Th-AM-Sym1 α-HELIX FORMATION BY SHORT PEPTIDES IN WATER, Robert L. Baldwin, Department of Biochemistry, Stanford University School of Medicine, Stanford, CA 94305.

Chemically synthesized analogs of the C-peptide (residues 1-13) or ribonuclease A are used to investigate specific sidechain interactions that enhance α-helix stability in H_2O. There is a major charged-group effect on helix stability, which causes the stability to depend strongly on pH between pH 2 and pH 8. In addition, the charged-group effect gives rise to large and specific changes in helix stability with ionic strenth. The charged-group effect has been assigned to two residues near opposite ends of the helix, $Glu2^-$ and $His12^+$, by pH titration and by making the substitutions Glu2 → Ala and His12 → Ala. Current work indicates that helix stability is increased both by the interactions of these charged groups with the helix dipole and by a $Glu2\cdots Arg10^+$ salt bridge. The changes in helix stability cannot be predicted from rules based on host-guest experiments: His^+ is a strong helix breaker in host-guest experiments. By combining suitable amino acid substitutions, it has been possible to double the fraction helix shown by C-peptide in optimal conditions (pH 5, 0°C). The peptides have been synthesized and characterized by Eunice J. York and John M. Stewart (University of Colorado Medical School, Denver) and these experiments have been made by Kevin R. Shoemaker and Peter S. Kim.

Th-AM-Sym2 THE THERMODYNAMICS OF PROTEIN DENATURATION: SINGLE AND MULTIPLE AMINO ACID VARIANTS OF BACTERIOPHAGE T4 LYSOZYME

W.J. Becktel, W.A. Baase, B.L. Chen, D.C. Muchmore, C.G. Schellman and J.A. Schellman
Institute of Molecular Biology, Univ. of Oregon, Eugene, OR 97403-1229

Variants of bacteriophage T4 lysozyme have been constructed by means of single and multiple amino acid replacements and their thermodynamics of unfolding investigated. The aim is to determine the role which individual amino acid side chains play in the overall stability. Both randomly generated and site directed mutations have been studied. The investigation includes the determination of the Tm of the variants, their free energy, enthalpy and entropy of unfolding as a function of temperature, and the dependence of these parameters on pH. Approximately thirty variants have been investigated. Almost all of them lower the stability of the protein. The exceptions are a series of variants containing an SS bridge which is absent in the wild type [L.J. Perry & R. Wetzel (1984) Science 226, 555-557]. Changes in enthalpy and entropy from a single point mutation can be quite large, but they are also invariably compensatory so that free energy changes are much smaller. The double and higher amino acid replacements which have been studied so far have shown an additivity in the thermodynamic changes.

The eventual goal is to correlate these stability changes with the structural studies being performed by B.W. Matthews and his associates.

(This work has been supported by PHS grant GM 20195 and by NSF grant PCM 8104339.)

Th-AM-Sym3 A SCALE OF HYDROPHOBICITY DERIVED FROM PROTEINS OF KNOWN STRUCTURE,

George D. Rose, Department of Biological Chemistry, Hershey Medical Center, Pennsylvania State University, Hershey, PA 17033.

The hydrophobic effect has long been a topic of intense chemical interest. After publication of Kauzmann's seminal review (Adv. Prot. Chem. 1959, 14, 1) it was widely accepted that hydrophobicity serves as the major driving force for the self-assembly of globular proteins. Kauzmann's arguments were based upon the thermodynamics of model compounds. Does the transfer of a hydrocarbon molecule from liquid hydrocarbon to water adequately reflect the change in side-chain accessibility when a protein unfolds? Calculations using proteins of known structure (Chothia, J. Mol. Biol. 1976, 105, 1; Janin, Nature, 1979, 277, 491) concluded that the answer to this question is "no"; hydrophobic groups were found to have only a weak tendency to be buried within the molecular interior, sequestered from the surrounding solvent.

These earlier findings are now reassesed in the light of a recent empirical study (Rose et al., Science, 1985, 229, 834). Here, the average area that residues bury upon folding is calculated and shown to be well correlated with solution measurements of the free energy of transfer of residue side-chains from water to organic solvent. A characteristic quantity, the mean fractional area loss, can be defined as the average area that a residue loses upon folding normalized by the standard state area for that residue. Residues are found to divide neatly into three groups based upon the derivative of their mean fractional area loss.

Th-AM-Sym4 DESIGN OF A SYNTHETIC FOUR-HELICAL BUNDLE PROTEIN. W. F. DeGrado, S. P. Ho, P. C. Weber, F. R. Salemme, W. Wilcox, S. Eshita, P. Pryciak, and D. Eisenberg, E. I. du Pont de Nemours & Company, Central Research & Development Department, Experimental Station, Wilmington, DE 19898, and Molecular Biology Institute, University of California, Los Angeles, CA 90024.

An artificial protein of four antiparallel alpha helices has been designed and is being synthesized by chemical methods. It resembles the four alpha-helical bundle class of naturally occurring proteins, described by Weber and Salemme (Nature, 287:82-84 (1980)). To simplify synthesis the protein has been designed in 9 modular units: four identical helices, three identical linkers which join two of the helices, and N- and C-terminal units. Thus the entire structure of 90 residues can be represented as: N-term + Helix + Link + Helix + Link + Helix + Link + Helix + C-term. Each helix consists of 16 residues designed to fold into a highly amphiphilic alpha-helix, with leucyl residues along one face and lysyl and glutamyl residues along the opposite face. In models, the leucyl residues from four helices pack tightly in a hydrophobic interaction, exposing all charged side chains to solvent. The first stage of the work involved the optimization of the sequence of the helices. A number of peptides were prepared which cooperatively self-assembled into tetramers or trimers of alpha-helices, and the free energy for tetramerization of the best of these peptides was app. -25 kcal/mol. X-ray grade crystals of this 16-residue peptide and a slightly shorter analogue have been obtained and are currently being analyzed. In a second stage, we are attempting to join two helices into a hairpin with an appropriately designed linker. Approaches to design and synthesis of these structures will be discussed.

Th-AM-A1 POTASSIUM, SODIUM AND CALCIUM CURRENTS IN EMBRYONIC CULTURES OF DROSOPHILA NEURONS. Lou Byerly, Section of Neurobiology, Department of Biological Sciences, University of Southern California, Los Angeles, CA. 90089-0371.

Whole-cell patch clamp studies have been performed on cultured Drosophila neurons. Cultures were prepared by disaggregating whole gastrulating embryos and plating the cells in serum-enriched Schneider's medium. Nerve cell bodies of 5 to 8μm in diameter were studied. Well-rounded cells with fine processes were selected in order to improve the quality of the clamp. The whole-cell I-V relation was linear from -140 to -40mV with resistances of 2 to 10GΩ. Steps to potentials more positive than -40mV activated outward currents. These currents were primarily carried by K^+, since the tail currents reversed below -65mV and the outward current rapidly disappeared after the patch broke when the pipet was filled with a CsCl solution. The magnitude of this voltage-dependent K conductance was 2-6nS at +20mV for most cells. At +20mV this K current activated to half maximum in 1-2ms and usually decayed by 40% from the maximum at the end of a 80ms pulse. Inactivation was not complete; 50% of the current remained at 800ms.

When K^+ was removed from internal and external solutions (CsCl in pipet), depolarizing steps evoked inward currents in some cells. A rapidly-inactivating inward current which activated at -25mV was lost when external Na^+ was replaced by $Tris^+$, and so is probably carried by Na^+. A prolonged inward current was activated by steps to 0mV in Na-free external solution. This current appears to be a Ca current since it increased when the external Ca^{2+} concentration was increased and was blocked by 0.1mM Cd^{2+}.

Supported by NIH grant NS15341 and RCDA NS00797.

Th-AM-A2 VOLTAGE-DEPENDENT K^+ CHANNEL IN THE YEAST PLASMA MEMBRANE. Michael C. Gustin, Boris Martinac, Yoshiro Saimi, Michael R. Culbertson and Ching Kung (Intr. by G. Nicol) Laboratory of Molecular Biology, University of Wisconsin, Madison, WI 53706

Ion channels are found in a wide variety of tissues and organisms. However, except in Paramecium and Drosophila, combined genetic and biophysical investigation of ion channels is often difficult or impossible. Because the yeast, Saccharomyces cerevisiae, can be genetically manipulated with ease, a genetic approach coupled with the ability to record ion channel activity in the plasma membrane would serve to advance our knowledge of channel structure, function and regulation.

Toward this goal, we have begun to study the native ion channels in the plasma membrane of yeast spheroplasts using the patch-clamp technique. Spheroplasts from diploid cells were obtained by zymolyase digestion of the cell wall. Gigaohm seals against the cell membrane were obtained using vigorous suction and were generally slow to develop (tens of seconds). Several different ion conductances were observed both in a whole-cell recording mode and in excised patches. The most prominent conductance was analyzed in detail and has the following characteristics: (i) activation by depolarization, (ii) slope conductance of 20-30 pS, (iii) high selectivity for K^+ over Na^+, (iv) inhibition by external TEA^+ or Ba^{2+} but not by TMA^+ or Ca^{2+} (v) bursting behavior of single channels with rapid flickers within a burst. The successful recording and analysis of this voltage-dependent K^+ conductance suggest that foreign channels introduced by transformation into yeast might be similarly studied. (Supported by NIH GM-22714, NIH GM-26217 and NSF BNS-8216149).

Th-AM-A3 POTASSIUM CURRENTS IN DEVELOPING CULTURED COCKROACH NEURONS. B.N. Christensen, T. Shimahara, Y. Pichon, D. Beadle and Y. Larmet, Département de Biophysique, Laboratoire de Neurobiologie Cellulaire du C.N.R.S., 91190 Gif sur Yvette, France.

The patch voltage clamp technique was utilized in either whole cell or patch mode to study the K^+ currents in cultured cockroach neurons. Under whole cell clamp conditions, hyperpolarization of the cell membrane did not produce any voltage dependent increase in membrane current. Cell membrane depolarizations produced a voltage dependent increase in outward current that could be blocked by 4-AP and TEA and was therefore, considered to be a K^+ current. This outward current was present in cells as early as two days after the start of the culture. In some cells the outward current was biphasic, with an early phase superimposed on a delayed current. The early current reached a peak within a few msec, was activated at holding potentials between -65 and -45 mV and in this sense, is unlike the rapidly activated K^+ current (I_A). In other cells the rapidly activated current was absent and the remaining outward current was similar to the delayed K^+ current. In some cells, this delayed current inactivated for voltage steps lasting 100 msec or longer. Step variations of currents corresponding to the opening and closing of individual K^+ channels were recorded in the cell attached mode. In a 14 days old culture channel conductance was estimated to be 16.4 and 13 pS at 70 and 30 mV depolarizations respectively. A histogram of channel open times from this cell contained two peaks corresponding to channel open times of 1.6 and 2.1 msec. In a two day old culture, long and short channel open times were observed. A frequency spectrum of those data could be fitted with two Lorentzians. The mean open time calculated from the corner frequencies were 13.26 and 0.4 msec. This suggests that during cell maturation the channel open time is modified.

Th-AM-A4 SINGLE K^+ CHANNELS FROM RAT AND HERMISSENDA BRAIN INCORPORATED INTO LIPID BILAYERS ON PATCH-CLAMP PIPETTES. J.Farley and B.Rudy, Prog. in Neurosci., Princeton Univ., Princeton, NJ 08544 and Dept. of Physiol. Biophysics, New York Univ. Med. Ctr., NY, NY 10016.

We describe here the use of planar lipid bilayers on patch-clamp electrodes to detect and characterize K^+ channels from rat brain synaptosomes and crude homogenates from Hermissenda nervous systems. Artificial membranes (2-40 G) were formed from a 70:30 molar ratio of bovine brain lipids (PE:PS). We have identified two different K^+ channels from rat brain, on the basis of their selectivity, unitary conductance, gating kinetics, and sensitivity to calcium and TEA ions. The most commonly observed channel displays: 1) a unitary conductance of 220 pS in a symmetrical 100 mM KCl/200 M CaCl solution, 2) cation selectivity (Pk=Pna 100:1), 3) voltage-dependent block by 1mM TEA applied to the intracellular (cis) face of the channel, 4) modest voltage-sensitivity: at 200 M Ca^{2+} concentrations, the channel is often open at -80 mV, and 5) low sensitivity to free Ca^{2+}. This channel appears to be similar to the "BK" channels described by others. A second intermediate-sized channel displayed: 1) a unitary conductance of 130 pS, 2) cation selectivity, 3) complex gating characteristics with at least two closed states, 4) resistance to intracellular TEA (10mM) and 4-AP (1 mM) block, 5) strong voltage-dependency, and 6) high-sensitivity to intracellular Ca^{2+}. This channel has also been reconstituted from Hermissenda brains. The open-state probability of the 130 pS channel from rat brain (at 100 M Ca^{2+}) was drastically reduced when protein kinase C (PKC) was added to the cis side of the bath. This effect was not observed when imipramine was present, a known inhibitor of PKC. These results are very similar to those we have previously described for the macroscopic TEA-resistant Ca^{2+}-dependent K^+ current in Hermissenda cells.

Th-AM-A5 IS THE SEMIPERMEABLE PROPERTY OF THE RESTING MEMBRANE DETERMINED BY EXCITABLE K CHANNEL? D. C. Chang, Dept. Physiol. & Mol. Biophys., Baylor Coll. of Med., Houston, TX 77030.

Although it is well established that the resting membrane is semipermeable, it is not clear that whether such semipermeability is determined by the number of Na and K channels that remain open at the resting state, or, that there may exist other kinds of "resting pathways" at the membrane which are selectively permeable to the K^+ ions. Our experiment is aimed to test it. The permeability ratio of (P_K/P_{Na}) of the resting membrane of the squid axon was determined by measuring the effect of removal of external Na^+ on the resting potential. We determined the permeability ratio both before and after suppressing the excitable K conductance (g_K) by one of the following ways: (1) internal perfusion with 20 mM tetraethylammonium (TEA), (2) internal perfusion with 2 mM 4-amino-pyridine (4-AP) plus 100 mM Cs^+, and (3) prolong perfusion of K-free NaF internal solution. Our voltage-clamp measurements indicated that these treatments reduced the g_K by 42, 118 and 12 fold, respectively. If the K channel is the major pathway of the resting K current and thus P_K is directly related to g_K, then suppression of g_K by those treatments should decrease P_K/P_{Na} many fold. Such a prediction was not observed in our experiment. Our measurement on 18 axons showed that the P_K/P_{Na} ratio after treatments of TEA and prolong NaF perfusion is only reduced to 0.83 and 0.69 of the control value, respectively. These results indicate that the P_K/P_{Na} ratio of the resting membrane are not proportional to the ratio of g_K/g_{Na}, suggesting that the resting K current probably pass through pathways different than the excitable K channel. (Work partially supported by ONR contract N00014-85-K-0424 and NSF grant BNS-8406932).

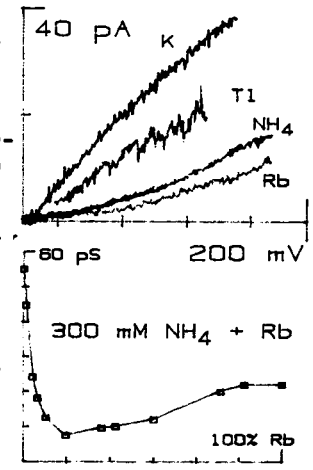
Th-AM-A6 ACETYLCHOLINE INHIBITS A-CURRENT IN DISSOCIATED CULTURED HIPPOCAMPAL NEURONS. Y. Nakajima, S. Nakajima, R.J. Leonard and K. Yamaguchi, Department of Biological Sciences, Purdue University, West Lafayette, IN 47907.

We have studied the effects of acetylcholine (ACh) on dissociated cultured hippocampal neurons using the whole-cell clamp. The culture methods are similar to those reported previously (Nakajima et al., 1985, PNAS, 82, 6325). The CA1 region of the hippocampus was removed from brain slices of postnatal rats (12 to 19 days old). The tissue was incubated in a papain solution, dissociated, and cultured on a glial feeder layer for 2 to 6 days. When depolarized under voltage clamp, the neurons exhibited fast transient outward currents (A-currents), which were inhibited by 4-amino-pyridine (4-AP; 2.5 mM). We found that ACh (0.1 μM) also suppressed the A-current as did the muscarinic agonists bethanechol (250 μM) and muscarine (20 μM). The effect of ACh (0.1 μM) on A-current was reduced by atropine (0.5 μM). Analysis of the steady-state inactivation curve and the activation curve revealed that both were shifted in the depolarizing direction by ACh and by 4-AP. As expected from their inhibition of A-current, ACh and 4-AP both increased the amplitude of the action potential and prolonged its duration. We propose that the muscarinic excitatory influence of ACh is partly derived from the suppression of A-current. In this sense ACh mimics 4-AP, a convulsant that produces its excitatory influence through the inhibition of A-current. (Supported by a NIH grant AG06093 and by an Alzheimer's Disease and Related Disorders Association Grant).

Th-AM-A7 THE HIGH-CONDUCTANCE Ca^{++}-ACTIVATED K^+ CHANNEL: CURRENT-VOLTAGE BEHAVIOR AND MULTI-ION PERMEATION PROPERTIES. R. Latorre, C. Miller, and G. Eisenman. Centro de Estudios Cientificos, Santiago, Chile; Grad. Dept. of Biochem., Brandeis U., Waltham, MA 02254; Physiol. Dept., UCLA Medical School, L.A., CA 90024.

Single Ca-activated K channels from rat skeletal muscle plasma membranes were inserted into planar bilayers formed from neutral phospholipids; and open-channel I-V curves were determined in solutions containing K, Rb, Tl, and NH_4 ions, over a wide range of concentrations (50-3000 mM) and voltages (-160 to 220 mV). Over the entire concentration range, I-V curves in symmetrical solutions were sublinear for K and Tl and supralinear for NH_4 and Rb (cf. upper figure for 300 mM). This suggests, in the context of a conduction pathway energy profile with two ion binding sites, that a central barrier is a more significant rate-determining step for NH_4 and Rb than for K and Tl. The I-V curves for NH_4 and Tl show substantial rectification in symmetrical solutions; from this we conclude that the ion conduction pathway is asymmetrical in structure. The conduction mechanism involves the simultaneous occupancy of the channel by at least two ions. This is shown by a striking conductance minimum in varying mixtures of K/Rb and of NH_4/Rb at constant total concentration (cf. lower figure).

Supported by NIH grants GM-31768 and GM-24749, NSF grant BNS-8411033, and a Tinker Foundation grant.



Th-AM-A8 BARIUM ACTIVATES TWO CALCIUM-DEPENDENT POTASSIUM CURRENTS OF SPINAL NEURONS IN CULTURE. A.B. Ribera and N.C. Spitzer (Intr. by D. Gruol). Biology Department, University of California, San Diego, La Jolla, CA 92093

Amphibian spinal neurons in culture possess both rapidly inactivating (I_{Kci}) and sustained(I_{Kcs}) voltage- and calcium-dependent potassium currents, similar to those described for other cells. Ba- or Ca-dependent whole cell outward currents were isolated by subtracting the voltage-dependent component of the potassium current produced in the presence of Cd (100-500 μM) from the currents obtained without Cd but in the presence of Ba or Ca (50-100 μM). These concentrations of divalent cations were low enough to avoid contamination by macroscopic inward currents through Ca channels; sodium currents were blocked by TTX. The two outward currents elicited by Ca in mature cells (19-26 hrs in vitro) could be distinguished by several criteria: I_{Kcs} was activated at potentials positive to -30 mV and did not show inactivation; I_{Kci} was activated at more depolarized potentials (-10 mV) and could be inactivated either by depolarized holding potentials or by a depolarizing prepulse. The ionic dependence of each current on potassium was defined by examination of the tail currents observed at different holding potentials following its maximal activation. Comparisons made on the same cell revealed that Ba was generally more effective than Ca in eliciting both currents. Ca and Ba activated I_{Kci} and I_{Kcs} in young neurons(6-8 hrs); Ba was again more effective. Ba influx appeared to suppress the voltage-dependent potassium current in most cells. Ca was often seen to have a similar effect in young neurons. The absence of a strict requirement for Ca in the activation of potassium currents raises the possibility that they can be activated by other, endogenous divalent cations. A.B.R. is a postdoctoral fellow of the USPHS. Supported by NS15918 from the NIH.

Th-AM-A9 BLOCKADE OF A CA-ACTIVATED K CHANNEL BY QUATERNARY AMMONIUM IONS. A. Villarroel, O. Alvarez, and R. Latorre. Department of Biology, Faculty of Sciences, Univ. of Chile, Santiago, and Centro de Estudios Cientificos de Santiago, Santiago, Chile.

The blockade of Ca-activated K channel incorporated into planar lipid bilayers by mono (QA) and divalent (BisQn) quaternary ammonium ions has been characterized. QA ions block the channel from cytoplasmic side and from the external side of the channel, but they do so in a strikingly different manner. From the cytoplasmic side all QA ions block by binding to a site located at a fractional electrical distance (δ) of about 0.27 and the dissociation constant (Kd) decrease as the QA compound is made more hydrophobic. From the external side QA ions block by binding to a site located at a δ=0.14 and it shows a high affinity for tetraethylammonium. Other QA block with larger Kd's. BisQn compounds are also able to block the channel. From the cytoplasmic side they block with a "δ" that is a function of the hydrocarbon chain length that separates the two charges. For BisQ2 "δ" is 0.45; it decreases to 0.3 for BisQ4 and then increases monotonically for compounds with a chain length larger than 6 carbon atoms. For BisQ10 "δ" is 0.55. We conclude that the gross architecture of the cytoplasmic and external mouths of the channel is different and that the cytoplasmic mouth is about 1nm deep followed by a constriction of less than 0.5nm in diameter.

This work was supported by Universidad de Chile, Departamento de Investigación y Bibliotecas, Grant B-1985/8413, Fondo Nacional de Investigación Grant 1299, and a grant from The Tinker Foundation, Inc., New York.

Th-AM-A10 BLOCKING AGENTS OF THE Ca^{2+}-ACTIVATED K^+ CHANNELS IN CULTURED RABBIT MEDULLARY THICK ASCENDING LIMB CELLS (MTAL). S.E. Guggino, N. Green and B. Sacktor (Intr. by W.B. Guggino). LBC, GRC, NIA, NIH, Baltimore, Maryland 21224 and LKEM, NHLBI, Bethesda, MD 20892.

We previously reported the presence of a 135 pS Ca^{2+}-activated K^+ channel observed in both cell-attached and cell-excised patches of cultured MTAL cells (Biophys. J. 47(2) 387a, 1985). Ba^{++}, 10 μM applied at the intracellular face of inside-out patches, causes a 7 fold decrease in fractional open time. At 100 μM, Ba^{++} causes a 2.5 and 3 fold decrease in both mean and average open times accompanied by long Ba^{++} blocked segments with mean and average blocked times of 750 ms and 3.8s respectively. TEA, applied at the external face of outside-out patches causes a flickery block in concentrations as low as 40 μM. At 400 μM, external TEA causes a 70% reduction in apparent single channel conductance. Charybdotoxin (CTX) 20 nM in the pipette of cell-attached or excised inside-out patches, abolishes channel fluctuations. Perfusion of CTX onto outside-out patches also causes block. Apamin, 100 nM in the pipette, does not affect the activity of this channel in cell-attached or excised patches. The results of ligand block dose-dependency suggest that cultured MTAL cells express a K^+ channel which is homologous to the maxi Ca^{++}-activated K channel found in muscle cells.

Th-AM-A11 BARIUM-ACTIVATED OUTWARD CURRENT IN A MOLLUSCAN EGG CELL. Sally Krasne, Karen McKeown, and Jianghong Ye*, Department of Physiology, UCLA, Los Angeles, CA. 90024.

Current-voltage relationships have been measured in unfertilized eggs from the Giant Keyhole Limpet, Megathura crenulata, using microelectrode voltage-clamp and patch-clamp techniques. Egg jelly was removed by washing in acidified seawater; vitelline membranes were removed through incubation in seawater containing a lytic agent obtained from the sperm of this species. No other treatments were used to clean the egg membrane. In ASW containing either 10mM Ca^{2+} and 50mM Mg^{2+} or 60mM Ca^{2+}, voltage-clamp experiments revealed an inward-rectifying potassium current (anomalous rectifier) and an outward rectifying potassium current which was blocked by TEA; chloride currents were also observed in some eggs. In ASW containing either 60mM Ba^{2+} or 10mM Ca^{2+}, 50mM Mg^{2+} and small amounts of Ba^{2+} (e.g. 2-4 mM), the inward rectifier was blocked, and an inward current followed by a slowly developing outward current was observed for large depolarizing pulses. Both of these latter currents were blocked by 50μM Cd^{2+} whereas only the outward current was blocked by external TEA. Single-channel outward currents were observed following depolarization of excised, inside-out membrane patches in 10mM Ca^{2+}, 50mM Mg^{2+}-ASW. These currents were also present when the bath contained 60mM Ba^{2+} suggesting that the Ba-stimulated outward currents observed in the voltage-clamp experiments did not simply result from a release of internal Ca^{2+}, and subsequent opening of Ca-stimulated potassium channels, following an influx of Ba^{2+}.

Supported by grants from NIH (HL20254) and the Muscular Dystrophy Association.

Th-AM-A12 LIGHT-INDUCED CALCIUM-DEPENDENT K^+ CHANNELS RECORDED FROM PATCHES OF NON-RHABDOMERIC SOMA MEMBRANE OF PHOTORECEPTORS. M. Sakakibara, H. Peter-Höpp, and D.L. Alkon. Lab. of Biophysics, NINCDS-NIH, MBL, Woods Hole, MA 02543.

The photoreceptors of the nudibranch mollusc Hermissenda crassicornis have four structural compartments: rhabdome, soma, axon, and terminal branches, on which are all sites of synaptic contact. Two microelectrode voltage-clamp of the soma and rhabdome compartments (isolated by axotomy) previously revealed three voltage-dependent outward K^+ currents: I_A, $I_{Ca^{2+}\text{-}K^+}$, and I_K (not appreciable at $V_m < 0$ mV, absolute). Light activates an inward Na^+ current, I_{Na^+}, and $I_{Ca^{2+}\text{-}K^+}$ (via intracellular release of Ca^{2+}). Patch-clamp (cell-attached configuration) of trypsin-treated somata revealed at least two populations of voltage-dependent outward channels (reversal potential of -60 to -70 mV, absolute), most likely I_A and $I_{Ca^{2+}\text{-}K^+}$, and light-induced outward channels, most likely $I_{Ca^{2+}\text{-}K^+}$, and no light-induced inward Na^+ channels. One type of outward channel (I_A) had a conductance of 2-5 ps, occurred with greater frequency at the onset of depolarization, was not activated from holding potentials $\geq$ -40 mV and was blocked by 10 μM 4-AP. The other type ($I_{Ca^{2+}\text{-}K^+}$) could be activated at more positive holding potentials and had a conductance of 10-20 ps. These latter channels, recorded with inside-out configuration, showed increased amplitude and frequency when Ca^{2+} was elevated from 1.6 to 11.5 μM. In 0 Na^+-ASW, light elicited (only in the cell-attached configuration) outward channels with the same properties as the voltage-dependent 10-20 ps channels ($I_{Ca^{2+}\text{-}K^+}$). Since at -60 to -40 mV in 0 Na^+-ASW light elicits no change of photoreceptor membrane potential, the light-elicited $I_{Ca^{2+}\text{-}K^+}$ cannot be a consequence of voltage-dependent $I_{Ca^{2+}}$ activation, and thus could result from diffusion of Ca^{2+} from the rhabdome to the soma.

Th-AM-B1 **Workshop: Fluorescence imaging of calcium in single cells**

Organizer: W.J. Lederer, Dept. Physiology, Univ. Maryland Sch. Med., 660 W. Redwood St., Baltimore, MD 21201
(301) 528-3852

Speakers

R.Y. Tsien: New Indicators -- one year later
W.J. Lederer: Cardiac Myocytes -- Part I
W.G. Wier: Cardiac Myocytes -- Part II
F. Fay: 2-D and 3-D imaging and more

One or two additional speakers will participate in the program and others are invited to make brief reports of new findings. Please contact W.J.Lederer before the meeting so that a final schedule of presentations can be made and distributed with registration.

Th-AM-C1 MECHANISTIC STUDIES ON THE ERYTHROCYTE ANION TRANSPORT PROTEIN, BAND-3. R.J. Pace, Research School of Chemistry, Australian National University, Canberra ACT Australia 2601

^{35}Cl NMR, T_2 relaxation has been used to assay Cl^- binding to Band-3 in washed membranes and whole cells. Chemical modification studies have shown that a single lysine and one or more arginine side chains are directly involved in the anion transport binding site (s). The lysine group is modifiable from the outside by 4,4'-diisothiocyano-stilbene-2,2' disulfonate (DIDS) and in the protein interior by 2,4 dinitro fluorobenzene (DNFB). The arginine(s) is accessible mainly from the inside with α diketone reagents, although modification of at least one external arginine group inhibits transport without affecting Cl^- binding to the (probably inward facing) transport site. Cl^- inhibits α diketone reaction at the internal site with an affinity appropriate for the transport site. This suggests a mechanism in which lysine and arginine side chains operate in tandem to transport monovalent anions in neutral complexed form through a relatively hydrophobic protein interior. At any time one of these side chains forms an internal salt bridge to a carboxylic group from aspastic or glutamic acid. Reaction of ghosts and whole cells with the carbodiimide EDC shows behaviour consistent with internal crosslinking of the lysine and carboxylic groups, locking the transporter into an inward facing form. EDC plus an exogenous amine, glycine ethyl ester (GEE), expose binding sites at both sides of the membrane, as would be expected from the proposed mechanism. Labelling studies with ^{14}C GEE indicate that the carboxylic group is located very near the end of the 17kDa fragment, as is the DIDS binding lysine. Assuming α helical membrane spanning regions, the results are consistent with the recently published sequence of murine Band-3 (Kopito & Lodish, Nature 316, 234 (1985)).

Th-AM-C2 H^+ (OH^-) FLUXES ACTIVATED BY INORGANIC IONS ON THE ANION EXCHANGER (BAND 3) OF HUMAN RED CELLS. R. B. Gunn, Department of Physiology, Emory University School of Medicine, Atlanta, GA 30322.

The initial steady state influx of proton equivalents was measured in the absence of CO_2-HCO_3^- in a pH-stat at 20°C. The cells had an initial internal pH_i = 7.4-7.6 and Cl_i = 110 mM. The influx of proton equivalents (or efflux of hydroxyl equivalents) was activated by external protons with a $pK_{\frac{1}{2}}$ = 4.1-4.2. The flux into cells suspended in Cl-free sucrose or media with 98 μM dinitro stilbene disulfonate (DNDS) and Cl_o = 0-160 mM (pH_o = 5.0) was the same: 6 μEq/(g Hgb · min). The Cl-sensitive, DNDS-inhibitable proton influx was activated by Cl_o when substituting for sucrose. At pH_o = 5, $K_{\frac{1}{2}}$-Cl = 73 ± 13 mM and V_{max} = 217 ± 18 μEq/(g Hgb · min) and at pH_o = 4, $K_{\frac{1}{2}}$-Cl = 35 ± 5 mM and V_{max} = 340 ± 15 μEq/(g Hgb · min). When H = OH, the influx was a hyperbolic function of H with initial slope = $P_{(H+OH)}$ = 1.3 x 10^{-2} cm/sec which is 10^3-10^4 times the value in planar lipid bilayers and vesicles. When H_o/H_i was held constant at 10, the flux increased only 4-fold as pH_o decreased from 7 to 4, and H increased 1,000-fold. The proton influx was equal to the sulfate influx from a 125 mM K_2SO_4 media into normal Cl cells between pH_o = 6 and pH_o = 3.3. These data quantitate the findings of M. Jennings (J. Membr. Biol. 40:365, 1978) that proton fluxes can be mediated by band 3, are inorganic anion-dependent, and stilbene-sensitive. They provide additional insight into the mechanisms of band 3 protein. Supported in part by USPHS grant HL28674.

Th-AM-C3 ASYMMETRY OF THE HUMAN RED BLOOD CELL ANION TRANSPORT SYSTEM AT 38°C. Philip A. Knauf and Jesper Brahm, Dept. of Biophysics, Univ. of Copenhagen, DK-2200 Copenhagen N, Denmark.

The band 3 anion transport protein (known as capnophorin) can exist in at least two different conformations: E_i, in which the transport site faces the cytoplasm, and E_o, in which it faces the external medium. Anion transport involves a spontaneous change from one conformation to the other, which can only take place when a suitable anion (such as Cl^- or HCO_3^-) is bound to the transport site. At 0°C the system is highly asymmetric, such that the ratio of E_i to E_o is between 5 and 15, with equal Cl^- concentrations inside (Cl_i) and outside (Cl_o) the cell. We have applied a rapid-flow method (Brahm, J. Gen. Physiol. 70: 283) to determine whether or not the system exhibits similar asymmetry under more physiological circumstances at 38°C. For intact red cells with an average internal Cl^- concentration of 107 mM, the external Cl^- concentration required to half-saturate the transport system, $K_{\frac{1}{2}o}$, was 2.8 mM, as determined from a Lineweaver-Burk plot of ^{36}Cl exchange data. $K_{\frac{1}{2}}$, the concentration of Cl^- which half-saturates the system with Cl_i=Cl_o, was measured in resealed ghosts with various internal KCl concentrations. From a Lineweaver-Burk analysis, the value of $K_{\frac{1}{2}}$ is 304 mM, far larger than the $K_{\frac{1}{2}}$ value at 0°C, 65 mM. Thus, the overall affinity of the anion exchange system for Cl^- is far lower at 38°C than at 0°C and the system is not saturated with Cl^- under physiological circumstances. From the values of $K_{\frac{1}{2}}$ and $K_{\frac{1}{2}o}$ the ratio of E_i to E_o at 38°C (with Cl_i=Cl_o) is about 29, so the system is more asymmetric at 38°C than at 0°C. This implies that the dissociation constants for Cl^- binding and/or the rate-constants for the transporting conformational change have different temperature dependencies. Supported by Fogarty Fellowship TW00975 and by N.I.H. Grants AM 27495 and HL 18208.

Th-AM-C4 TEMPERATURE DEPENDENCE OF LATERAL DIFFUSION by J. Eisinger, J. Flores (AT&T Bell Laboratories, Murray Hill, NJ 07974) and J. B. A. Ross (Dept. of Biochemistry, Mt. Sinai School of Medicine, NY, NY 10029).

It has been suggested that long-range diffusion of lipid analogue probes in membranes, as measured in fluorescence photo-bleaching recovery (FPR), is drastically impeded by membrane proteins.[1] This suggestion is based on random walk computer simulations and the finding that the local lateral diffusion coefficient (D) of excimeric membrane probes (e.g. pyrene dodecanoic acid, PDA) is considerably greater than D obtained by FPR: The diffusion range of PDA probes is typically 1-10 nm, and of the order of 1 μm in FPR. We have measured the temperature dependence (2-35°C) of the local D for PDA in intact erythrocytes and the experimental values fit an Arrhenius plot with an effective energy barrier of 6.1± 0.5 kcal M^{-1}. This is half the activation energy reported for long-range diffusion in a similar system,[2] but similar to the activation energy for rotational diffusion of diphenyl hexatriene (DPH) in erythrocyte membranes.[3,4] This means that the energy barrier for nearest neighbor spatial exchanges is similar to that for rotational diffusion, in accord with the theoretical relationship between translational and rotational mobilities of lipid analogue probes.[5]

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Th-AM-C5 POTASSIUM LEAK IN SS ERYTHROCYTES: DEPENDENCE ON RED CELL MCHC, EXTRACELLULAR pH, AND DEOXYGENATION, MM Connolly and ME Fabry, Department of Medicine, Division of Hematology, Albert Einstein College of Medicine, Bronx, New York, 10471.

Red cells from sickle cell anemia patients contain hemoglobin S (HbS) which polymerizes when deoxygenated. Deoxygenated SS cells assume the classic sickled shape and leak potassium (K^+) by an unknown mechanism. It is generally assumed that K^+ leak is proportional to the extent of HbS polymerization inside the cell. At a given pO_2, the extent of polymerization is directly proportional to the mean corpuscular hemoglobin concentration (MCHC) which is in turn directly proportional to the red cell density. We have isolated density (MCHC) defined fractions from Percoll Stractan continuous density gradients and determined their rate of K^+ leak under carbon monoxide (control) and deoxy conditions. We find that at pH 7.5 the discocyte fraction (F2, MCHC=33-35 gm/dl) leaks K^+ faster (17.6 mEq/L cells/hr) than the densest fraction (F4, MCHC=41-47 gm/dl) (1.8 mEq/L cells/hr). Since this is, in part, due to their lower initial K^+ content, the rate of loss in K^+ loaded cells was also studied. K^+ leak was studied as a function of extracellular pH and was found to be higher at pH 8 (13 mEq/L cells/hr) than at pH 7 (1 mEq/L cells/hr). The rate of K^+ leak of F2 as a function of deoxygenation was also studied; after the percent deoxyhemoglobin reached a level of 40%, an abrupt increase in the rate of K^+ leak was observed. The last finding is in agreement with the hypothesis that K^+ leak increases with polymerization. However, since the first two observations pair low rates of K^+ leak with conditions (low pH and high MCHC) leading to a high degree of intracellular polymerization, we conclude that a simple correlation between the rate of K^+ leak and the extent of intracellular polymerization does not exist.

Th-AM-C6 VOLUME-DEPENDENT AND NEM-STIMULATED K+Cl TRANSPORT IS ELEVATED IN SS,SC AND CC HUMAN RED CELLS. Mitzy Canessa, Anda Spalvins and Ronald Nagel. Endocrine-Hypertension Dept, Brigham and Women's Hospital,Boston,Ma 02115 and Dept. of Medicine,A.Einstein College of Medicine, New York,N.Y.

A wide variety of cell densities have been observed in SS,SC and CC red cells. We re port here studies on the KCl cotransport system involved in the regulation of cell volume.

We have studied the effect of bumetanide (BS),chloride removal (NO3 substitution,CHLD),osmotic and acid pH induced swelling (pH7-8) and NEM (1 mM) on ouabain-resistant K efflux. All cells were loaded by nystatin (12 Na+98 K.mmol/l cell) and K efflux (mmol/l cellxhr=FU) was measured into (mM) 140 Na, 0.1 ouabain, 10 glucose, 1 Mg, 10 tris-Mops pH 7, 7.4, and 8, w/o 0.01 bumetanide (BU). The basal CHLD K efflux was significantly higher (M±SD,FU) in SS cells (4.5 ±3,n=11), SC (1,n=2) and CC (1.4,n=1) than in AA (0.3+0.3,n=4). Osmotically-induced swelling markedly stimulated BU-resistant,CHLD K efflux from SS (6.2 ± 4), SC (4.4) and CC (8.5),slightly in AA (1.2 ±0.28) and not in fetal cells (FEC,n=3), while BU-sensitive flux was not significantly different. Swelling induced by acid pH also promoted a CHLD, BU-resistant K efflux is SS (4.8±3.7) and CC (8), but not in AA and FEC cells. NEM also stimulated a CHLD K efflux in SS (18±7.6), SC (14),CC (15), AA (7.4±3) and FEC (3.8±1.8) cells. The present results indicate that oxygenated SS cells exhibit a BU-resistant Cl and volume-dependent K efflux, stimulated by NEM which is only slyghtly active in normal AA cells inactive in FEC and which is shared by Hb CC and SC cells.The KCL transporter might be related to the low density fraction of younger cells or in some way to the HBS polymerization or HB C aggrega tion phenomena displayed by these genetic variants of hemoglobin.

Th-AM-C7 MEMBRANE AND CYTOPLASMIC RESISTIVITY PROPERTIES OF NORMAL AND SICKLE RED BLOOD CELLS.
G.V. RICHIERI AND H.C. MEL, Department of Biophysics and Medical Physics, University of California, Berkeley 94720

The cytoplasmic resistivities and membrane breakdown potentials of normal (AA), sickle cell trait (AS), and sickle (SS) red blood cells have been measured by the biophysical methodology of resistive pulse spectroscopy, over a range of osmolalities. At isotonicity the average membrane breakdown potentials are virtually identical for the three types of cells, occuring at about 1150 V/cm. Average isotonic cytoplasmic resistivities are somewhat higher for the SS cells 166.7 ± 7.49 ohm-cm as compared with the normals (147.6 ± 1.98 ohm-cm) or AS cells (148.7 ± 1.79 ohm-cm). As medium osmolality is varied, the differences in resistive properties becomes enlarged, especially at very low and very high osmolalities. At high osmolalities both types of sickle cells show a large increase in internal resistivity, as compared to the normals; at low osmolalities the SS samples exhibit a distinctly different membrane breakdown characteristic, decreasing in this parameter while the other two groups increase.

Out of 15 SS samples tested, 3 displayed much higher cytoplasmic resistivities at isotonicity: 218.2 ± 5.25 ohm-cm, as compared to an average of 153.5 ± 3.46 ohm-cm for the other 12. The relationship between these high resistivities and the subfraction of irreversible sickle cells in the samples is discussed.

Th-AM-C8 CONTROL OF RED CELL UREA AND WATER PERMEABILITY BY SULFHYDRYL REAGENTS. Michael R. Toon and A. K. Solomon, Biophysical Laboratory, Dept. of Physiology and Biophysics, Harvard Medical School, Boston, MA 02115.

Chemical relaxation equations have been used to characterize the binding of the sulfhydryl reagent, pCMBS (p-chloromercuribenzene sulfonate), to the water transport inhibition site in NEM (n-ethylmaleimide)-treated human red cells. The kinetics are those of a slow bimolecular association (k_{on} = 6 x 10^3 M^{-1} s^{-1}) followed by a faster conformation change with $K_{I\ app}$ = 150 μM. The reaction is reversible with cysteine. pCMBS inhibits urea transport at another site in NEM-treated cells with much higher affinity ($K_{I\ app}$ = 0.09 μM) and similar, but much faster, kinetics (k_{on} = 1 x 10^6 M^{-1} s^{-1}). This site can not be reversed by cysteine. Another sulfhydryl reagent, DTNB (5,5'-dithiobis(2-nitrobenzoic acid)), binds covalently to band 3 in NEM-treated cells but covalent binding has no effect on water or urea flux or their inhibition by pCMBS. NEM binds to 5 of the 6 cysteine residues on band 3 leaving only 1 free (Rao and Reithmeier, 1979, JBC 254, 6144). The conclusion that DTNB binds to this SH group means that there are no further SH groups left on band 3 to which pCMBS could bind to inhibit water and urea transport. Stilbene anion transport inhibitors bind specifically to band 3 where they alter the time course of pCMBS inhibition of water transport, showing that water transport is modulated by band 3 or a protein complex including band 3. The observation that the reflection coefficient (Chasan and Solomon, 1985, BBA, in press) for urea is 0.7 links urea and water transport. These observations suggest that the pCMBS inhibited urea and water transport sites are either not SH groups or are on another protein, itself linked to band 3. (Supported by NIH GM 34099).

Th-AM-C9 OSMOTIC FRAGILITY MODEL FOR RED CELL POPULATIONS

Hugo A. Massaldi, Gary V. Richieri and Howard C. Mel. Department of Biophysics and Medical Physics, University of California, Berkeley, CA 94720.

A model that predicts the osmotic fragility curve of a red cell population is developed by combining the equation of Ponder with the size distribution of the cells, as determined by resistive pulse spectroscopy. Two of the parameters involved, namely the normalized osmotic volume correction, B, and the swelling index, k, are determined from the experimental average properties of the cell population. A new parameter, n, a measure of the excess surface area-distribution of the cells, is incorporated through a simple function that relates the critical volume to the size of the cells, and is shown to be linked to the other parameters. The model is used to fit and interpret fragility data obtained in this laboratory, and to determine the values of the parameters B, k, and n, for normal and sickle cell samples.

From the values of B and k, critical volumes are estimated to be 10-12% higher than the first spherical volume for normal cells, whereas a value of 6-7% is found for the corresponding difference in the case of sickle cells. The value of n, determined for normal cells, indicates an essentially constant surface to volume ratio within a given individual's cell population, in agreement with direct observations reported in the literature. This is contrasted with the different trend observed in the case of the sickle cell distribution.

Th-AM-C10 MEMBRANE COMPARTMENTALIZED ATP IN HUMAN RED CELL GHOSTS. F. PROVERBIO, D.G. SHOEMAKER AND J.F. HOFFMAN (Intr. by W.K. Chandler). Yale Univ. Sch. of Med. Dept. of Physiol. New Haven CT 06510 & IVIC-CBB, Aptdo 1827, Caracas 1010A (Venezuela).

The membrane-bound phosphoglycerate kinase (PGK) and Na,K-ATPase appear to be directly linked via a compartmentalized form of ATP. The membrane ATP pool can be filled either by incubating porous ghosts for 30 min at 37°C in the presence of Mg and ATP or by running the PGK reaction forward in order to synthesize ATP. The membrane ATP pool can be emptied either by running the PGK reaction backward or by incubating the membranes for 15 min at 37°C in a medium containing Mg, Na and K. If the membrane pool is filled with non-radioactive ATP and the porous ghosts assayed for Na-stimulated ^{32}P-incorporation from bulk [γ-^{32}P]ATP, no incorporation is seen. The enzyme preferentially utilizes the ATP present in the pool rather than bulk ATP. If the pool is emptied before the assay, then ^{32}P-incorporation into the Na-phosphointermediate is seen. When the pool was loaded with caged-ATP and the membranes assayed for Na-stimulated ^{32}P-incorporation before and after exposure to UV-light, the Na-stimulated ^{32}P-incorporation was only seen with membranes not exposed to the UV-light. After exposure to UV-light, when the photoreleased ATP present in the pool was then emptied, the Na-stimulated ^{32}P-incorporation was seen again. It was also found that pool ATP, in the absence of bulk ATP, could 1) markedly stimulate the ouabain binding rate to the ghosts, resulting in inhibition of the Na,K-ATPase under these circumstances and 2) prevent the ouabain-sensitive stimulation of p-nitrophenyl phosphatase activity by K. This latter effect could be overcome by adding Na to the medium or by emptying the ATP pool. These results indicate possible functional roles for ATP compartmentalized within the membranes. (Supported in part by NIH Grants HL-09906 and AM-17433).

Th-AM-C11 OUABAIN-SENSITIVE Na/K PUMP MEDIATED EFFLUX OF Na AND INORGANIC PHOSPHATE (Pi) STIMULATED BY ADP AND INHIBITED BY ATP IN HUMAN RED CELL GHOSTS. R. MARIN AND J.F. HOFFMAN. Yale Univ. Sch. of Med. Dept. of Physiol. New Haven CT 06510 & IVIC-CBB, Aptdo 1827, Caracas 1010A (Venezuela).

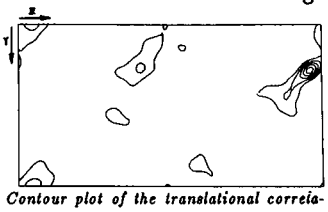
The Na,K-pump catalyzes different transport reactions that have common requirements such as dependence on Mg^{++} and inhibition by ouabain. One of these modes is the "uncoupled" Na efflux that occurs when cells or ghosts are incubated in the absence of both Na_o and K_o. This flux is inhibited by Na_o and by the removal of internal ATP. Human red cell ghosts were assayed for "uncoupled" Na efflux in the presence of increasing concentrations of ADP and/or Pi. The "uncoupled" Na efflux was inhibited by either ADP or Pi or both. But when ATP was removed (by incorporating hexokinase before resealing and incubation with glucose during the flux assay) a new ouabain-sensitive Na efflux was found to occur. This new Na efflux: 1) requires ADP+Pi ($K_{\frac{1}{2}}^{ADP}$ = 200 µM; $K_{\frac{1}{2}}^{Pi}$ = 2.5 mM), 2) is inhibited by the presence of 2-5 µM ATP, 3) is 100% inhibited by 10 mM Na_o, 4) is stimulated by about 50% by 20 mM K_o (only in ghosts prepared with no K_i) and 5) is coupled to an ouabain-sensitive Pi efflux (equal to about one-half of the ouabain-sensitive Na efflux) that is inhibited by Na_o or by K_o. (Supported in part by NIH Grants HL-09906 and AM-17433).

Th-AM-C12 COMPARTMENTATION OF PHOSPHATE UPTAKE IN HUMAN RED CELLS. David G. Shoemaker and Robert B. Gunn, Dept. of Physiology, Emory University School of Medicine, Atlanta, GA 30322.

Influx of ^{32}-phosphate (^{32}P) was measured into human red cells suspended in a medium containing (mM): 140 NaCl; 5 KCl; 1 Na_2HPO_4; 1 $MgCl_2$; 10 Dextrose; 10 HEPES; pH 7.4 @ 37°C. Control cells exhibited an initial rate of ^{32}P influx of 2.29 ± 0.08 mmoles/(Kg Hb·hr) (SEM, n=10). The inhibition of phosphate transport by DNDS (4,4'-dinitro stilbene-2,2'-disulfonate) at 37°C produced nonlinear Dixon plots with a maximal inhibition of 76 ± 1% (n=4), but at 20°C, pH 7.6 and 125 mM phosphate, 95% of ^{32}P influx is DNDS-inhibitable. The DNDS-insensitive influx was completely inhibited by the replacement of external sodium with N-methyl-D-glucamine. The rate of incorporation of extracellular ^{32}P into adenosine nucleotides was measured in perchloric acid extracts of hemolysates with Norit-A decolorizing carbon adsorption and determined to be 1.20 ± 0.08 mmoles/(Kg Hb·hr) (n=7). Depletion of cellular ATP levels by incubation overnight in the absence of substrate inhibited total phosphate influx by 57% while inhibiting incorporation of extracellular phosphate into the nucleotides completely. Membranes prepared by exhaustive washing of hemolysates and analyzed by thin layer chromatography had 69% of the transported ^{32}P associated with the membrane in a compound which migrated with an R_f value equivalent to ADP with only 21% and 10% associated with AMP and ATP, respectively. Inhibition of glyceraldehyde-3-phosphate dehydrogenase with iodoacetic acid reduced ^{32}P uptake by only 27% (1.67 ± 0.15), but reduced incorporation into the nucleotide fraction by 66%. These results indicate that inorganic phosphate transport under physiological conditions may take place by two independent transport mechanisms: 1) DNDS-sensitive (75%) and 2) Na-dependent (25%), which deliver extracellular phosphate to different intracellular pools. Supported by AHA Grant-in-Aid (DGS) and NIH grant HL 28674 (RBG).

Th-AM-D1 PRELIMINARY ELECTRON DENSITY MAPPING OF THE REACTION CENTERS FROM R. SPHAEROIDES USING THE MOLECULAR REPLACEMENT METHOD. J.P. Allen, G. Feher, UCSD, La Jolla, CA 92093; T.O. Yeates, D.C. Rees, D.S. Eisenberg, UCLA, Los Angeles, CA 90024; J. Deisenhofer, H. Michel, R. Huber, Max Planck Institut, 8033 Martinsried, W. Germany.

The recently determined structure of the reaction center (RC) from R. viridis (1) was used in the Patterson Search technique (2) to analyse the diffraction data obtained from crystals of RC from R. sphaeroides. A complete data set cut off to 5Å resolution was measured on crystals (3) (space group $P2_12_12_1$) with a multiwire area detector. A calculation of the real space rotation function gave a clear orientation of the R. viridis model (excluding the cytochrome) in the unit cell. Three independent pairs of translation vectors were determined (see Fig.); they gave consistent results. Preliminary phases were calculated using the correctly positioned model. These phases are bein refind by the use of isomorphous replacement with a mercury derivative. The results indicate a strong homology between the structures of the two species. Collection and analysis of data of higher resolution are in progress.



Contour plot of the translational correlation function at Harker section $x = 1/2$.

Work supported by NIH, and the Searle Scholar Program.

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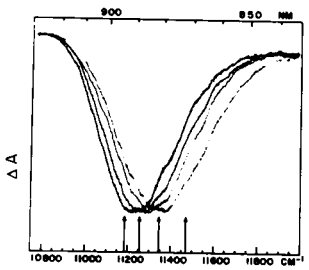
Th-AM-D2 CORRELATION OF PARAMAGNETIC STATES AND CHROMOPHORE STRUCTURE IN CRYSTALS OF BACTERIAL PHOTOSYNTHETIC REACTION CENTERS, David E. Budil and James R. Norris; Intr. by David M. Tiede, Chemistry Division, Argonne National Laboratory, Argonne, Illinois, 60439

The availability of both magnetic resonance and x-ray data from crystals of bacterial reaction centers makes possible a detailed and highly accurate assignment of the structure of paramagnetic states in photosynthesis to the molecular framework of the reaction center. In R. viridis, the orientation of the primary donor triplet state determined by EPR most closely approaches that of a monomer bacteriochlorophyll triplet localized on the L subunit half of the P_{890} dimer. The EPR measurement may be reproduced from the x-ray structure to within experimental error by including about 22% special pair charge-transfer character in the $^3P_{890}$ state. Calculation of the zero-field parameters of $^3P_{890}$ and the EPR linewidth of the P_{890} cation from this model are in excellent agreement with experimental values. The implications of this electronic structure for the mechanism of charge separation in bacterial photosynthesis, and comparison with data from reaction center crystals of R. sphaeroides R-26 will be discussed.

(This work was supported by the Division of Chemical Sciences, Office of Basic Energy Sciences of the U.S. DOE.)

Th-AM-D3 PHOTOCHEMICAL HOLEBURNING IN PHOTOSYNTHETIC REACTION CENTERS: INSIGHT INTO ULTRA-FAST REACTION DYNAMICS. Thomas R. Middendorf, David J. Lockhart, and Steven G. Boxer, Dept. of Chemistry, Stanford University, Stanford, CA 94305

Photochemical holeburning experiments have been performed at low temperature on reaction centers from R. spheroides, R-26.1 mutant. Holes are obtained by exciting P870 to its lowest excited state and measuring the change in absorption before charge recombination between $P870^+$ and Q^-. The hole spectra have a FWHM of about 400 cm^{-1}. The holewidth and position depend on burn wavelength: the holes get narrower and the deepest point in the hole shifts as the burn wavelength is tuned through the P870 absorption band (see Figure). The holes are temperature independent between 1.5 and 2.1K and do not change shape over a wide range of burn power. This result can be interpreted as suggesting an ultra-fast decay process following photoexcitation of P870 (time scale tens of femtoseconds). An alternative explanation is that the zero-phonon line is suppressed and the hole is due to unresolved phonon and vibronic structure. This interpretation requires a substantial change in the potential energy surface between the ground and excited state and strong excited state electron-phonon coupling. This may occur if the excited state of P870 has substantial charge transfer character.



Th-AM-D4 EFFECTS OF NUCLEAR POLARIZATION ON REACTION DYNAMICS IN PHOTOSYNTHETIC REACTION CENTERS
R.A. Goldstein and S.G. Boxer, Dept. of Chemistry, Stanford University, Stanford CA 94305

Singlet-triplet mixing in the initial radical-pair state of bacterial reaction centers (P^+I^-) is due to the hyperfine mechanism at low magnetic fields and both the hyperfine and Δg effect at high magnetic fields (>1kG). Since the hyperfine field felt by electron spins in P^+I^- is dependent upon the nuclear spin state, the relative probabilities for formation of ^3P and the ground state after charge recombination will depend on the nuclear spin configuration. As a result, the reformed ground and triplet states of P will have non-equilibrium distributions of nuclear spin states (nuclear spin polarization).

We have shown that ^3P decays largely through repopulation of P^+I^- at room temperature [1]; this decay path involves a second passage through the S-T mixing process. The initial S-T mixing enriches ^3P in nuclear spin states which favor S-T mixing; consequently S-T mixing on the second passage will be more effective, and the observed ^3P decay rate will be higher than expected. The effect should go away at high magnetic field as the Δg mechanism starts to dominate S-T mixing. This result can be used to explain differences observed in the magnetic field dependence of the ^3P yield and decay rate [1]. Experiments involving reexcitation of the sample will also be influenced by the residual ground state nuclear polarization. Due to unequal nuclear relaxation rates in the triplet and ground states, these polarizations can exist even after the triplet state has completely decayed and can persist for very long times. Results of calculations will be presented demonstrating that this polarization can cause unexpected changes in ^3P, P^+I^-, and fluorescence yield and decay kinetics, as well as high rep-rate or long flash experiments and saturation measurements. [1]Chidsey et al. (1985) PNAS 82,6850.

Th-AM-D5 DIRECT MEASUREMENT OF THE LOWEST EXCITED SINGLET STATE LIFETIME OF β-CAROTENE AND RELATED CAROTENOIDS.
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There has been general disagreement as to the lifetime of the lowest excited singlet states of carotenoids. Picosecond transient absorbance spectroscopy was used to measure directly the absorbance spectra and lifetimes of the lowest excited singlet states of β-carotene, β-8'-apocarotenal, and canthaxanthin in toluene. A 3 ps laser pulse at 510 nm was used to electronically excite the carotenes. The transient absorbance of each sample was probed between 400 nm and 700 nm using a 1 ps white light pulse. The 475 nm band of β-carotene bleaches immediately upon excitation and is accompanied by a positive absorbance change with a maximum at 550 nm. These absorbance changes follow single exponential decay kinetics with τ = 8.4 ± 0.6 ps back to ground state. No longer-lived species are formed. Similarly, excitation of β-8'-apocarotenal and canthaxanthin result in bleaching of their respective visible absorption bands followed by a positive absorbance change in the 550 nm region. The excited state of β-8'-apocarotenal decays to ground state in 25.4 ± 0.2 ps, while that of canthaxanthin decays in 5.2 ± 0.6 ps. These observations are interpreted as arising from decay of the lowest excited singlet state of the carotenes. These results show that there exists an inverse relationship between the lifetime of the lowest excited singlet state of the carotenoid and the degree of unsaturation in the carbon framework. These results are discussed in terms of previous attempts to determine the excited singlet state properties of carotenoids and the role of carotenoids as accessory antenna pigments in photosynthetic organisms. (This work was supported by the Division of Chemical Sciences, Office of Basic Energy Sciences of the U.S. DOE.)

Th-AM-D6 TRIPLET CAROTENOID FORMATION IN REACTION CENTERS FROM RHODOPSEUDOMONAS SPHAEROIDES:
Stephen V. Kolaczkowski, Michael K. Bowman and David E. Budil; Intr. by Marion C. Thurnauer

Carotenoid containing reaction centers from the photosynthetic bacterium Rhodopseudomonas sphaeroides that cannot transfer an electron beyond the primary acceptor bacteriopheophytin undergo a back-reaction forming ground state P870 and excitation of the carotenoid to its first excited triplet state (CarT). Through the use of Reaction Yield Detected Magnetic Resonance (RYDMR) we have determined that triplet state Pf is not quenched by the carotenoid. Nanosecond optical difference spectroscopy at cryogenic and physiological temperatures (9-295K) have identified the different molecular species formed during the reaction and fits to the optical transients have yielded rate constants for the formation of CarT by two paths. CarT is formed from an intermediate triplet state between triplet Pf and Pr (in carotenoidless RCs Pr is formed from this intermediate triplet species) at all temperatures with a temperature dependent yield. CarT is also formed from Pr at low temperatures and not at higher temperatures due to competition between the Pr forming path and the CarT forming path from the unidentified intermediate triplet between Pf and Pr. These experiments have led to a clearer understanding of CarT formation and reconciled previous observations.

(This work was supported by the Division of Chemical Sciences, Office of Basic Energy Sciences of the U.S. DOE.)

Th-AM-D7 MICROWAVE POWER AND TEMPERATURE DEPENDENCE OF THE REACTION YIELD-DETECTED MAGNETIC RESONANCE SPECTRUM OF THE PRIMARY RADICAL PAIR OF BACTERIAL PHOTOSYNTHESIS, David E. Budil, Jau H. Tang and James R. Norris, Chemistry Division, Argonne National Laboratory, Argonne, Illinois, 60439

The reaction yield-detected magnetic resonance (RYDMR) spectrum of the primary radical pair in quinone-depleted photosynthetic reaction centers from the bacterium R. sphaeroides R-26 is presented as a function of microwave power and temperature. The RYDMR signal at intermediate powers conforms well to the "inverted wing" lineshape predicted by model calculations. Significant changes occur in the RYDMR intensity, linewidth, and lineshape in the temperature range 290K to 220K, reflecting a temperature dependence in decay kinetics or electron spin-spin interactions of the radical pair. At low and intermediate microwave powers, a decrease in temperature results in a more positive RYDMR signal, whereas at high power, lower temperature gives a more negative signal. This demonstrates that changes in the RYDMR lineshape arise primarily from a decrease in the triplet radical pair decay rate, and not spin-spin interactions, with temperature. The microwave effect upon radical pair lifetime is combined with analysis of the RYDMR linewidth to provide the temperature dependence of both singlet and triplet radical pair decay rate constants.

(This work was supported by the Division of Chemical Sciences, Office of Basic Energy Sciences of the U.S. DOE.)

Th-AM-D8 THE SUPERHYPERFINE STRUCTURE OF ^{14}N IN IRON DEPLETED RCs FROM R. SPHAEROIDES R-26.1 RECONSTITUTED WITH ^{65}Cu: DETERMINATION OF THE NUMBER OF NITROGEN TO METAL LIGANDS. G.Feher, R.A.Isaacson, R.J.Debus, M.Y.Okamura, UCSD, La Jolla, Ca 92093*.

The EPR spectrum of Fe-depleted RCs reconstituted with ^{65}Cu (1) exhibits a hyperfine (hf) structure characteristic of ^{65}Cu (I=3/2); the hf lines are further split by superhyperfine interactions with ^{14}N (Fig.1). Expansion of the m_I=-3/2 resonance reveals 7 lines split by 10.9 Gauss (Fig.2). The number of lines suggests 3 equivalent nitrogens ligated to Cu^{2+} [i.e., 2nI+1 lines; I(^{14}N)=3/2,n=3] (2). Replacement of ^{14}N with ^{15}N yielded 4lines, also in accord with 3 nitrogens [I(^{15}N)=1/2]. In R. viridis Fe^{2+} is believed to form 4 nitrogen ligands (3). This discrepancy may be due to: i) the difference in bacterial species, ii) the difference between Cu^{2+} and Fe^{2+} or iii) a much smaller hf splitting of the 4th nitrogen. ENDOR and X-ray diffraction studies should resolve this question. *Work supported by NSF and NIH.

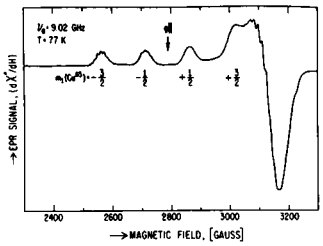


Fig. 1. *EPR spectrum of RCs with $^{65}Cu^{2+}$ instead of Fe^{2+}.*

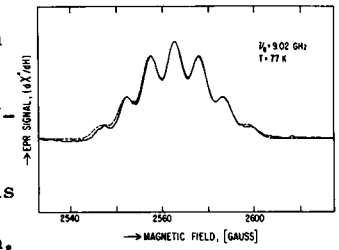


Fig. 2. *Expanded m_I=-3/2line. Dashed line is theoretical fit for S ^{14}N.*

1)R.J.Debus, G.Feher, M.Y.Okamura (1985) Biochem. (submitted). 2)H.L.VanCamp, R.H.Sands, J.A.Fee, (1981)J.Chem.Phys. 75,2098. J.H.Hyde, W.E.Antholine, W.Froncisz, R.Basoni, Proc.Intl.Symp.,Siena, Italy (in press). 3)J.Deisenhofer, O.Epp, K.Miki, R.Huber, H.Michel (1985) Nature (in press).

Th-AM-D9 PHOTOCHEMISTRY IN IRON-DEPLETED REACTION CENTERS FROM RPS. SPHAEROIDES R-26.1. D. Holten,[a] C. Kirmaier[a], R.J. Debus,[b] M.Y. Okamura[b] & G. Feher,[b] [a]Dept. of Chemistry, Washington University, St. Louis, MO 63130 & [b]Dept. of Physics, U.C.S.D., La Jolla, CA 92093.

The photochemistry of Fe-depleted[1] reaction centers (RCs) from Rps. sphaeroides R-26.1 was studied by transient absorption spectroscopy. Excitation of Fe-depleted RCs with 30-ps flashes produced the initial charge separated state P^+BPh^-Q with a yield and a visible/near-infrared absorption difference spectrum indistinguishable from that observed in native Fe-containing RCs. In native reaction centers, the subsequent electron transfer reaction, $P^+BPh^-Q \rightarrow P^+BPhQ^-$, occurs with unity quantum yield and a 205-ps time constant. In contrast, the lifetime of state P^+BPh^-Q in Fe-depleted reaction centers was found to be ~4 ns. A study of the decay paths of P^+BPh^-Q using longer-duration (10 ns and 400 ns) flashes showed that 40% undergo reverse electron transfer to give the ground state, 30% form the triplet state P^R (which has a lifetime of ~ 30 μs), and 30% undergo the electron transfer reaction $P^+BPh^-Q \rightarrow P^+BPhQ^-$. Reconstitution of the Fe-depleted RCs with Zn^{2+} restored the "native" kinetics. Thus, the absence of a divalent metal ion decreases the electron transfer rate from BPh^- to Q by a factor of ~ 50.

Work supported by the NSF.

1) R.J. Debus, M.Y. Okamura and G. Feher, Biochemistry (submitted).

Th-AM-D10 PICOSECOND STUDY OF THE $P^+I^-Q \rightarrow P^+IQ^-$ ELECTRON TRANSFER REACTION IN Rps. VIRIDIS REACTION CENTERS. C. Kirmaier,[a] D. Holten[a] & W.W. Parson,[b] Departments of [a]Chemistry, Washington Univ., St. Louis, MO 63130 & [b]Biochemistry, Univ. of Washington, Seattle, WA 98195.

We have measured the kinetics of the conversion of state P^+I^-Q to state P^+IQ^- in Rps. viridis reaction centers (RCs) at 285, 76 and 5 K by monitoring the time evolution of the visible and near-infrared absorption changes. The time constant for electron transfer from I^- to Q at all three temperatures is 175±10 ps, measured by recovery of bleaching in the bacteriopheophytin (BPh) Q_X band near 540 nm and by decay of the broad I^- absorption band centered near 690 nm. The 175±10 ps time constant in Rps. viridis RCs is comparable to the value of 205±15 ps measured at 285 K for Rps. sphaeroides RCs. However, the observation that the rate of electron transfer is independent of temperature in Rps. viridis is in contrast to recent results on Rps. sphaeroides, in which the time constant decreases from 205 ps at 285 K to 100 ps near 100 K and then remains constant down to 5 K.[1] A further difference between the RCs of the two species is that the observed kinetics in Rps. sphaeroides RCs show a dependence on the visible/near-infrared detection wavelength at both room and low temperature,[1] whereas in Rps. viridis RCs this phenomenon is not observed at 285 K. Thus, although the overall photochemistry is similar in the two species, there are significant differences in the details of the primary electron transfer reactions.

Work supported by the NSF.

1) C. Kirmaier, D. Holten and W.W. Parson, Biochim. Biophys. Acta (in press).

Th-AM-D11 ELECTRON TRANSFER REACTIONS IN REACTION CENTER PROTEIN FROM RPS. SPHAEROIDES.

M.R. Gunner, P.L. Dutton, U. of PA, Phila, PA; N.W. Woodbury, W.W. Parson, U. of WA, Seattle, WA

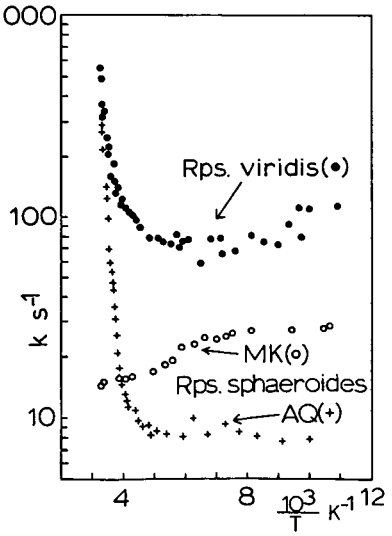
In reaction center protein with the native UQ_{10} as Q_A, the quantum yield for electron transfer from BPh^- to Q_A approaches unity, and charge recombination in the $(BChl)_2^+Q_A^-$ state occurs by direct electron tunneling. We have replaced UQ in the Q_A site by other quinones permitting comparison of the rates and extents of internal electron transfers. The in situ $E_{1/2}$ of the Q_A^-/Q_A couple was determined by measurement of the intensity of delayed fluorescence, which is proportional to the ΔG between $(BChl)_2^*$ and $(BChl)_2^+Q_A^-$; (2) the rate of an additional temperature dependent route for charge recombination accessible to low potential quinones which was found to be proportional to the fluorescence intensity. Conclusions include: (1) The correlation between solution and in situ $E_{1/2}$ depends on the structure of the quinone (e.g. with UQ=0 the $E_{1/2}$ of 2-methyl AQ is -235 in DMF, -250 as Q_A; however, tetramethyl BQ is -150 in DMF, +30 as Q_A). (2) Quantum efficiency is >90% for Q_As with midpoints as much as 180 mV lower than UQ. Below this is an unexpectedly steep decline. (3) The correlation of the rate of the thermal charge recombination route with the in situ $E_{1/2}$ for Q_A suggests that the reaction occurs via equilibration of $(BChl)_2^+Q_A^-$ with a quinone independent intermediate. The energy level of this state appears to be more than 100 mV lower than that of $(BChl)_2^+BPh^-$ measured on the ns time scale. (4) The rate of the direct tunneling reaction between $(BChl)_2^+$ and Q_A^- at 10°K is independent of the midpoint Q_A to at least 250 mV below UQ, inconsistent with expectations from electron tunneling theory.

Supported by NSF PCM 82-17270.

Th-AM-D12 TEMPERATURE DEPENDENCE OF CHARGE RECOMBINATION IN REACTION CENTERS FROM Rhodopsedomonas viridis

R.J. Shopes and C.A. Wraight, University of Illinois, Urbana

Illumination of reaction centers from Rps. viridis containing a single menaquinone induces the charge separated state $P^+Q_A^-$. When the bound donor cytochromes are oxidized before the flash, $P^+Q_A^-$ recombines with monophasic kinetics. The decay rate decreased with decreasing temperature between 310K and 220K but increased slightly as the temperature was lowered from 220K to 90K leveling off to a value of about 110 s^{-1}. This temperature dependence is consistent with a direct electron tunnelling route in competition with an indirect decay pathway, via a higher energy state, with a positive activation energy of about 30 kJ/mol. The activated pathway dominates at high temperature and the direct route dominates at low temperature. This result is in contrast to similar experiments in Rps. sphaeroides, with either menaquinone or the native ubiquinone functioning as Q_A, for which the rate increases only slightly on lowering the temperature from 310K to 90K. However, when anthraquinone is incorporated as Q_A in Rps. sphaeroides the temperature dependence of the kinetics is more similar to that seen for Rps. viridis. Supported by NSF PCM-83-16487.



Th-AM-D13 DEUTERIUM ISOTOPE EFFECT ON ELECTRON TRANSFER IN REACTION CENTERS FROM R. SPHAEROIDES.. M.Y.Okamura and G.Feher, UCSD, La Jolla, CA 92093*. ENDOR studies of the primary quinone, Q_A^- in bacterial RCs showed the presence of 2 hydrogens bonded to the oxygens of Q_A (1). They could be exchanged in D_2O with a characteristic exchange time of ∿2hr. We have investigated the effect of deuteration on the electron transfer rate $D^+Q_A^- \xrightarrow{k} DQ_A$. The rate was monitored optically (λ=865nm) in RCs at different times, t_{incub}, after dilution in D_2O buffer (0.025% cholate, pH8, T=20°C). The small changes in rate were quantitated by subtracting the kinetic traces at time t_{incub} from the trace obtained at t=0. The differential decay curve peaks at $t=k^{-1}$ (130ms) as expected theoretically (Fig.a). The amplitude of the peak, ΔA_m, is related (for small changes) to the change in rate by $\Delta k/k \simeq (2.7)(\Delta A_m/A_o)$, where A_o is the amplitude normalized to -1. The time course of ΔA_m agrees with the proton exchange time determined by ENDOR. The data (Fig.b) show an increase of the electron transfer rate by ∿6% upon deuteration, suggesting that the vibrations of the H-bond play a role in the electron transfer.

*Work supported by the NSF.

1)W.Lubitz, E.C.Abresch, R.J.Debus, R.A.Isaacson, M.Y.Okamura, G.Feher (1985) Biochim. Biophys. Acta 808, 464.

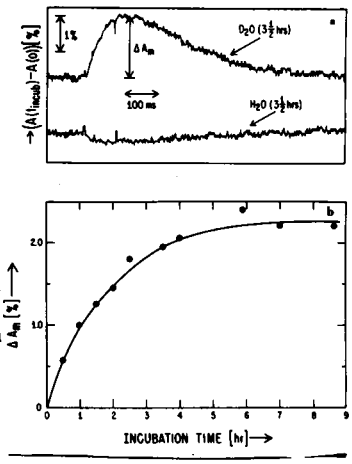


Fig.a,b. *Isotope effect of the charge recombination kinetics* $D^+Q^- \xrightarrow{k} DQ$.

Th-AM-E1 **IDENTIFICATION OF THE [^{14}C]-DANTROLENE AND [^{3}H]-RYANODINE BINDING SITES IN ISOLATED SARCOPLASMIC RETICULUM USING DIRECT PHOTOLABELING.** Abdul J. Rasool, John Sutko and Kevin P. Campbell,(Intro. by Byron A. Schottelius), Dept. of Physiology and Biophysics, The University of Iowa, Iowa City, IA 52242.

Junctional sarcoplasmic reticulum vesicles exhibit a Ca^{2+} uptake which is stimulated by either ryanodine or ruthenium red. The increase in Ca^{2+} uptake by these vesicles is presumably due to an inhibition of a Ca^{2+} efflux mechanism from the junctional sarcoplasmic reticulum. Dantrolene, a skeletal muscle relaxant, also acts by inhibition of Ca^{2+} release. We are using direct photolabeling of sarcoplasmic reticulum vesicles with high intensity UV irradiation in the presence of [^{14}C]-dantrolene and [^{3}H]-ryanodine in an attempt to identify the binding sites for these inhibitors of Ca^{2+} release. Analysis of preliminary photolabeling experiments with 1-10 uM-[^{14}C]-dantrolene, revealed specific incorporation of [^{14}C]-dantrolene into a 94,000 Da protein. Initial experiments with 100 nM [^{3}H]-ryanodine have shown that several sarcoplasmic reticulum proteins can be photolabeled with [^{3}H]-ryanodine. Immunoprecipitation with monoclonal antibodies specific for the junctional sarcoplasmic reticulum proteins is being employed to further characterize the photolabeled proteins. Junctional sarcoplasmic reticulum vesicles are first photolabeled with [^{14}C]-dantrolene or [^{3}H]-ryanodine, detergent solubilized and junctional specific proteins are immunoprecipitated with monoclonal antibodies. Monoclonal antibodies to the 60,000 Da, 90,000 Da, 94,000 Da and 300-350,000 Da proteins are being investigated. The identification of proteins involved in dantrolene and ryanodine binding should lead to a greater understanding of the mechanism of Ca^{2+} release within the sarcoplasmic reticulum. (Supp. by NIH NS18814, MDA and IIE)

Th-AM-E2 **JUNCTIONAL-SPECIFIC SARCOPLASMIC RETICULUM PROTEINS: IDENTIFICATION AND CHARACTERIZATION USING MONOCLONAL ANTIBODIES.** Kevin P. Campbell, Carol Reynolds Raab and Steven D. Kahl. Dept. of Physiology and Biophysics, The University of Iowa, Iowa City, IA 52242.

A hybridoma screening procedure using a double-dot immunoassay has been developed for the production of monoclonal antibodies specific for the junctional sarcoplasmic reticulum membrane of rabbit skeletal muscle. Hybridoma cells were prepared by cell fusion of mouse myeloma cells with spleen cells from mice immunized with isolated sarcoplasmic reticulum vesicles enriched in junctional membrane (heavy sarcoplasmic reticulum vesicles or isolated triads). Selection of hybridoma colonies that secreted monoclonal antibodies directed against the junctional sarcoplasmic reticulum membrane was based upon the ability of hybridoma culture supernatants to react with heavy sarcoplasmic reticulum vesicles and not with light sarcoplasmic reticulum vesicles in an immunodot screening assay. Selected hybridoma colonies were further analyzed against isolated triads and isolated transverse tubular membrane vesicles. Identification of the epitope for each monoclonal antibody was examined by indirect immunoperoxidase staining of protein blots and/or immunoprecipitation of ^{125}I-labeled sarcoplasmic reticulum proteins. Monoclonal antibodies have identified proteins of 22,000 Da, 35,000 Da, 60,000 Da, 63,000 Da (calsequestrin), 90,000 Da, 94,000 Da and 300-350,000 Da as junctional-specific. Structural characterization using proteolytic and glycosidase treatments has shown that these junctional-specific proteins (except calsequestrin) are exposed to the cytoplasm and that the 94,000 Da protein is an Endo H sensitive glycoprotein. The effects of the junctional-specific monoclonal antibodies on Ca^{2+} release from isolated sarcoplasmic reticulum vesicles is currently being determined. Supported by NIH (NS 18814), MOD and MDA

Th-AM-E3 **ULTRASTRUCTURAL LOCALIZATION OF JUNCTIONAL-SPECIFIC SARCOPLASMIC RETICULUM PROTEINS USING MONOCLONAL ANTIBODIES.** Barry G. Timms, Carol Reynolds Raab and Kevin P. Campbell, (Intr. by Robert E. Fellows), Dept. of Physiology and Biophysics, The University of Iowa, Iowa City, IA 52242.

Monoclonal antibodies to heavy sarcoplasmic reticulum vesicles and isolated triads have identified several proteins as components of the junctional sarcoplasmic reticulum membrane in rabbit skeletal muscle. Ultrastructural localization of these sarcoplasmic reticulum proteins was performed by indirect immunogold labeling of ultrathin acrylic resin sections of rabbit skeletal muscle. The aim of these studies is to determine the distribution of these proteins within the junctional sarcoplasmic reticulum membrane and to possibly identify protein components of the "SR feet". Both longitudinally and tangentially sectioned profiles of the triad region were found to be labeled with purified monoclonal antibodies to a 60,000 Da protein, a 90,000 Da protein and a 94,000 Da glycoprotein. In order to examine the junctional membrane at a higher level of morphological resolution and to improve the visualization of the "SR feet", we have used sarcoplasmic reticulum vesicles enriched in junctional sarcoplasmic reticulum membrane for immunocytochemical localization of the junctional proteins. A consistent labeling pattern was observed in the membrane regions associated with the junctional gap between the sarcoplasmic reticulum and the transverse tubule vesicles with the above junctional-specific antibodies. In addition, monoclonal antibodies to a 300-350,000 Da protein labeled this specific region of the isolated triads. Results suggest that there are several junctional-specific proteins with a specific structure/function relationship in this specialized region of the sarcoplasmic reticulum. (Supp. by NIH (NS 18814), MOD and MDA).

Th-AM-E4 **Ca^{2+} + Mg^{2+}-ATPase LOCALIZATION IN GASTROINTESTINAL SMOOTH MUSCLE FROM RABBIT AND OPOSSUM.** Barry G. Timms, Rick Roberts, James Christensen and Kevin P. Campbell, (Intr. by Charles C. Wunder), Depts. of Physiology and Biophysics, and Internal Medicine, The University of Iowa, Iowa City, IA 52242.

Ca^{2+}, which is essential to the normal contraction-relaxation cycle in smooth muscle, enters the intracellular space from both the extracellular medium and the sarcoplasmic reticulum. Ca^{2+} + Mg^{2+}-ATPase is the major membrane protein of skeletal sarcoplasmic reticulum and is responsible for the active transport of Ca^{2+} across the sarcoplasmic reticulum membrane. Recent biochemical evidence has demonstrated a Ca^{2+}-stimulated ATPase activity in microsomal fractions from smooth muscle cells. Our approach to understanding the structure and function of sarcoplasmic reticulum in gastrointestinal smooth muscle is through the structural and functional characterization of the protein components of smooth muscle sarcoplasmic reticulum using monoclonal antibodies. Monoclonal antibody characterization has included immunoblot analysis together with immunocytochemical localization by indirect immunogold labeling of acrylic resin sections. Extracts of smooth muscle were subjected to immunoblot analysis with monoclonal antibodies to rabbit skeletal and canine cardiac Ca^{2+} + Mg^{2+}-ATPase. A positive cross reactivity to the ATPase was observed using the monoclonal antibody to the cardiac ATPase but not with the striated muscle ATPase. The monoclonal antibody to the cardiac ATPase was subsequently used to determine the subcellular localization of Ca^{2+} + Mg^{2+}-ATPase of sphincter and non-sphincter smooth muscle samples from the gastrointestinal tract. Preliminary observations indicate that Ca^{2+} + Mg^{2+}-ATPase is associated with membrane profiles of the sarcoplasmic reticulum. (Supp. by NIH AM-11242, AM-34986 and NS-18814).

Th-AM-E5 MONOCLONAL ANTIBODY TO THE CA^{2+}+MG^{2+}-DEPENDENT ATPase (ATPase) OF CARDIAC SARCOPLASMIC RETICULUM (SR) CROSSREACTS WITH TYPE I (SLOW) BUT NOT TYPE II (FAST) SKELETAL MUSCLE FIBERS. A.O. Jorgensen* and K.P. Campbell**, *Department of Anatomy, University of Toronto, Toronto, Canada, **Department Physiology and Biophysics, University of Iowa, Iowa City, USA.

Phospholamban originally described as a cardiac SR protein, has recently been shown to be present in Type I (slow) but not Type II (fast) skeletal muscle fibers (Jorgensen and Jones, Fed.Proc. 44, 1373 (1985)). Since phospholamban is believed to interact with the ATPase of the cardiac SR to modulate the Ca^{2+} fluxes across the SR, one might expect that the ATPases of the SR in cardiac and slow skeletal fibers also have common structural characteristics not present in Type II (fast) myofibers. To investigate this possibility, a monoclonal antibody to the ATPase from cardiac SR was used to stain transverse cryostat sections of canine gracilis muscle (34% Type I (slow) myofibers) by indirect immunofluorescence labeling. Immunoblotting showed that this monoclonal antibody binds specifically to the ATPase of the SR from cardiac and soleus muscle tissues but does not crossreact with the ATPase of the SR from fast skeletal muscle. The results of the immunofluorescence staining showed that some myofibers were strongly labeled while others were labeled only at the level of the background. Staining of adjacent sections for the alkali stable ATPase, specific for Type II (fast) myofibers showed that all the Type I (slow) myofibers were specifically labeled with the monoclonal antibody to the cardiac ATPase while the Type II (fast) myofibers were unlabeled. We conclude that the ATPases of the SR from cardiac and Type I (slow) myofibers have at least one epitope in common, not present on the ATPase of the Type II (fast) myofibers. (Supported by *HSFO grant T445 and **NIH grant NS-18814).

Th-AM-E6 **PRODUCTION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES SPECIFIC TO CANINE CARDIAC SARCOPLASMIC RETICULUM PROTEINS.** David R. Pepper, Carol Reynolds Raab and Kevin P. Campbell, Dept. of Physiology and Biophysics, The University of Iowa, Iowa City, IA 52242.

Monoclonal antibodies have been used to investigate the structure and function of skeletal sarcoplasmic reticulum proteins. Several monoclonal antibodies to the skeletal 53,000 Da glycoprotein cross-react with the 53,000 Da glycoprotein of cardiac sarcoplasmic reticulum but monoclonal antibodies to skeletal Ca^{2+} + Mg^{2+}-ATPase do not cross react with the cardiac Ca^{2+} + Mg^{2+}-ATPase. To further examine the similarities and differences between cardiac and skeletal sarcoplasmic reticulum proteins, we are developing a library of monoclonal antibodies to canine cardiac sarcoplasmic reticulum. Mice were immunized with canine cardiac sarcoplasmic reticulum vesicles and hybridoma cells were produced by cell fusion of spleen cells with mouse myeloma cells. Selection of hybridoma colonies was based upon the ability of hybridoma culture supernatants to react with intact cardiac sarcoplasmic reticulum vesicles using an immunodot assay. Identification of the epitope for each monoclonal antibody by an immunoblot assay has shown that monoclonal antibodies produced are directed against the Ca^{2+} + Mg^{2+}-ATPase, 53,000 Da glycoprotein and 35,000 Da protein of canine cardiac sarcoplasmic reticulum. Immunoblots have indicated that monoclonal antibodies to the 53,000 Da glycoprotein and 35,000 Da protein react with both cardiac and skeletal muscle extracts. In contrast, the monoclonal antibody to the cardiac Ca^{2+}+ Mg^{2+}-ATPase recognizes the cardiac ATPase but not the skeletal ATPase. The differences in structure of the cardiac and skeletal ATPases as revealed by the monoclonal antibodies may reflect functional differences, e.g., the proposed regulation of the cardiac ATPase by phospholamban. (Supp. by NIH NS 18814).

Th-AM-E7 PHOSPHORYLATION OF PROTEIN AND LIPID COMPONENTS OF TRANSVERSE TUBULE MEMBRANES ISOLATED FROM SKELETAL MUSCLE. M. Angélica Carrasco, Enrique Jaimovich and Cecilia Hidalgo[a]. Depto. Fisiol. y Biofis. Fac. Med., U. de Chile and [a]Muscle Dept. Boston Biomed. Res. Inst. and Dept. Neurol., Harvard Med. Sch.

Transverse tubule (T-T) membranes, isolated from frog and rabbit skeletal muscle,were phosphorylated with (γ-^{32}p) ATP. Six protein bands were phosphorylated in the T-T membranes isolated from rabbit muscle (sub-unit molecular weights 135 ; 120; 94; 34 and 19 Kd). The phosphorylation of the 135 and 34 Kd proteins was absolutely dependent on exogenously added c-AMP; the phosphorylation of the other proteins, while stimulated by c-AMP, did not require c-AMP. Addition of c-AMP dependent protein kinase did not modify the phosphorylation pattern, suggesting that the isolated T-T membrane contain endogenous protein kinases. In contrast to c-AMP, addition of calcium and of calmodulin did not modify protein phosphorylation.

The lipid fraction extracted from the T-T membranes (frog), showed phosphorylation of two minor phospholipid components, tentatively identified as phosphatidyl - inositol phosphate and biphosphate. The phosphorylation of both components peaked at 2 min at 25°C,and decreased significantly after 5 minutes. These results suggest that the isolated T-T membranes have the enzymes involved in the phosphorylation and dephosphorylation of phosphotidylinositol, a requirement in the proposed physiological role of inositol 1,4,5, trisphosphate as the messenger in excitation contraction coupling (Vergara, Tsien and Delay, Proc. Natl. Acad. Sci. 82, 1985).

Supported by U. de Chile DIB grants B2123 and B2149 and by NIH grant HL23007.

Th-AM-E8 GENERATION OF A RESTING MEMBRANE POTENTIAL IN ISOLATED TRANSVERSE TUBULE VESICLES BY THE ACTION OF THE Na,K-ATPase. Troy J. Beeler, Dept. of Biochemistry, Uniformed Services University of the Health Sciences, Bethesda, MD 20814-4799

Muscle contraction is initiated by the release of Ca from the terminal cisternae of the sarcoplasmic reticulum following depolarization of the transverse tubule (T-tubule) by the action potential. For Ca release to occur, the T-tubule membrane must first have a physiological resting membrane potential (70-90 mV). Therefore we investigated the effects of ion gradients and ion transport on the membrane potential of isolated T-tubule vesicles as a step towards reconstituting the Ca release process in vitro. The Na,K-ATPase activity of T-tubule vesicles was relatively high (1.1 µmol/mg min) and the vesicles were able to accumulate ^{22}Na in an ATP-dependent manner (120 nmol/mg). Addition of ATP to T-tubule vesicles resulted in the formation of an inside-positive membrane potential (measured spectrophotometrically using oxonol VI). The potential change was dependent on both the Na^+ and K^+ concentration, and was blocked by inhibitors of the Na,K-ATPase. By measuring the effect of various ionophores on the membrane potential of T-tubule vesicles we concluded that the ATP-dependent potential change is due to the generation of a K^+ diffusion potential and to electrogenic transport by the Na,K-ATPase. Under optimal conditions, the resting membrane potential of the T-tubule vesicles was greater than 80 mV. In isolated triads, depolarization of the T-tubule membrane did not induce the release of Ca indicating that the coupling mechanism is inactivated or that a required factor is lost during the triad preparation.

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Th-AM-E9 BLOCK OF SR CALCIUM CHANNELS BY ORGANIC POLYCATIONS. P. Palade, Department of Physiology and Biophysics, University of Texas Medical Branch, Galveston, Texas 77550.

Using conditions previously developed for studying drug-induced calcium release from triadic SR (Biophys. J. 47, 453a, 1985), caffeine, thymol and many other drugs (but not inositol 1,4,5-trisphosphate; IP_3) cause net Ca^{++} release. We have determined that drugs causing Ca release do so by specifically increasing unidirectional Ca efflux, presumably by opening SR Ca channels. These determinations involve preloading the SR with ^{45}Ca and then, at the time of drug addition, adding a buffer mixture of unlabelled Ca and EGTA which minimizes reuptake of released ^{45}Ca and fixes the free Ca^{++} outside the SR at 0.6 µM. Submicromolar concentrations of polycations such as ruthenium red, protamine, polylysine, neomycin and gentamycin, micromolar concentrations of streptomycin and kanamycin and submillimolar concentrations of spermine inhibit net release of Ca induced by many drugs. We have tested the effects of ruthenium red and neomycin on the increase in unidirectional ^{45}Ca efflux induced by two of these drugs, 10 mM caffeine and 300 µM thymol. We find that at concentrations similar to those which inhibit net Ca release, ruthenium red and neomycin inhibit drug-induced increases in unidirectional ^{45}Ca efflux. These polycations do not affect Ca release induced by certain other drugs nor do they stimulate ATPase activities; therefore we do not believe that the effects of polycations on Ca release are mediated by increases in the rate of Ca pumping. We conclude that these polycations block the SR Ca channels opened by caffeine and thymol, and that caution must be exerted in interpreting experiments using such polycations as specific phosphoinositide blockers, since very low polycation concentrations block SR Ca^{++} channels that are not sensitive to IP_3 under the same experimental conditions.

Th-AM-E10 **CHARACTERIZATION OF A PROTEIN PHOSPHATASE ACTIVITY ASSOCIATED WITH CARDIAC SARCOPLASMIC RETICULUM.** Evangelia G. Kranias and Joseph Di Salvo. Dept. of Pharmacology and Cell Biophysics and Dept. of Physiology and Biophysics, Univ. of Cincinnati College of Medicine, Cincinnati, OH 45267.

The calcium pump in cardiac sarcoplasmic reticulum (SR) appears to be under reversible regulation through phosphorylation/dephosphorylation reactions on phospholamban, a 22,000 dalton polymeric proteolipid. Phospholamban is phosphorylated by cAMP-dependent and Ca^{2+}•calmodulin-dependent protein kinases and both phosphorylations are associated with stimulation of the initial rates of Ca^{2+}-transport. The stimulatory effects of the two protein kinases on the Ca^{2+} pump can be reversed by an endogenous protein phosphatase(s). The SR associated protein phosphatase activity is not due to contamination by cytosol, mitochondria, or sarcolemma. The protein phosphatase was solubilized by triton X-100 and effectively extracted into a 105,000 × g supernatant. The solubilized enzyme could use both ^{32}P-phosphorylase *a* and heat denatured ^{32}P-SR as substrates. Chromatography of the solubilized preparation on Mono Q HR 5/5 revealed a single peak of activity which eluted at 0.3 M salt and was coincident for ^{32}P-phosphorylase *a* or ^{32}PSR. When the peak activity fractions were subjected to chromatography on polylysine-Sepharose 4B one peak of activity was again observed eluting between 0.4-0.6 M salt. Further analysis of the enzyme preparation using sucrose density gradient ultracentrifugation indicated that the protein phosphatase has an apparent M_r of 46,000. These findings are consistent with the hypothesis that the SR associated phosphatase may participate in regulating the state of phosphorylation of phospholamban. Supported by NIH grants HL 26057, HL 22619 and HL 20196.

Th-AM-F1 PHOSPHORYLATION OF CARDIAC SARCOLEMMA VESICLES ENRICHED IN THE DIHYDROPYRIDINE RECEPTOR OF THE Ca^{2+} CHANNEL. Albert T. Leung, Toshiaki Imagawa, Alan Sharp and Kevin P. Campbell, Dept. of Physiology and Biophysics, The University of Iowa, Iowa City, IA 52242.

Cardiac muscle contraction is initiated and regulated by Ca^{2+} influx across the sarcolemma via voltage-dependent Ca^{2+} channels. It has been postulated that the cardiac Ca^{2+} channel is regulated through phosphorylation by cAMP-dependent protein kinase. 1,4-Dihydropyridines (e.g. nifedipine, nitrendipine) are potent blockers of the cardiac Ca^{2+} channel and it is believed that the high affinity dihydropyridine receptor is a component of the cardiac Ca^{2+} channel. In order to study the phosphorylation of the cardiac Ca^{2+} channel, we have used Nifedipine-Sepharose to isolate sarcolemma membrane vesicles that are enriched in the dihyropyridine receptor. In control sarcolemma vesicles, the major protein substrates phosphorylated by the cAMP-dependent protein kinase exhibited molecular weights of 155,000, 100,000, 55,000, 25,000 (phospholamban contaminant) and 15,000. Sarcolemma vesicles enriched in the dihydropyridine receptor have been shown to have two major substrates of molecular weights 155,000 and 55,000 under non-reducing conditions and 150,000 and 50,000 under reducing conditions. Our results on cardiac sarcolemma are consistent with Curtis and Catterall's observations (PNAS USA, 82:2528-2532, 1985) that the skeletal muscle dihydropyridine receptor has two phosphorylated subunits of molecular weights 165,000 and 55,000 under non-reducing conditions and 150,000 and 50,000 under reducing conditions. We are currently using CHAPS-solubilized sarcolemma and Nifedipine-Sepharose to further investigate the phosphorylation of the cardiac dihydropyrine receptor. (A.T.L. is an AHA Med. Student Research Fellow, T.I. is a MDA Postdoc. Fellow. Supported by AHA and NIH-NS18814.)

Th-AM-F2 INTERACTION OF CHOLERA TOXIN WITH SUPPORTED MONOLAYERS CONTAINING GANGLIOSIDE G_{M1}. R.A. Reed, J. Mattai, D.L. Gantz, and G.G. Shipley. Biophysics Institute, Boston University School of Medicine, Boston, MA 02118.

Lipid monolayers containing various mole fractions (0.01 to 0.1) of ganglioside G_{M1} in egg yolk phosphatidylcholine formed at the air-water interface have been transferred using the Langmuir-Blodgett technique to (a) glass slides coated with octadecyltrichlorosilane or (b) carbon-coated electron microscope grids. Monolayer transfer has been demonstrated using fluorescence microscopy, by the transfer of a fluorescent phospholipid analogue NBD-phosphatidylethanolamine incorporated into the lipid monolayer. Incubation of supported monolayers with solutions of fluorescein-labeled cholera toxin and its B-subunit resulted in specific binding of the toxin to monolayers containing G_{M1}, as revealed by fluorescence microscopy. In separate studies, G_{M1}-containing monolayers supported on electron microscope grids were incubated for increasing times with solutions of unlabeled cholera toxin followed by negative staining using 1.0% uranyl acetate. For low incubation times, electron micrographs showed staining of individual particles (diameter ~60Å) representing cholera toxin bound to its receptor G_{M1} in the monolayer. At longer incubation times (18-36 hrs) the electron micrographs showed patches of stained cholera toxin molecules in crystalline, 2-dimensional arrays. The dynamic properties (e.g. lateral diffusion) and the structure of these receptor (G_{M1})-ligand (cholera toxin) complexes are amenable to study by fluorescence and image-reconstruction methods, respectively.

Th-AM-F3 REGULATION OF [^{3}H]FORMYL-METHIONYL-LEUCYL-PHENYLALANINE ([^{3}H]fMLP) BINDING BY PHORBOL 12-MYRISTATE 13-ACETATE IN HUMAN GRANULOCYTES. K. Williamson and J. Navarro. Dept. of Physiology, Boston University School of Medicine, Boston, MA 02118.

Formyl-Methionyl-Leucyl-Phenylalanine (fMLP) is a chemotactic agent in human granulocytes (neutrophils and a differentiated leukemia cell line (HL-60)). These cells exhibit high affinity sites for [^{3}H]fMLP which are specific and saturable. We have found that the prior treatment of granulocytes with phorbol 12-myristate 13-acetate (PMA) at 37°C caused a dramatic inhibition of the specific binding of [^{3}H]fMLP. This effect was concentration and temperature dependent. Pretreatment of granulocytes at 4°C by PMA did not affect the specific binding. An inactive analog of PMA, 4α phorbol 12,13 didecanoate did not reduce the binding. The addition of PMA to isolated granulocyte membranes was without effect, while membranes from PMA-treated cells exhibited a dramatic reduction in [^{3}H]fMLP binding.

These observations suggest that the effect of PMA is specific and requires the integrity of the granulocytes. Since PMA activates Protein Kinase C it is possible that the loss of [^{3}H]fMLP binding may be caused by modification of the fMLP receptor via protein phosphorylation and/or the inhibition of recycling of the receptor. (This work was supported by NSF grant DCB-8511671.)

Th-AM-F4 CISPLATIN BINDING TO NEOPLASTIC GH3/B6 PITUITARY CELLS USING TERBIUM FLUORESCENCE. Robert G. Canada. Department of Physiology and Biophysics, Howard University College of Medicine, Washington, D.C. 20059.

The fluorescent properties of terbium (Tb^{3+}) were used to study the binding of cisplatin (cis-dichlorodiammineplatinum II) to GH3/B6 pituitary tumor cells. The fluorescence intensity of the Tb^{3+}-GH3/B6 complex was quenched in the presence of cisplatin. The IC_{50} for cisplatin inhibition of Tb^{3+}-GH3/B6 fluorescence was 190 μM. Eadie-Scatchard analysis revealed that cisplatin interferes with the cellular binding of Tb^{3+} in a noncompetitive manner, causing a dramatic decrease in the maximum number of high-affinity Tb^{3+} binding sites, without appreciably affecting their binding affinity. Association kinetics were investigated under pseudo-first order conditions; and, the halflife for the cellular binding of cisplatin was determined to be 2.7 min. The data suggest that GH3/B6 cells possess a specific cisplatin binding receptor in their plasma membrane.

Th-AM-F5 LIGAND-INDUCED CONFORMATIONAL TRANSITIONS OF THE NICOTINIC ACETYLCHOLINE RECEPTOR. Michael P. McCarthy and Robert M. Stroud. Dept. of Biochemistry and Biophysics, Univ. of California, San Francisco, CA 94122.

The nicotinic acetylcholine receptor (AChR) from Torpedo californica responds to a variety of naturally-occurring ligands in several different ways. Agonists induce transient opening of the ion channel and cause desensitization upon prolonged exposure, antagonists block agonist binding, and local anesthetics specifically modulate the AChR by a number of as yet undefined mechanisms. We have used the technique of tritium-hydrogen exchange to monitor the conformational transitions of purified AChR present in native membrane vesicles at pH 7.8 and 4C. Binding of the agonist carbamylcholine over a concentration range of 10 μM - 10 mM or the agonists suberyldicholine, hexamethonium and decamethonium over a more limited range of concentration causes no change in the global exchange kinetics. This suggests that the resting and desensitized states of the AChR are very similar in overall conformation. The local anesthetic procaine causes a slight retardation of exchange at low concentration, but at concentrations of 1 mM or more, procaine or the local anesthetic tetracaine slightly accelerate exchange. The latter response may be due to the solubilizing effect of local anesthetics observed at high concentrations in membranous systems. Antagonists such as α-bungarotoxin, and to a lesser extent tubocurarine, retard exchange upon binding. This effect is too large to be caused simply by screening of the AChR by these ligands, and indicates that antagonist binding does induce a conformational change in the AChR.

Th-AM-F6 THIOL-GROUP MODIFICATIONS OF TORPEDO CALIFORNICA ACETYLCHOLINE RECEPTOR: SUBUNIT LOCALIZATION AND EFFECTS ON FUNCTION. Amy S. Yee and Mark G. McNamee, Department of Biochemistry and Biophysics, University of California, Davis, CA 95616

The effects of thiol-group modifications on acetylcholine receptor (ACHR) function were measured using purified ACHR reconstituted into Asolectin vesicles. N-Phenylmaleimide (NPM) was used to modify sulfhydryl groups on ACHR in the absence of any prior reduction by dithiotheitol, so that only the functional relevance of free sulfhydryls was examined. Modification by NPM led to the inhibition of ion channel activity without a detectable effect on ligand binding. The ion flux inhibition by NPM primarily affected channel activation, since the initial rates of activation decreased over a wide range of carbamylcholine concentrations. The ^{3}H-NPM subunit labelling pattern of ACHR (a multisubunit membrane protein with $\alpha_2\beta\gamma\delta$ stoichiometry) revealed that there was preferential labelling of the γ subunit. At high NPM concentrations, the number of sulfhydryl groups on the γ subunit that could be modified with NPM was approximately two. Detergent was required during labelling for functionally relevant thiol group modifications, and most of the label was protected from protease digestion in the reconstituted membranes. These results are consistent with the presence of the NPM modification in a bilayer and/or cytoplasmic domain.

Th-AM-F7 EFFECT OF PROTEIN SURFACE CHARGE ON BINDING OF LOCAL ANESTHETICS TO THE ACETYLCHOLINE RECEPTOR. J.P. Earnest[1,2], H.P. Limbacher, Jr.[1], M.G. McNamee[2], and H.H. Wang[1]. [1]Dept. of Biology, University of California at Santa Cruz, and [2]Dept. of Biochemistry and Biophysics, University of California at Davis.

We have shown that spin-labeled amine local anesthetics interact with several components of the reconstituted acetylcholine receptor membrane: at least one population is electrostatically immobilized by the acetylcholine receptor (AChR), and two other populations are associated with lipid components (Earnest et al, BBRC, 1984). Our present study demonstrates that the size of the protein-associated local anesthetic population is affected by ionic strength and pH. Affinity-purified AChR from **T. californica** was reconstituted in dioleoylphosphatidylcholine. Binding of a spin-labeled local anesthetic ("C6SLMeI", a quaternary derivative of intracaine) was measured from pH 6.5-9.5 and from .1-.45 M NaCl by resolution of the electron-spin-resonance (ESR) spectrum. The degree of change observed in the contribution of the AChR-associated component to the composite C6SLMeI spectrum is attributed to the change in the concentration of C6SLMeI at the extracellular aqueous surface of the AChR, as predicted by the Gouy-Chapman and Boltzmann relations. This suggests that the highly negative surface potential on the extracellular domain of the AChR is important for binding of quaternary amine local anesthetics to site(s) on the receptor and/or ion channel. The dissociation constant was estimated by multiequilibrium modeling of the membrane-bound, protein-bound, and aqueous species of C6SLMeI from the ESR data. The ability to isolate and quantify multiple populations of local anesthetics in a defined lipid bilayer which itself contributes no surface potential makes the reconstituted AChR membrane ideal for further study on the role of receptor surface potential.

Th-AM-F8 **RECEPTOR-MEDIATED Ca^{2+} MOBILIZATION IN THE DDT_1 SMOOTH MUSCLE CELL LINE: COMPARATIVE EFFECTS OF ALPHA$_1$-ADRENERGIC AND P_2-PURINERGIC AGONISTS.** George R. Dubyak* and Elwood E. Reynolds+, Depts. of Biochem/Biophys.* and Pharmacology+, U. of Pa., Philadelphia, PA 19104.

Cytosolic [Ca^{2+}] was measured in DDT_1 smooth muscle cells loaded with 50-100 μM fura 2; both cell suspensions and cell monolayers attached to plastic coverslips were used. Consistent with previous studies using quin 2-loaded DDT_1 cells (Reynolds and Dubyak, Biochem. Biophys. Res. Comm. 130:627, 1985), norepinephrine (NE) (EC_{50}= 300 nM) stimulated both a rapid release of Ca^{2+} from intracellular stores and an enhanced influx of Ca^{2+} across the plasma membrane. However, the time courses of the Ca^{2+} transients were considerably faster than those in quin 2-loaded cells, consistent with reduced effects of Ca^{2+} buffering due to high indicator concentration. The effects of NE on both Ca^{2+} mobilization and Ca^{2+} influx were completely inhibited by prazosin (0.1 μM), phentolamine (10 μM), or by brief pre-incubation (1 min) with 30 nM phorbol myristate acetate (PMA). Similar Ca^{2+} transients were also observed when the cells were treated with low concentrations of extracellular ATP (EC_{50}= 2μM). The ATP-induced transients were resistant to inhibition by prazosin or phentolamine but were reduced (>60% inhibition) by pre-treatment of the cells with PMA. With respect to both agonist selectivity and sensitivity, these effects of extracellular ATP are identical to those characterized in Ehrlich tumor cells (Dubyak and DeYoung, J. Biol. Chem. 260:10653, 1985) and consistent with the presence of so-called P_2 purinergic receptors. As is the case for alpha$_1$-adrenergic receptors, the putative purinergic receptors on DDT_1 cells appear to be coupled to the inositol phospholipid signalling cascade. Supported by NIH grant HL-15835.

Th-AM-F9 RED BLOOD CELL MEMBRANE - A MODEL FOR STUDYING MUSCARINIC CHOLINERGIC AGONISTS AND ANTAGONISTS. Lily C. Tang, Carol A. Bean, WRAIR, Washington, DC. and Dennis Jones, ICD, Aberdeen Proving Ground, MD. (Intr. by June M. Whaun).

The presence of muscarinic receptors on the human red blood cell (RBC) membrane has been demonstrated via direct binding of radiolabelled quinuclidinyl benzilate (^{3}H-QNB); this binding can be blocked by the specific muscarinic antagonist atropine. Erythrocytes exposed to cholinergic agents exhibit membrane structural changes. To test our proposal that the human RBC ghost may be a model for studying muscarinic cholinergic agonists and antagonists, we performed the radiolabelled binding assay on a number of recently synthesized atropine analogues and other proposed cholinergic antagonists. Fresh human RBC were collected and prepared free of hemoglobin. Various levels of each test compound were assayed to obtain the IC_{50}. These results were compared with the efficacy of the same compounds, in place of atropine, against the organophosphate soman. Our preliminary data indicate a correlation between the inhibition of ^{3}H-QNB binding and the survival of mice when treated with the compounds and 2-PAM against 2 LD_{50} of soman. The current experiments support our previous findings. This inhibition of ^{3}H-QNB binding to human RBC membrane can serve as a probe for further investigations of membrane structural changes due to cholinergic stimulation.