observed inhibition of endonuclease activity as high pressure. In addition, the specificity of EcoRI for Mg^{2+} appears to be relaxed at elevated pressure.