W-PM-G5

DYNAMIC ENERGY LOCALIZATION IN BACTERIAL ANTENNA COMPLEXES: THE INFLUENCE OF NUCLEAR MOTION ON THE MECHANISM OF EXCITATION TRANSFER ((**R. Kumble**, S. Palese, R. W. Visschers, P. L. Dutton and R. M. Hochstrasser)) Department of Chemistry and the Johnson Research Foundation, University of Pennsylvania, Philadelphia, PA 19104.

Ultrafast pump-probe spectroscopy has been applied to follow excitation dynamics within the core antenna complex (B873) from *Rs. rubrum* and its dimeric subunit (B820) in the visible, near-infrared and infrared spectral regions. These measurements have revealed fast relaxation processes within the dimeric subunit which imply a strongly scattered mechanism for excitation transfer amongst the bacteriochlorophyll (BChl) pigments within the antenna aggregates: both the Stokes shift and dephasing between the BChl dimer exciton levels of B820 occur on a sub-50 fs timescale. Transient spectral properties in the near-IR and IR regions have been studied to gain insight into (a) the detailed exciton level structure of antenna complexes from location of inter-exciton and charge-transfer transitions; (b) contributions of static and dynamic localization phenomena from monitoring the evolution of vibrational difference spectra in the 1600-1750 cm^{-1} range. The relevance of vibrational properties in understanding the mode of energy transfer will be discussed, elaborating upon the information obtained from the observation of coherent wavepacket states and from transient infrared measurements.

W-PM-G6

CHEMICAL COMPLEMENTATION IDENTIFIES A PROTON ACCEPTOR OF REDOX-ACTIVE Y_D IN PHOTOSYSTEM II.
((**Sunyoung Kim**, Richard J. Debus, and Bridgette A. Barry)) Dept. of Biochemistry, University of Minnesota, St. Paul, MN 55108 and Dept. of Biochemistry, University of California, Riverside, CA 92521

Photosystem II (PSII) contains a stable, light-induced, deprotonated tyrosine radical, $D^{\bullet}$. Using site-directed mutagenesis and chemical rescue, we have identified a proton acceptor for redox-active tyrosine D. Effects of mutagenesis and chemical rescue on the proton acceptor were monitored by difference FT-IR spectroscopy, and effects on the tyrosyl radical were monitored by EPR spectroscopy. We have acquired a vibrational spectrum associated with oxidation of tyrosine D and the protonation of the acceptor. The 3600-3100 cm^{-1} region of the spectrum contains N-H stretching vibrations, demonstrated by isotopic labeling. Mutagenesis of histidine 189 of D2 to leucine (HL189D2) alters the FT-IR and EPR spectra. In attempt to biochemically complement side-chain function, imidazole or 4-methylimidazole was then reconstituted into HL189D2 PSII. Both FT-IR and EPR spectra of imidazole-reconstituted samples support the conclusion that an accessible cavity has been generated in the mutant and that functional reconstitution has occurred. Imidazole-reconstituted samples showed an increase in $D^{\bullet}$ yield that is consistent with reconstitution in approximately 40% of centers. The FT-IR spectrum of 4-methylimidazole-reconstituted HL189D2 samples demonstrates that this species does not act as a reversible proton acceptor/donor partner for the radical; this result may be due to either the larger steric bulk or higher basicity of 4-methylimidazole, as compared to imidazole. We conclude that histidine 189 of D2 acts as a proton acceptor for redox-active tyrosine D.

W-PM-G7

A DIFFERENCE INFRARED STUDY OF THE PHOTOSYNTHETIC WATER-OXIDIZING COMPLEX
((**Jacqueline J. Steenhuis** and Bridgette A. Barry)) Department of Biochemistry, University of Minnesota, St. Paul, MN 55108

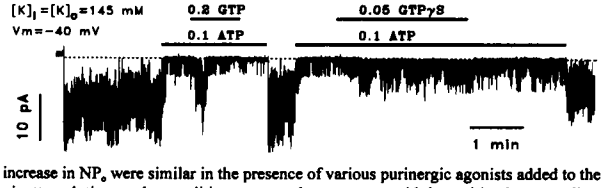
We have studied the two forms of the S_2 state of the manganese-containing catalytic site of photosystem II using difference infrared spectroscopy. With this method we can test the hypothesis that there are protein conformational differences between the two forms of the S_2 state, which are known as the g=4.1 and the multiline state. A light-minus-dark difference spectrum was constructed at 200 K, 130 K, and 80 K. These illumination temperatures generated the S_2 multiline state, the S_2 g=4.1 state, and a chlorophyll cation radical, respectively. Our data show that the g=4.1 form of the S_2 state arises from a unique protein conformation. Also, these spectra show that formation of the S_2 multiline state perturbs the vibrational spectrum of a carboxylic acid residue. This residue may be in the vicinity of the manganese cluster. A change in hydrogen bonding or effective dielectric constant upon formation of the S_2 state can explain this perturbation. This carboxylate residue is conserved in cyanobacterial and plant photosystem II.

K-ATP CHANNELS

W-Pos1

AGONIST-INDEPENDENT, GTP-MEDIATED STIMULATION OF CARDIAC K_{ATP} CHANNELS. ((A.Babenko and G.Vassort)) INSERM U390, Montpellier 34090 FR.

Hypothesis on agonist-dependent $G_{i,o}$ protein-mediated activation of cardiac K_{ATP} channels via membrane-delimited mechanism is based on observations that GTP added to ATP-containing solution bathing inside-out patches in the presence of adenosine or acetylcholine at the outer side of membrane and application of $G_{\alpha i-1,2}$ and $G_{\beta\gamma}$ subunits increase the channel open probability (NP_o). We report now that GTP stimulates K_{ATP} channels in inside-out sarcolemma fragments from rat ventricular cells under similar experimental conditions but without any agonist added to the pipette solution. The probability of observation of the GTP-induced K_{ATP} channels stimulation and relative



increase in NP_o were similar in the presence of various purinergic agonists added to the pipette solution, under conditions expected to prevent rapid desensitization as well as uncontrolled presence of ATP at the outer side of patches due to possible ATP transport from "intracellular" solution, and/or P_1-purinoceptor activation by adenosine derived from ATP hydrolysis. The effect was not inhibited by pertussis and cholera toxins treatment. In conclusion, GTP stimulates K_{ATP} channels independently of extracellular agonists via a membrane-delimited $G_{i,o}$ protein-independent mechanism. Supported by RFBR.

W-Pos2

BIOPHYSICAL CHANGES OF SKELETAL MUSCLE K_{ATP} CHANNELS IN K^+ DEPLETED RATS AND PHARMACOLOGICAL INTERVENTIONS.
((D. Tricarico, R. Mallamaci, V. Tortorella* and D. Conte Camerino)) Dept. of Pharmacobiology, and Dept. of Medicinal Chemistry*, Faculty of Pharmacy, University of Bari, Bari, ITALY.

Recently, mutations in the gene encoding the α1-subunit of the skeletal muscle Ca^{2+}-channel have been found in patients affected by hypokalemic periodic paralysis (HOPP) (Sipos et al., *J.Physiol.* 483.2:299, 1995). However, the link between the fiber depolarization, the paralysis and the Ca^{2+} channel mutation is still obscure. The administration of ATP sensitive K^+ channels (K_{ATP}) openers, pinacidil and cromakalim, to HOPP patients prevents the muscle paralysis. In the present work we investigated the properties of K_{ATP} channels of skeletal muscle fibers of K^+ depleted rats (Hypo K^+), the animal model of HOPP. In these rats, we tested cromakalim, and vanadate and mexiletine, drugs that have been shown to open K_{ATP} channels of cardiac cells. A treatment of male Wistar rats with K^+ free diet for 38-45 days led to a drop of serum K^+ level from 5.0±0.1 meq/L in the normokalemic rats (normo K^+) to 2.6±0.2 meq/L in the Hypo K^+ rats. In these animals, the resting potential of the extensor digitorum longus (EDL) muscle fibers of the Hypo K^+ rats, recorded by the two microelectrode technique, was drastically reduced. Further depolarization occurred after "in vivo" and "in vitro" administration of insulin. Similar phenomenas occurs in HOPP patients. Patch clamp recordings, showed that the mean current of K_{ATP} channel was reduced in the Hypo K^+ rats. Two types of K_{ATP} channels have been found in the Hypo K^+ rats. The first type, had a low single channel conductance (γ) of 29±4 pS. Whereas, γ was 71±1 pS in the normo K^+ rats. The second type had a K_{ATP} channel with normal γ but an altered selectivity to K^+ ion. Both types of channels partially lost the sensitivity to both MgATP and MgADP. Cromakalim (10-100 μM), vanadate (500 μM) and mexiletine (100-500 μM) restored the K_{ATP} conductance and prevented the fiber depolarization induced by insulin in Hypo K^+ rats. Our data indicate that closure of K_{ATP} channels contributes to the fiber depolarization in the Hypo K^+ rats, and that this animal model is suitable to search for therapeutic strategies in HOPP. (Telethon-Italy, project n° 579).

W-Pos3

ACTIVATION OF NATIVE AND CLONED POTASSIUM CHANNELS BY PIP_2. ((C.-L. Huang[#] and D.W. Hilgemann[*])), Departments of Medicine[#] and Physiology[*], Southwestern Medical Center, Dallas, TX, 75235.

K_{ATP} and inwardly rectifying K-channels are strongly activated by PIP_2 and inhibited by its hydrolysis in cardiac membranes (Hilgemann & Ball, Science 273,956,1996). We examined the PIP_2 sensitivity of several native and cloned potassium channels, using PIP_2 antibodies and cationic agents (pentalysine, neomycin, and micomolar Al^{3+} in the presence of 10 mM EGTA) to bind PIP2, and using liposomes to modify membrane composition. Ca-activated potassium conductances in giant membrane patches from tracheal smooth muscle and pituitary tumor cells (GH4C1) were almost insensitive to the PIP_2-modifying interventions. IRK1 and ROMK1 channels, expresed in Xenopus oocytes and studied in giant excised patches, were highly sensitive to the PIP_2-modifying interventions: PIP_2 strongly increased and stabilized the K-channel conductances. Channel run-down was blocked by 0.2 mM fluoride in the presence of 0.5 mM magnesium, and the cationic peptides strongly inhibited the conductances. PIP_2 antibodies induced irreversible channel run-down. Al^{3+}reversed the effects of PIP_2; after Al^{3+}had been removed for > 1min, fluoride reversed the inhibitory effect of Al^{3+}. Thus, PIP_2 may play an important regulatory and/or structural role in the function of inwardly-rectifying potassium conductances.

W-Pos4

A DISRUPTER OF ACTIN MICROFILAMENTS IMPAIRS SULFONYLUREA-INHIBITORY GATING OF CARDIAC K_{ATP} CHANNELS. ((**Peter A. Brady,** Alexey E. Alekseev, Luba A. Aleksandrova, Luis A. Gomez, Andre Terzic)). Mayo Clinic, Rochester MN. (Spon. by J. Lipsky).

The efficacy with which sulfonylureas inhibit cardiac ATP-sensitive K^+ (K_{ATP}) channels is reduced during metabolic compromise and cellular contracture. Disruption of the actin-microfilament network, which occurs under similar conditions, reduces the sensitivity of the channel towards intracellular ATP. To investigate whether a disrupter of actin-microfilaments could also affect the responsiveness of the K_{ATP} channel to sulfonylureas, single channel currents were measured in the inside-out configuration of excised patches from guinea-pig ventricular myocytes. Treatment of the internal side of patches with DNase I (100 μg/ml), which forms complexes with G-actin and prevents actin-filament formation, antagonized sulfonylurea-induced inhibition of K_{ATP} channels which was coupled with a loss of sensitivity to ATP. The apparent K_d and Hill coefficient for the inhibitory effect of glyburide, a prototype sulfonylurea, on K_{ATP} channel opening were respectively 0.13 μM and 0.95 prior to and 2.7 μM and 0.98, following DNase-treatment. DNase did not alter intraburst kinetic properties of the channel. When denatured or co-incubated with purified actin (200 μg/ml), DNase I no longer decreased glyburide-induced channel inhibition. This suggests that sulfonylurea-inhibitory gating of cardiac K_{ATP} channels may also be regulated through a mechanism involving sub-sarcolemmal actin-microfilament networks.

W-Pos5

OPENING OF K_{ATP} CHANNELS IN GUINEA-PIG CARDIOMYOCYTES BY 2,4-DINITROPHENOL SEPARATE FROM METABOLIC INHIBITION. ((**Alexey E. Alekseev,** Luis A. Gomez, Luba A. Aleksandrova, Peter A. Brady, Andre Terzic)). Mayo Clinic, Rochester, MN

Opening of ATP-sensitive K^+ (K_{ATP}) channels by the uncoupler of oxidative phosphorylation, 2,4 dinitrophenol (DNP), has been assumed secondary to metabolic inhibition and reduced intracellular ATP levels. In guinea-pig ventricular cardiomyocytes, DNP (200 μM) induced K_{ATP} channel opening, despite millimolar concentrations of ATP (1-2.5 mM) present not only in the pipette solution under whole-cell conditions, but also on the internal side of inside-out patches under cell-free conditions. Despite a sulfonylurea-insensitivity, DNP-induced currents had a single channel conductance (71 pS), inward rectification, reversal potential, and intraburst kinetic properties (open time constant, τ_{open}: 4.8 ms; fast closed time constant, τ_{closed}: 0.33 ms) characteristic of K_{ATP} channels suggesting that DNP did not affect the pore region of the channel. A DNP analog, with the pH-titrable hydroxyl- replaced by a methyl-group, lacked K_{ATP} channel opening ability. In the whole-cell mode, alkalinization of the extracellular milieu to pH 8.0 antagonized DNP-induced K_{ATP} current, whereas acidification to pH 6.4 enhanced DNP's action consistent with the pH-dependence in the membrane permeability of DNP (pK~4.1). In the inside-out configuration, the pH-dependence of the effect of DNP on channel opening was opposite in direction to that obtained under the whole-cell mode suggesting that transfer of protonated DNP across the sarcolemma is essential for activation of K_{ATP} channels. Thus, DNP can induce opening of cardiac K_{ATP} channels through an apparent functional uncoupling of the ATP/(sulfonylurea)-dependent inhibitory gating. We conclude that the use of DNP for metabolic stress-induced K_{ATP} channel opening should be reevaluated.

W-Pos6

TITRATING KATP CONDUCTANCES INTO SINGLE INSULIN-SECRETING CELLS VIA DYNAMIC CLAMPING. ((**T.A. Kinard,** L.S. Satin, G. de Vries and A. Sherman)). Dept. of Pharm. and Tox., Med. Coll. of VA., Richmond, VA. 23298 and Math. Res. Branch, NIDDK, Bethesda, MD. 20892

Pancreatic islets burst in elevated [glucose], but the ion channel mechanisms mediating bursting are incompletely understood (see Satin and Smolen, 1994; Endocrine 2:677-87). A new approach, dynamic clamping, was used to test how specific changes in ionic conductance affect the firing properties of single insulin-secreting cells. Membrane potential (Vm) was rapidly sampled in current clamp, scaled appropriately, and used to calculate and inject an artificial ion current into a patch-clamped cell in real time (DClamp, Dyna-Quest Tech.). We investigated the effects of a simple conductance, GKATP, known to be important for ß-cell electrical activity, on cell firing. Vm was measured in mouse ß-cells or HIT cells at room temperature using whole-cell or perforated-patch techniques and standard HEPES-buffered physiological solutions. Adding increasing amounts of GKATP progressively hyperpolarized cells. The mean change in HIT cell Vm on increasing GKATP to 0.1, 0.5 or 1 nS was -13.9 ± 1.8 mV (n = 14), -28 ± 4.6 mV (n = 6), or -45.2 ± 6.4 mV (n = 4). For mouse ß-cells, these same conductance changes hyperpolarized cells by -10.3 ± 1.7 mV (n = 8), -27.8 mV ± 1.9 mV (n = 4) or -34.0 ± 4.8 mV (n = 5). Hyperpolarization due to increased GKATP slowed or abolished electrical activity. In some cases hyperpolarization due to injected inward current could change firing patterns from continuous-firing to burst-firing. Burst period was variable and fast, <10 sec. These results suggest that dynamic clamping may be a useful new approach to understand islet cell bursting.

W-Pos7

EFFECTS OF NICORANDIL ON ISOLATED CELLS OF THE RAT MESENTERIC ARTERY ((**C.S. Davie,** J.A. Millar, N.B. Standen)) Ion Channel Group, Dept. Cell Physiology & Pharmacology, University of Leicester, UK

The vasodilator nicorandil has two mechanisms of action; it acts as a ATP-sensitive K^+ (K_{ATP}) channel opener and activates guanylyl cyclase. We have used dissociated cells of the mesenteric artery to study the nicorandil-activated current. Adult Wistar rats were killed by cervical dislocation. Small mesenteric arteries were removed and arterial rings were mounted in a myograph. Nicorandil (1 to 300μM) produced a concentration dependent relaxation, with a log EC_{50} of -4.77±0.07 (17μM). The relaxation was blocked with 10μM glibenclamide, log EC_{50} -4.32±0.05 (48μM). For patch clamping, smooth muscle cells from these branches were enzymatically isolated and currents were measured using the whole-cell configuration. Nicorandil activated an inward current in 140mM $[K^+]_o$ solution, at -60mV. The current was unaffected by IbTX (100nM) but substantially blocked by glibenclamide (10μM). The current-voltage relationship was near-linear and reversed at ~0mV, suggesting the activation of a voltage independent K^+ current and indeed, when 6mM $[K^+]_o$ solution was used it reversed near -80mV. We conclude that the relaxant action of nicorandil in the mesenteric artery is, in part, a result of its ability to open K_{ATP} channels.
Supported by the MRC. We thank Chugai Pharmaceuticals and Rhône-Poulenc Rorer for the gift of nicorandil.

W-Pos8

EFFECT OF POTASSIUM CHANNEL MODULATION ON HYPOXIC PULMONARY VASOCONSTRICTION. ((**S. A. Barman**)) Department of Pharmacology and Toxicology, Medical College of Georgia, Augusta, GA 30912. (Spon. by G. O. Carrier)

The role of ATP-sensitive potassium (K^+) channels and calcium - activated K^+ channels on the hypoxic pressor response in the canine pulmonary circulation was studied in isolated blood perfused dog lungs using 1) 10^{-5} M glibenclamide, a blocker of ATP-sensitive K^+ channels, 2) 10^{-5} M cromakalim, an opener of ATP-sensitive K^+ channels, and 3) 10^{-3} M tetraethylammonium ions (TEA), a blocker of calcium - activated K^+ channels. Hypoxia (Po_2 < 50 mm Hg) increased total pulmonary vascular resistance and capillary pressure by increasing precapillary resistance and postcapillary resistance. Pretreatment with cromakalim inhibited the hypoxic vasoconstrictor response while both glibenclamide and TEA potentiated the pressor effect of hypoxia. These data indicate that both ATP - sensitive K^+ channels and calcium - activated K^+ channels play a significant role in modulating the canine pulmonary vasoactive response to hypoxia.

W-Pos9

PROPERTIES OF INWARD RECTIFIER K^+ CURRENTS IN SMOOTH MUSCLE CELLS FROM RABBIT CORONARY ARTERIES.((**X. Xu**, S.J. Rials, Y. Wu, R.A. Marinchak, R.A. Filart, P.R. Kowey)) Lankenau Hospital & Medical Research Center, Wynnewood, PA 19096. (Spon. by Z.J. Bosnjak)

In smooth muscle cells enzymatically isolated from rabbit coronary arteries (rabbit CSMC), an inward rectifier K^+ current (I_{K1}) was identified. The current-voltage (I/V) relationships of I_{K1} showed a strong inward rectification with negligible outward current when cells were dialyzed with pipette solution containing 1 mM Mg^{2+}. However, dialyzing the cells with nominal Mg^{2+}-free pipette solution reveal a significant outward current hump in I_{K1} I/V relationship with a negative slope conductance typically observed in I_{K1} of ventricular myocytes, suggesting the strong inward rectification of I_{K1} in rabbit CSMC is partly due to the inhibitory effects of internal Mg^{2+}. I_{K1} of rabbit CSMC was inhibited by extracellular Ba^{2+}, but was insensitive to TEA, glyburide or 4-AP. I_{K1} conductance varied significantly among individual cells, ranging from 0.29 nS to 5.5 nS with an average of 1.9±0.3 nS (n=17) in physiological K^+ gradient. Like I_{K1} in ventricular myocytes, I_{K1} induced in rabbit CSMC inactivated during very negative hyperpolarizing steps, which was largely due to a block by external Na^+. I_{K1} in rabbit CSMC was not potentiated by 10^{-5} M acetylcholine, but was inhibited by α-adrenergic stimuli. Norepinephrine produced a 41±10 % (n=3) inhibition at 10^{-4} M and methoxamine produced a 81±3 % (n=6) inhibition at 10^{-3} M. We concluded that I_{K1} is present in rabbit CSMC and it shares many common properties with I_{K1} in ventricular myocytes.

W-Pos10

BLOCK OF CARDIAC INWARD RECTIFIER POTASSIUM CURRENT BY CHLOROETHYLCLONIDINE. ((**A. Stadnicka**, W.M. Kwok, G.C. Rehmert, Z.J. Bosnjak)) Department of Anesthesiology, Medical College of Wisconsin, Milwaukee, WI 53226

The α_{1B}-adrenergic receptor antagonist chloroethylclonidine (CEC) increases cardiac arrhythmias during coronary reperfusion and contributes to development of abnormal automaticity in ischemic Purkinje fibers. CEC sensitive α_{1B}-receptors modulate automaticity and prevent the development of abnormal rhythm. CEC impedes the stimulating action of α_1-agonists on Na^+-K^+ pump, increases membrane depolarization and the incidence of arrhythmias. Not only the Na^+-K^+ pump but also inward rectifier potassium current (I_{KIR}) is an important contributor to the total membrane current at diastolic potentials. In this study, CEC interaction with I_{KIR} was investigated. Whole-cell voltage-clamp was used to record I_{KIR} from isolated guinea pig ventricular myocytes. Propranolol (1 μM) excluded β-adrenergic input. Externally applied CEC blocked the steady-state I_{KIR} in both the inward and outward directions in a dose-dependent manner (1 μM-100 μM). K_d values were 5.5 ± 0.3 μM and 5.4 ± 0.3 μM, the Hill coefficients were 1.4 ± 0.1 and 1.7 ± 0.1 for currents in the inward and outward directions, respectively. The block was voltage-independent, and the steady-state activation was not affected. CEC, at 10 μM, decreased the whole-cell conductance by 44.5 ± 6% at hyperpolarized and depolarized membrane potentials. Complete block of I_{KIR} by 70-100 μM CEC was fully reversed upon washout. These results suggest that inhibition of cardiac I_{KIR} and hence an increase in cell excitability may contribute to arrhythmogenic action of CEC.

W-Pos11

SULFONYLUREA RECEPTOR 2B AND KIR 6.1 FORM A SULFONYLUREA-SENSITIVE BUT ATP-INSENSITIVE K+ CHANNEL.
((**M. Yamada**, S. Isomoto, S. Matsumoto, C. Kondo, Y. Horio, T. Shindo and Y. Kurachi)) Dept. of Pharm. II, Faculty of Med., Osaka Univ., 2-2 Yamada-oka, Suita, Osaka 565, Japan.

The channel current in a mammalian cell line HEK293T cotransfected with the sulfonylurea receptor 2B (SUR2B) and an inwardly-rectifying K+ channel clone uK_{ATP}-1 (Kir 6.1) was analyzed at the single channel level by using the patch clamp technique. In the cell-attached configuration, K+ channel openers such as pinacidil and nicorandil activated ~33 pS K+ channels in the presence of ~145 mM external K+. These channels were inhibited by sulfonylurea glibenclamide. Surprisingly, the channels did not spontaneously open on patch excision but were activated by intracellular nucleoside diphosphates such as UDP and GDP. The nucleoside diphosphate-induced channel activity was inhibited by glibenclamide but not intracellular ATP. Intracellular ATP on its own activated the channels in a Mg^{2+}-dependent manner. K+ channel openers and various intracellular nucleoside triphosphates synergistically activated the channel. From these data, the SUR2B/Kir 6.1 channel is not the classical ATP-sensitive K+ channel but closely resembles the nucleotide diphosphate-dependent K+ channel found in vascular smooth muscle cells that is sensitive to sulfonylurea drugs but not to ATP. These data further indicate that not only SUR but Kir significantly influences the response of SUR/Kir channels to intracellular nucleotides.

W-Pos12

A MOUSE PANCREATIC β CELL $K_{ir}4.1$ CHANNEL: CLONING, SEQUENCE, AND ANALYSIS OF INWARD RECTIFICATION PROPERTIES IN *XENOPUS* OOCYTES.
((**P. Drain**, L. Li, N. Chehab, and C. Engle)) Departments of Physiology, and Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia, PA 19104-6085.

Numerous inward rectifier K channel subunits are expressed in insulin-secreting β cell lines. We have cloned from the mouse β cell line mβHC9 a K channel subunit designated m$βK_{ir}4.1$ that is highly homologous to the rat $K_{ir}4.1$ subunit. The putative transmembrane segment M2 contains an E in the homologous position of other inward rectifer K channels that predicts expression would result in strong inwardly rectifying K currents. Functional expression and cell-attached patch clamp recording in *Xenopus* oocytes, however, indicates that m$βK_{ir}4.1$ can give rise to K channels that pass relatively large outward currents. Mutant analysis of $K_{ir}2.1$ (IRK1) led Yang et al. (1995) to the proposal of a second C-terminal amino residue E224 controlling the strong rectification of $K_{ir}2.1$. Substitutions at this position, including E224G, exhibited attenuated inward rectification compared to wild-type. In the homologous position of m$βK_{ir}4.1$, the E is naturally replaced by a G, suggesting that at least part of the rectification properties of m$βK_{ir}4.1$ could result from the presence of a G at the C-terminal position. Analysis of the sequence and rectification properties of m$βK_{ir}4.1$ are presented.

W-Pos13

FUSION OF A SULFONYLUREA RECEPTOR WITH INWARD RECTIFIERS MAKES FUNCTIONAL K_{ATP} CHANNELS. ((**J.P. Clement IV**[1], G. Gonzalez[1], K. Kunjilwar[1], L. Aguilar-Bryan[2] and J. Bryan[1])), Departments of Cell Biology[1] and Medicine[2], Baylor College of Medicine, Houston, TX

β-cell ATP-sensitive K^+ channels, K_{ATP}, are heteromultimers of the high affinity sulfonylurea receptor, SUR1, and $K_{IR}6.2$, an inwardly rectifying K^+ channel subunit in an unknown stoichiometry. Ligation of SUR1 and $K_{IR}6.2$ cDNAs through six glycine codons generates a SUR1~$K_{IR}6.2$ fusion protein with a defined 1:1 ratio. Expression of SUR1~$K_{IR}6.2$ in COSm6 cells generates homomeric K_{ATP} channels comparable to unfused channels, with activation by metabolic poisoning and diazoxide, inhibition by sulfonylureas and ATP, and moderate rectification in Mg^{2+}. The result shows a 1:1 stoichiometry is sufficient for channel formation. Digitonin solubilized, ^{125}I-azidoglibenclamide labeled complexes of SUR1~$K_{IR}6.2$ co-sediment with SUR1 plus $K_{IR}6.2$ on sucrose gradients with a mass of ~ 1000 kDa. A triple fusion, SUR1~$(K_{IR}6.2)_2$, does not form K_{ATP} channels when expressed alone, but is rescued by co-expression with SUR1 monomers showing that a 1:1 ratio of SUR1-to-$K_{IR}6.2$ is also necessary for active channel formation. A truncation of SUR1, which removes the second nucleotide binding fold, and causes persistent hyperinsulinemic hypoglycemia of infancy, does not rescue the triple fusion indicating four functional receptors are needed for channel activity. The data indicate K_{ATP} channels are hetero-octamers, consisting of four SUR1 receptors interacting with four $K_{IR}6.2$ subunits, $(SUR1/K_{IR}6.2)_4$. Funded by NIH, JDFI and ADA grants to JB & LAB.

W-Pos14

STOICHIOMETRY OF THE K_{ATP} CHANNEL COMPLEX ((Shyng, S.-L., Clement IV, J., Bryan, J. and **C.G. Nichols**)) Departments of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, MO 63110 and Cell Biology, Baylor College of Medicine, Houston, TX 77030

K_{ATP} channels were expressed in COSm6 cells after transfection with cloned sulfonylurea receptor (SUR) and inward rectifier (Kir6.2) subunits. Coexpression of SUR with wild type and N160D mutant Kir6.2 (Shyng *et al.*, 1996, *J. Physiol.* **494**, P51) generates channels with mixed rectification properties consistent with the K_{ATP} pore being formed as a heteromer of four Kir6.2 subunits. Expression of SUR-Kir6.2, or SUR-Kir6.2[N160D] fusion proteins, which force a SUR:Kir6.2 ratio of 1:1, generated K_{ATP} channels with identical spermine sensitivity to channels formed by co-expression of the parent monomers. Reducing the SUR:Kir6.2 ratio, by co-expression of monomeric Kir6.2 with the fusion protein, decreased K_{ATP} conductance. This dominant-negative effect was reversed by compensatory co-expression of monomeric SUR subunits. The phenotype of channels formed by mixing fusion proteins and Kir6.2 monomers was predominantly that expected of the fusion protein, in apparent ratio of fusion:monomer of 7:1. When SUR monomers were additionally transfected, the phenotype became predominantly that of the monomeric Kir6.2 component, with an apparent ratio of dimer:monomer contribution to functional channels of 1:6 (or 7:42), i.e. additional monomeric SUR expression caused a 42-fold increase in the apparent contribution of monomeric Kir6.2 subunits to functional channels. The results indicate that K_{ATP} channels are probably formed as an octomeric complex.

W-Pos15

K_{ATP} CHANNEL GATING: CONTROL OF ATP SENSITIVITY BY A RESIDUE IN THE M2 REGION OF Kir6.2. ((**S.-L. Shyng**, T. Ferrigni, and C. G. Nichols)) Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, MO 63110.

ATP-sensitive potassium channels are complexes of two proteins: the sulfonylurea receptor, a member of the ABC transporter family, and Kir6.2, a member of the small inward rectifiers. Wild-type K_{ATP} channels display weak inward rectification induced by polyamines and Mg^{2+}. Point mutations in Kir6.2 at amino acid position 160 from N to D or E causes the channel to become a strong inward rectifier (Shyng *et al.*, 1996, *J. Physiol.***494**, P51). The mutations also decrease the apparent sensitivity of the channel to inhibition by ATP. Substituting the N160 residue by A, D, E, or Q results in channels with $K_{1/2}$ ([ATP] causing half maximal inhibition) of ~6, 46, 18, 63μM, respectively, compared to wild type $K_{1/2}$ of 10μM. Further analysis demonstrates a negative correlation between the $K_{1/2}$ and maximal open probability of the channel. Using noise analysis, the estimated open probability of wild type channels is 0.72 in the absence of ATP. The N160A, N160D, N160E, N160Q mutant channels have open probabilities of 0.78, 0,86, 0.81, and 0.89, respectively. Since the inward rectifier Kir6.2 does not have an ATP-binding consensus sequence, it is unlikely that the N160 mutations cause changes in ATP sensitivity by altering the ATP binding directly. Instead, we propose a kinetic model in which mutations at position 160, which lines the channel pore, cause changes in the open probability of the channel, thereby alter the apparent sensitivity to ATP.

W-Pos16

A MODEL FOR REGULATION OF THE β-CELL ATP-SENSITIVE K^+-CHANNEL BY NUCLEOTIDES AND DIAZOXIDE. ((**Stephen J. Tucker**, Fiona M. Gribble, Stefan Trapp and Frances M. Ashcroft.)) University Laboratory of Physiology, Oxford University, Parks Road, Oxford OX1 3PT, United Kingdom.

The ATP-sensitive K^+-channel (K-ATP) plays a key role in insulin secretion from pancreatic β-cells. It is closed by glucose metabolism and sulphonylureas, which stimulate secretion, and opened by diazoxide, which inhibits release. Metabolic regulation is mediated by changes in ATP and and MgADP, which inhibit and potentiate channel activity respectively. The K-ATP channel consists of a pore-forming subunit, K_{ir} 6.2, and a regulatory subunit, SUR1. To investigate the role of nucleotides and diazoxide in channel activation we have made mutations in the nucleotide-binding domains (NBD) of SUR1 which are predicted to abolish the ability of these domains to hydrolyse nucleotides. Our results are consistent with the idea that hydrolysis of MgATP at NBD1 (but not NBD2) is essential for channel activation by diazoxide. The stimulation of channel activity by MgADP may also involve nucleotide hydrolysis since the ability of MgADP to stimulate the mutant channels is impaired and in wild-type channels non-hydrolysable ADP analogues and Mg^{2+}-free ADP are not potentiatory. In addition wild-type and mutant channels show no differences in ATP sensitivity or refreshment of channel activity by MgATP. Using this data we present a model based upon similar schemes for the catalytic cycles of related ABC-transporters. To further investigate this model we are currently examining the role of structural domains within SUR1 and K_{ir} 6.2 which appear to be involved in coupling the action of diazoxide and nucleotides to the channel pore.

W-Pos17

CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR CONFERS THE SENSITIVITY TO SULFONYLUREAS ON KIR6.1, AN INWARDLY-RECTIFYING K CHANNEL, EXPRESSED IN NIH3T3 CELLS. ((A. Ishida-Takahashi, **M. Horie**, T. Washizuka, H. Otani, S. Sasayama)) Department of Cardiovascular Medicine, Kyoto University Graduate School of Medicine, Kyoto 606-01, Japan (Spon. by K. Ishihara)

KIR6.1 is a ubiquitous type of inward rectifier K channels with two transmembrane spannings. Sulfonylurea receptor (SUR), a member of ATP-binding cassette (ABC) superfamily, has been shown to render the KIR6.1 sensitive to ATP and SU. Since CFTR also belongs to ABC superfamily and is sensitive to SU, we tested whether CFTR modulates the function of KIR6.1. Using $CaPO_4$ method, we transfected KIR6.1 alone or with CFTR into NIH3T3 cells and harvested stable cell lines by means of G418 and brastcidin selection. The 3T3 cells were voltage-clamped by using conventional whole-cell patch-clamp technique in the presence of 5.4 mM Ko and 150 mM Ki (~25°C). Both KIR6.1- and KIR6.1/CFTR-transfected cells displayed K conductances that showed weak inward rectification and were reversibly blocked by submillimolar Ba (IC_{50}=89.3 vs 67.3 μM). Currents recorded from KIR6.1-transfected cells were not affected by glibenclamide while the compound inhibited the K conductance in cotransfected cells (IC_{50}=36 μM). In the cell-attached mode with a 150 mM K-pipette solution, both KIR6.1- and KIR6.1/ CFTR-transfected cells displayed a class of K channels having weak inward rectification and γ of ~52 pS. In the inside-out mode, the channels recorded from both types of cells were not inhibited by ATP. However, similar to the whole-cell conductance, the channels in cotransfected cells became sensitive to glibenclamide. Thus coexpression with CFTR conferred the sensitivity to SU, but not to ATP, on KIR6.1, a putative cardiac type of ATP-sensitive K channel, suggesting both molecules may interact in native cell membrane.

W-Pos18

HOMOMERIC K_v-3.4 CHANNELS ARE CARBACHOL-SENSITIVE AND EXHIBIT MECHANOSENSITIVITY. ((S. Ji, S. A. John, **J. N. Weiss**)) UCLA Cardiovascular Res. Lab., UCLA Sch. Med., Los Angeles, CA 90095.

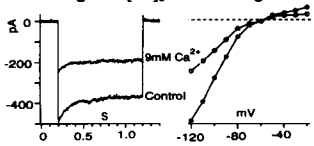
The K_v-3.4, or CIR (cardiac inward rectifier), potassium channel has been reported to form homomeric channels poorly on its own but readily co-assembles with K_v-3.1 to give rise to heteromeric K_{ACh} channels. We have successfully expressed homomeric K_{ir}-3.4 channels at high levels in *Xenopus* oocytes without co-injection of K_v-3.1. Whole-oocyte K_v-3.4 currents, recorded using the two-electrode voltage (TEV) clamp, exhibited strong inward rectification, high K selectivity, and sensitivity to block by Ba and Cs. In cell-attached patches with 100 mM $[K]_o$, K_v-3.4 channels had a single channel conductance of ~33.2 ± 0.3 pS (n=4). Co-expressed with the m2 receptor, K_v-3.4 currents increased significantly (200 to 300%) after exposure to 10 μM carbachol. Unlike whole oocyte currents which were stable, K_v-3.4 currents in giant cell-attached patches ran down rapidly over several minutes. Patch excision caused immediate and irreversible rundown. To determine whether mechanical stress inherent to membrane patches might cause the rapid rundown, we exposed oocytes to hypotonic (50%) bath solution, which reversibly suppressed whole cell K_v-3.4 currents by ~27 ± 4% (n=5). Similar effects were observed with the carbachol-stimulated K_v-3.4 channels and K_v-3.1/K_v-3.4 heteromultimers, but not with K_v-1.1 or K_v-2.1 channels. We further examined if $I_{K,ACh}$ in the atrium possesses mechanosensitivity. Application of 4-cm H_2O positive pressure to the patch pipet decreased the carbachol-stimulated whole-cell $I_{K,ACh}$ by over 10%. These results demonstrate that K_v-3.4 can form homomeric carbachol-sensitive inwardly rectifying K channels and may contribute mechanosensitivity to $I_{K,ACh}$ in the atrium.

INWARD RECTIFIER K CHANNELS

W-Pos19

BLOCK OF INWARD RECTIFIER CURRENT IN HUMAN CAPILLARY ENDOTHELIAL CELLS BY EXTERNAL Ca^{2+}. (**F. Jow**, R. Numann, and T. Colatsky) Wyeth-Ayerst Research, Princeton, NJ08543.

The inward rectifier current (IK_{IR}) is a dominant current in human capillary endothelial cells, occurring in 90% (n=72) of all cells studied, with an average current density of -8.4 ± 0.9pA/pF. IK_{IR} is activated at potentials below -60mV and blocked by 50μM Ba^{2+}. Increasing extracellular Ca^{2+} ($[Ca]_o$) from 1.5mM to 9mM reduced the magnitude of IK_{IR} at -120mV by 38.3 ± 2.1% (n=5). This reduction in IK_{IR} was not voltage dependent (-80 to -120mV). Decreasing $[Ca]_o$ from 1.8mM to 0.5mM increased IK_{IR} by 6.9 ± 1.9% (n=5). The relationship between $[Ca]_o$ and the magnitude of IK_{IR} can be described by an exponential with a 66% reduction in peak current at 4.0 ± 0.6 mM $[Ca]_o$. These data indicate that relatively small changes in $[Ca]_o$ can have significant effects upon the magnitude of IK_{IR} in human capillary endothelial cells, suggesting that IK_{IR} plays a dynamic modulatory role in endothelial cell function.



W-Pos20

TETRAMERIC SUBUNIT STRUCTURE OF THE INWARDLY RECTIFYING POTASSIUM CHANNEL K_{ir} 2.2. ((**K. F. Raab-Graham** and C. A. Vandenberg)) Dept. of Molecular, Cellular, and Developmental Biology and Neuroscience Research Institute, University of California, Santa Barbara, CA 93106.

Previous electrophysiological studies have suggested that inwardly rectifying potassium channels are composed of four homologous subunits (Yang et al. (1995) *Neuron* 15:1441-7). We report physical evidence for the tetrameric organization of the inwardly rectifying potassium channel K_{ir} 2.2. Channel stoichiometry was determined by chemical crosslinking and confirmed by velocity sedimentation. K_{ir} 2.2 channels were partially purified from rat cortex plasma membranes and treated with the bifunctional crosslinker glutaraldehyde. Crosslinked products were separated by SDS PAGE followed by immunoblotting. In the absence of crosslinker, the channel protein migrated as a single band of apparent molecular weight ~60 kDa. The time course of crosslinking revealed three additional bands, corresponding to the sequential association of monomers to form dimers, trimers and finally tetramers. Fully crosslinked channel was present as a single band of tetrameric size. Relative mobility of each band plotted against the logarithm of its assigned copy number produced a linear relationship, indicating a direct stoichiometric relationship. In addition, velocity sedimentation of endogenous K_{ir} 2.2 channel proteins in H_2O and D_2O yielded a single sharp peak of high molecular weight indicating a multisubunit protein. Crosslinking studies in combination with the hydrodynamic characterization of the channel demonstrate that K_{ir} 2.2 channels are formed by the association of four subunits.

W-Pos21

THE INTRINSIC GATING MECHANISM IS FUNDAMENTAL TO INWARD RECTIFICATION IN THE IRK1 CHANNEL. ((J.-K. Lee, S. A. John, Y. Lu, R.-C. Shieh, J. N. Weiss)) UCLA Sch. of Med., Los Angeles, CA 90095. (Spon. By J. N. Weiss)

Inward rectification of IRK1 has been attributed to voltage-dependent pore block by Mg_i and polyamines (PA). Recently we reported an intrinsic gating mechanism sensitive to pH_i (Shieh *et al. J Physiol* 1996:494,363). To examine the relationship between intrinsic gating and block by PA, macroscopic currents were recorded from inside-out giant patches excised from *Xenopus* oocytes expressing wild type (WT) and mutant IRK1 channels during voltage clamps from -80 mV to 70 mV in Mg_i- and PA-free solution, at pH_i 7.2 and 9.0. In the single IRK1 mutants D172N and E224G, which have been shown to have intact but reduced affinity for block by Mg_i and PA, the kinetics of intrinsic gating were slower than in WT type IRK1 at pH_i 7.2, but accelerated at pH_i 9.0 similar to WT. In contrast, in the double mutant D172N-E224G, which has markedly reduced affinity for block by Mg_i and PA, intrinsic gating was also eliminated at both pH_i 7.2 or 9.0. To investigate the pH sensitivity of intrinsic gating, 3 candidate pH-sensitive cysteine residues in the M1and M2 transmembrane segments were mutated to serine. In C90S and C102S, the pH-sensitivity of intrinsic gating was absent; in C169S, inward and outward currents were markedly reduced at pH_i 9.0. Moreover, in WT IRK1, application of Ag (20 nM), which irreversibly forms S-Ag bonds with cysteine, eliminated both the pH_i sensitivity of the intrinsic gating mechanism and voltage-dependent block by spermine (up to 10 μM). These findings suggests that in addition to D172 and E224, cysteine residues in M1 and M2 play critical roles in both intrinsic gating and sensitivity to PA-block. Combined with recent evidence about Mg_i-block (Aleksandrov *et al. Biophys J* 1996:70:2680), they suggest that the intrinsic gating mechanism is fundamental to inward rectification, and that Mg_i and PA enhance inward rectification by binding to and increasing the effectiveness of the intrinsic gate, rather than by physically blocking the channel pore.

W-Pos22

EXPRESSION OF GREEN FLUORESCENT PROTEIN (GFP)-TAGGED INWARD RECTIFIER K^+ CHANNEL. ((E.N. Makhina, Q. Sha and C.G. Nichols)) Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, MO

A highly fluorescent version of GFP (Cormack et al., 1996, *Gene*, **173**, 33-38) was fused to the C-terminus of Kir1.1, Kir2.1 and Kir2.3 inward rectifier K^+ channels. Injection of chimeric Kir2.1-GFP cRNA into *Xenopus* oocytes indicated that functional channels could be expressed from such constructs. COSm6 cells were transfected with chimeric Kir2.3-GFP DNA under the control of a CMV promoter. Chimeric Kir2.3-GFP protein produces strong fluorescence permitting visualization of localization and processing of the channel in living cells. In contrast to the signal produced by GFP alone, which is uniformly distributed within the cell, Kir2.3-GFP fluorescence appears in clusters (~1 μm diameter), located in the perinuclear region and more widely within the cell, possibly reflecting movement from the Golgi compartment through the secretory pathway to the membrane.

W-Pos23

AN ENDOGENOUS INWARDLY RECTIFYING K^+ CURRENT IN XENOPUS OOCYTES. ((**M. Martínez**[1], R Gamboa[1], C. Plata[2], J. Ramos-Franco[1], L. Escobar[3], G. Gamba[2], and R. Mejía-Alvarez[1])) [1]Inst. Nal. de Cardiología. [2]Inst. Nal. de Nutrición. [3]Univ. Nal. Aut. de México. Mexico City, Mexico.

Xenopus laevis oocytes are widely used as an expression system of recombinant ion channels. In general, the lack of similar endogenous channels makes these cells a suitable system for structure-function studies. Nevertheless, *Xenopus* oocytes possess a wide diversity of ion conductances. An endogenous inward rectifier K^+ current (endogenous I_{RK}) has been previously reported by several groups, however its functional profile has been overlooked. The main goal of this work was to describe endogenous I_{RK} in terms of its biophysical properties and sensitivity to block by external Ba^{2+}. Endogenous I_{RK} was recorded in 55 defolliculated oocytes (from 8 frogs) with the two-microelectrodes voltage-clamp technique. Varying $[K^+]_o$ caused a shift in E_r and a change in the slope conductance, as expected for a K^+ current. Activation and inactivation were voltage dependent and displayed monoexponential time courses (τ_{act}=3 ms, τ_{inact}=121 ms, at -140 mV). The recovery from inactivation followed a biexponential time course (τ_1=134 ms, τ_2=892 ms). External Ba^{2+} blocked endogenous I_{RK} with low affinity. The blockade of the peak current was essentially voltage-independent, and had a k_d of 0.9 mM. In contrast, the effect of Ba^{2+} on the late current (80 ms) was both time- and voltage-dependent, with a K_d that displayed a monoexponential voltage dependence (τ=50 mV). These observations suggest the presence of two different binding sites for Ba^{2+}, one in the external vestibule and another lying within the pore. Our results indicate that although endogenous I_{RK} displays many similarities with other I_{RK}'s, it shows important unique characteristics, such as purely voltage-dependent gating kinetics.
Supported by the NIH-Fogarty TW00077-02 and CONACYT 8547 to RMA, and CONACYT 3840 to GG.

W-Pos24

ION CHANNELS FUSED TO GREEN FLUORESCENT PROTEIN ARE EXPRESSED NORMALLY AND RETAIN PHYSIOLOGICAL PROPERTIES ((S.A. John, **J.I. Goldhaber**, J.N.Weiss, B. Ribalet)) UCLA School of Medicine, Los Angeles, CA 90095

The inward rectifying channel IRK1 was fused to Green Fluorescent Protein (GFP). To examine the morphological distribution and physiological properties of the fusion protein, we transfected into HEK293 cells. The fusion protein was targeted to the plasma membrane as judged by confocal microscopy. Serial optical sectioning showed plasma membrane labeling in addition to some intracellular labeling; presumptively the golgi/ER membranes. Cell-attached patches showed the presence of an inward rectifying K channel. In inside-out patches excised into symmetrical 140 mM KCl, Mg_i-free solution, single channels had a conductance of ~20 pS and ran down rapidly. We also were able to express the fusion protein in Xenopus oocytes. With $[K]_o$=90 mM, two electrode voltage clamp pulses were applied from -80 to +80 mV, and revealed a characteristic inward rectifying K current, completely inhibited by 3 mM Ba_o, similar to the IRK1 wt channel. To test whether GFP fusion to other ion channels was also feasible, a connexin31/GFP fusion protein was also constructed. When transfected into HEK293 cells, a punctate labeling between transfected cells was observed, consistent with plasma membrane localization and the expected morphology/distribution of gap junctions. Some intracellular labeling was also observed. These findings demonstrate the utility of GFP fusion proteins as a marker for following the time course and cellular localization of ion channels expressed in mammalian and Xenopus oocyte systems.

W-Pos25

UDCG-212, A NEW POSITIVE INOTROPIC AGENT, BLOCKS INWARDLY RECTIFYING K^+ CHANNEL IN HUMAN VENTRICULAR MYOCYTES. ((**M. Oyaizu**, R. Sato, H. Kasugai, M. Saito and T. Narahashi)) Dept. Mol. Pharmacol. & Biol. Chem., Northwestern Univ. Med. Sch., Chicago, IL 60611.
(Spon. by C. H. Wu)

Inwardly rectifying K^+ channel current (I_{K1}) of cardiac muscle plays a critical role in the maintenance of the resting membrane potential and the rapid repolarization process of cardiac action potential. We investigated the effects of UDCG-212 (UD), a positive inotropic agent that increases the myofilament response to Ca^{++} and decreases the activity of phosphodiesterase III, on I_{K1} in freshly isolated human ventricular myocytes using the patch clamp technique. Under the conditions of perforated nystatin patch, bath application of 30 μM UD inhibited the peak I_{K1}. The current was similarly inhibited by isoproterenol. In contrast, UD failed to inhibit the current in the presence of the specific PKA inhibitor KT5720, suggesting that PKA is involved in the UD-induced inhibition of I_{K1}. In single channel recording from cell attached patches, bath-applied UD suppressed I_{K1} by decreasing the open probability without changing the unitary current amplitude. These results indicate that human ventricular I_{K1} may be inhibited via PKA-dependent pathways.

W-Pos26

Inward rectifier K^+ channels bind to members of the PSD/SAP family ((B.A. Wible*, E.A. Accilli▼, A.T. Dennis, and A.M. Brown▼) Rammelkamp Center, MetroHealth Campus, and Depts. of *Biochemistry, and ▼Physiology and Biophysics, CWRU, Cleveland, Ohio. 44109.

Native ion channel complexes consist of pore-forming α-subunits complexed with accessory proteins which may modulate their function. This is the case for the voltage-gated K^+ channel family, Kv1, for which a family of auxiliary subunits, Kvβ, has been described. Kv1 α-subunits also bind to members of the PSD/SAP family. The PSD/SAP proteins serve to cluster the Kv1 α-subunits in transfected cells by binding to their C-terminal regions. Thus far, no accessory proteins for inward rectifier K^+ channels (IRKs) have been described. We have used the yeast two-hybrid system to identify IRK binding proteins. The C-terminus of hIRK (IRK2), an inward rectifier cloned from human heart but also expressed in brain, was used to screen a rat brain activation domain fusion library. One of the positive clones from this screen was identical to a portion of SAP102. The C-termini of IRK1 and Kv1.4 also bound SAP102 while the C-terminus of GIRK1 did not. For Kv1.4-C, SAP/PSD binding is determined primarily by the last four amino acids, ETDV. The final four residues of hIRK and IRK1 are ESEI, while in GIRK1 they are DRFT. Our data suggest that the residues ESEI are the primary determinants of SAP/PSD binding in IRKs. Deletion of these residues in hIRK-C abolish binding to SAP102, while replacement of ETDV in Kv1.4-C with ESEI preserved binding. Recently, the C-terminus of IRK1 was used in a screen of the rat brain yeast activation domain library and isolated a portion of chapsyn-110, another member of this family. Further experiments are being performed to determine the functional consequences of IRK binding to members of the PSD/SAP family. (Supported by AHA/Northeast Ohio Affiliate and HL36930).

W-Pos27

N-GLYCOSILATION TAGS AND K^+ SELECTIVITY IN ROMK1.

(Ruth A. Schwalbe, **Laura Bianchi**, and Arthur M. Brown.), Rammelkamp Center, MetroHealth Campus,CWRU, Cleveland,OH.(spon. by Arthur M. Brown).

We have been mapping the topology of ROMK1 expressed in Sf9 cells using glycosylation site substitution mutagenesis. To our surprise, M128N and Q139N in the putative pore-forming segment H5, were glycosylated (Schwalbe et al. J.Biol.Chem. 271:24201-24206, 1996). Two characteristics of the pore of ROMK1 are the K^+ selectivity and the block by extracellular barium. Wild type ROMK1 is highly K^+ selective and block by barium is time-dependent. The mutant channels at 128 and 139 were non-selective for K^+ and the time-dependence of barium block was abolished. Unglycosylation of Q139N by tunicamycin had no effect. N-substitutions at putative C-terminus positions 199, 222 and 298 were unglycosylated and abolished K^+ selectivity and time-dependent barium block. Substitution at 259 produced wild-type behavior and in preliminary experiments, glycosylation. The putative C-terminus of ROMK1 appears to have sites that are near the pore and at least one extracellular site.

(supported by NIH grant HL36930 to Arthur M. Brown).

W-Pos28

HOMOMERIC ASSEMBLY OF ROMK1 CHANNELS VIA DISTINCT DOMAINS IN THE C-TERMINAL CYTOPLASMIC REGION AND TRANSMEMBRANE SEGMENTS

((Koster, J.C., Bentle, K.A., Nichols, C.G. and Ho, K.)) Department of Cell Biology & Physiology, Renal Division, and Department of Medicine, Washington University School of Medicine, St. Louis, MO 63110

Inwardly rectifying ROMK1 (Kir 1.1a) K^+ channels are proposed to function as homomeric tetramers (Glowatzki et al., 1995 *Proc. R. Soc.* 261, 251-261). To delineate the domains necessary for channel assembly, N-, C-terminal, and transmembrane ROMK1 deletion mutants, which by themselves are inactive, were coexpressed equimolar with full-length ROMK1 subunits in *Xenopus* oocytes. Deletion mutants lacking either the C-terminus (Δ203-389) or transmembrane M1 and M2 segments (Δ69-202), including the H5 segment, do not significantly alter full-length ROMK1 activity. In contrast, mutants that lack N-terminal regions (Δ3-68 or Δ39-68) strongly suppress ROMK1 currents in a dominant negative manner (control= 55.5 ± 14.3 μA at +50mV; coinjected Δ3-68= 5.1 ± 1.8 at +50mV; coinjected Δ39-68= 0.8 ± 1.0 at +50mV). Specificity of this inhibition is demonstrated by the inability of the Δ39-68 deletion mutant to attenuate glucose transporter activity (Glut1) in coinjected oocytes. Interestingly, a more limited N-terminal deletion (Δ3-38) results in current and rectification properties similar to full-length ROMK1 channel. Taken together, these findings suggest that distinct transmembrane and C-terminal domains are required for ROMK1 oligomerization and that, in contrast to a previous report (Fink et al., 1996 *FEBS Lett.* 378, 64-68), the N-terminus is not required for assembly.

W-Pos29

INHIBITION OF AN INWARD RECTIFIER POTASSIUM CHANNEL BY G-PROTEIN βγ SUBUNITS

((Q. Sha, N. Cohen*, E.N. Makhina, A. N. Lopatin, M.E. Linder, S. Snyder* and C. G. Nichols)) Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, MO 63110, and *Department of Neuroscience, Johns Hopkins University, Baltimore, MD 21205

A family of inward rectifier K channels (GIRKs, Kir3) are activated by Gβγ proteins and are responsible for the G-protein activation of Kir currents that underlie vagal slowing of the heart and certain neuronal processes. However, there is substantial evidence for G-protein inhibition of Kir currents underlying other processes, including TRH induced prolactin secretion in GH3 cells, microglia activation, and guard cell regulation of stomatal movement, and the molecular basis of this inhibition is unexplained. The Kir2 family of subunits encode constitutively active inward rectifier K channels. We report here that one member (Kir2.3) is inactivated by co-expression with G-protein β,γ subunits, whereas another member (Kir2.1), which shares 60% amino acid identity, is unaffected. Co-immunoprecipitation, and direct binding of G-protein βγ subunits, to Kir2.3, but not Kir2.1, suggests that the inhibition of Kir2.3 subunits results from a direct protein-protein interaction. When Kir2.1 and Kir2.3 subunits are coexpressed, the G-protein inhibitory phenotype of Kir2.3 is dominant. This raises the possibility that heteromeric expression of Kir2.3 with other Kir subunits could give rise to numerous type of inward rectifier current, with the capacity for down-regulation by G-proteins.

W-Pos30

A NOVEL, EXTRASENSITIVE METHOD OF K^+ CHANNEL LOCALIZATION.

((A.N. Lopatin and C.G. Nichols)) Department of Cell Biol. and Physiology, Washington University School of Medicine, St. Louis, MO

The high permeability of K channels to $Tl(I)^+$ ions, and the weak solubility of thallium halide salts was utilized to develop a simple, yet very sensitive, approach to study membrane localisation of inwardly rectifying potassium channels (Kir1.1, Kir2.1, Kir2.3). Kir channels were expressed in *Xenopus* oocytes, and oocytes were loaded with Br^- by microinjection. Oocytes were then voltage-clamped using two microelectrodes and Tl^+ was applied extracellularly. Under conditions favoring influx of $Tl(I)^+$ ions (negative membrane potentials), crystals of TlBr, visible under low power microscopy, formed under the membrane. Crystals did not form in uninjected oocytes, but formed with total Kir current densities as low as 10 μS. The number of observed crystals was much lower than the estimated number of functional ion channels, consistent with a clustered channel distribution. Channel clustering was confirmed by successful patch-clamping of regions in which clusters formed. Based on the pattern of crystal formation, Kir channels appear to be expressed around the point of cRNA injection. Similar experiments have been carried out with cultured COS cells transfected with Kir2.3 channels. TlBr crystals could be formed on the these cells, with highest densities appearing as a ring around the nucleus, close to the cell edge. With modifications this approach could be applied to other types of ion channels.

W-Pos31

INHIBITION OF IRK1 (KIR 2.1) CURRENT BY ALKYLAMINE ANALOGUES

((W.L. **Pearson** and C.G. Nichols)) Dept. of Cell Biology and Physiol.,Washington Univ. School of Medicine, St. Louis, MO 63110.

Inward rectifier K^+ channels display a high conductance for inward current and a very low conductance for outward current when measured in intact cells. Inward rectification is conferred by multivalent cations present in the cytoplasm (Mg^{2+} ions and the polyamines). Endogenous polyamines (putrescine, spermidine, and spermine) have simple chemical structures: 2 to 4 amine groups linked by 3 and 4 carbon alkyl chains. To investigate the relative contribution of charge and length of polyamine molecules to block of Kir 2.1 channels, we examined the effects of a series of n-monoaminoalkanes and diaminoalkanes (alkyl chains from 2 to 12 carbons) on Kir 2.1 channel activity. Short-chain monoamines (aminomethane to 1-aminobutane) did not block at concentrations up to 1 mM, while longer chain monoamines effectively blocked the channels at concentrations less than 100 μM. All diamines tested (diaminoethane to 1,12 diaminododecane, 100 μM) blocked Kir 2.1 channels in a voltage-dependent manner (>50% at 50 mV). Block by most diamines occurred almost instantaneously upon changing voltage, while block by the same concentration of monoamines of similar length occurred more slowly (eg. for 1-aminohexane τ_{block}= 2 ms, vs <0.1 ms for 1,6 diaminohexane, 50 mV, 100 μM). For both monoamines and diamines, increase in chain length increased the affinity of block. The results show that both charge and alkyl chain length contribute to the blocking process, suggesting both an electrostatic and hydrophobic interaction with the channel pore.

W-Pos32

SCANNING MUTAGENESIS OF PUTATIVE MEMBRANE-SPANNING SEQUENCES OF AN INWARD RECTIFIER K^+ CHANNEL

((**Anthony Collins**, Yuh Nung Jan & Lily Yeh Jan)) HHMI/Dept. of Physiology, UCSF, CA 94143-0724

Hydrophobic interactions between proteins and membrane lipids are thought to be generally of a non-specific nature. Therefore it may be expected that an integral membrane protein would tolerate a wide range of hydrophobic residues at positions which directly contact membrane lipids, but would not tolerate a charged residue at these positions due to the energy cost of burying a charge. Mutation of hydrophobic residues involved in the internal structure of the protein (within or between subunits, or in the active site) to either charged or to different hydrophobic amino acids would be expected to disrupt protein function. Standard hydropathy analysis of the amino acid sequence of $K_v2.1$ (IRK1) reveals two hydrophobic stretches of 22 residues (M1 and M2) which may span the membrane as α-helices. In an attempt to distinguish between lipid-contacting residues and internal residues in $K_v2.1$ we mutated positions in M1 and M2 individually and tested these mutants for inward rectifier function by injecting *Xenopus* oocytes with mRNA transcribed *in vitro* and measuring currents by two-electrode voltage clamp in 90 mM external K^+. Tolerated mutations were defined as those which produced measurable inwardly-rectifying currents. 17 out of 20 non-Trp M1 residues tolerated being mutated to Trp, while one of the two M1 Trp residues tolerated Ala. 19 M2 residues tolerated Trp. Helical wheel and net plots show that the non-tolerant positions are within a 120° segment and in the C-terminal half of M1, and within 60° and spread throughout the length of M2. More surprisingly, at least 14 of the Trp- and Ala-tolerant M1 residues also tolerate Asp.

W-Pos33

BLOCK OF INWARD RECTIFIER IRK1 BY SULFHYDRYL-SPECIFIC REAGENTS ((**C.Dart**, M.L.Leyland, R.Barrett-Jolley & P.R.Stanfield)) Ion Channel Group, Dept. Cell Physiology & Pharmacology, University of Leicester, PO Box 138, Leicester, UK.

We have investigated the effect of Ag^+ and other sulfhydryl-specific reagents on inwardly rectifying K^+ channels IRK1 (Kir 2.1) stably expressed in murine erythroleukaemia (MEL) cells. Membrane currents were recorded from single MEL cells using the conventional whole-cell clamp technique, $[K^+]_o$=70mM. Application of 100nM extracellular Ag^+ (radius of Ag^+ cation=0.12nm) led to a rapid reduction of steady-state inward current. Fractional remaining current (f) measured at -102mV following 90s exposure to Ag^+ was 0.31±0.05 (n=7) (mean±SEM). Silver block of IRK1 was independent of voltage, suggesting that the binding site for Ag^+ is near the extracellular face of the channel. External application of the charged methanethiosulfonate derivative, MTSEA (2.5mM; radius=0.18nm) led to a partial reduction in inward current (f=0.82±0.04; n=6), which could be reversed only by addition of 1mM dithiothreitol. A larger MTS reagent, MTSET (1-3mM; radius=0.29nm) had no discernible effect upon inward current (n=5). The thiolate group of cysteine is known to react avidly with sulfhydryl-specific reagents. Mutation of residue 149 in the pore-forming H5 region from cysteine to serine (C149S) resulted in channels that were insensitive to block by either Ag^+ (n=6) or MTSEA (n=6). The finding that sulfhydryl-specific reagents of different sizes produce varying degrees of block of wildtype IRK1 has implications as to the size of the pore at the level of cysteine 149.

Supported by Wellcome Trust

W-Pos34

DIFFERENTIAL CONTROL BY RECEPTOR STIMULATION OF RECOMBINANT HETEROMERIC KIR3.0 CHANNELS.

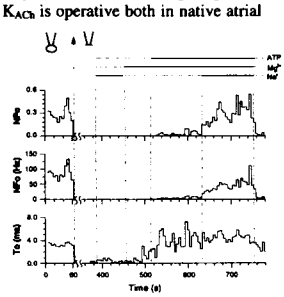
((**F. Döring**, E. Wischmeyer, A. Spauschus, E. Dissmann and A. Karschin)) Max-Planck-Institute for biophysical Chemistry, 37077 Göttingen, Germany. (Spon. by D. Schild)

Native G protein-activated K^+ channels in heart and brain may assemble as heterotetrameric polypeptides from different subunits of the Kir3.0 subfamily of K^+ inward rectifiers. When individually expressed in *Xenopus* oocytes recombinant Kir3.1-4 subunits gave rise to small current amplitudes or completely failed to form functional channels. In addition to biochemical evidence electrophysiological analysis of oocytes injected with all possible combinations of Kir3.0 subunit mRNA indicated that heteromeric channels assemble between certain subunits. The formation of heteromers was confirmed by functional expression of concatemers between Kir3.1 and Kir3.2-4. With G protein-coupled 5-HT_{1A} receptors coinjected, both basal (with 96 mM external K^+) as well as agonist-induced currents from heteromeric channels exhibited characteristic channel gating kinetics and were increased 2-25 fold in amplitude when compared to Kir 3.1 alone. In contrast, the Kir3.2-4 subunits among themselves yielded inward currents of only very small to moderate amplitudes. In oocytes potassium inward currents through heteromeric Kir3.0 channels were always activated in response to the receptor agonist 5-HT. However, when expressed in mammalian COS-7 cells, some heteromeric channels were activated by 10 µM 5-HT whereas others, composed of different subunits, appeared to be inhibited by 5-HT. We present data on the investigation of the molecular mechanisms that give rise to this differential regulation of Kir3.0 channels.

W-Pos35

Na^+ ACTIVATION OF THE MUSCARINIC K^+ CHANNEL BY A G-PROTEIN-INDEPENDENT MECHANISM. ((J.-L. Sui*, K. W. Chan, and D. E. Logothetis)) Dept. of Physiology and Biophysics, Mount Sinai School of Medicine, New York, NY 10029.

Muscarinic potassium channels (K_{ACh}) are composed of two subunits GIRK1 and GIRK4 and are directly gated by G proteins. We have identified a novel gating mechanism of K_{ACh}, independent of G-protein activation. This mechanism involves functional modification of K_{ACh} which requires hydrolysis of physiological levels of intracellular ATP and is manifested by an increase in the channel mean open time. The ATP-modified channels can in turn be gated by intracellular Na^+, begining at ~3 mM and with an EC_{50} ~ 40 mM. Na^+-gating of K_{ACh} is operative both in native atrial cells and in a heterologous system expressing recombinant channel subunits. Block of the Na^+ / K^+ pump (e.g. by cardiac glycosides) causes significant activation of K_{ACh}, consistent with mediation of the effect by intracellular Na^+ accumulation as evidenced by the time course of activation, and an increase in the frequency of channel opening, which is indistinguishable from that seen with Na^+ activation of K_{ACh}. These results demonstrate for the first time a direct effect of cardiac glycosides on atrial myocytes involving ion channels critical in the regulation of cardiac rhythm. (*Supported by the Aaron Diamond Foundation)



W-Pos36

IDENTIFICATION OF SPECIFIC DETERMINANTS OF ACTIVITY OF A G-PROTEIN-GATED K^+ CHANNEL WITHIN ITS HETEROMERIC SUBUNITS. ((K. W. Chan, J.-L. Sui, M. Vivaudou and D. E. Logothetis)) Dept. of Physiology and Biophysics, Mount Sinai Sch. of Medicine, CUNY, New York, NY 10029.

G-protein-gated inwardly rectifying K^+ (GIRK) channels, which are important regulators of membrane excitability both in heart and brain, appear to function as heteromultimers. GIRK1 is unique in the GIRK channel family in that although it is by itself inactive, it can associate with the other family members (GIRK2-GIRK5) to enhance their activity and alter their single-channel characteristics. We have identified F137 in the pore region of GIRK1, which critically controls channel activity. F137 is found only in GIRK1, while the remaining GIRK channels possess a conserved Ser residue in the analogous position. The single point mutant GIRK4(S143F) behaved as a GIRK1 analog, forming multimers with GIRK2, GIRK4 or GIRK5 channels that exhibited prolonged single-channel open-time duration and enhanced activity compared to that of homomultimers. Expression of the corresponding GIRK1(F137S) mutant alone resulted in appreciable channel activity with novel characteristics which was further enhanced upon coexpression with other GIRK subunits. Thus, although F137 of GIRK1 is a critical determinant of channel activity, additional determinants of activity do exist. Using deletion and chimeric constructs, we identified a carboxy-terminal region unique to GIRK1, which contributed to the production of large currents. Chimeras containing this GIRK1 region produced homomeric channels, exhibiting currents several fold greater than those from either wild-type subunit alone. Single-channel recordings of the active chimeras, containing the pore of GIRK4, exhibited patterns of activities with open-time kinetics and conductance representative of those of GIRK4.

W-Pos37

MODULATION OF THE CARDIAC GIRK1 BY OXIDATIVE STRESS.

((**G. Jeglitsch**, *P. Ramos, +A. Encabo, *H. Esterbauer, +K. Groschner and W. Schreibmayer.)) Departments of Medical Physics and Biophysics, *Biochemistry and +Pharmacology and Toxicology of the University of Graz, Austria.

Oxidative stress mediated by reactive oxygen species such as H_2O_2 and $O_2^{\cdot-}$ is centrally involved in the process of atherosclerosis and related cardiovascular diseases. In order to test the hypothesis, whether oxidative stress can modulate excitability proteins which are involved in the atherosclerotic process, we employed heterologous expression of the G-protein activated potassium channel from atrium (GIRK1) in *Xenopus laevis* oocytes. K^+-currents resulting from GIRK1/GIRK5 channels were recorded using the Two-Electrode-Voltage-Clamp (TEVC) technique. Oxidative stress was generated by 1mM hypoxanthine (HX) in combination with 4mU/ml xanthine oxidase (XO). The produced H_2O_2 and $O_2^{\cdot-}$ were quantitated by chemiluminiscence

Our results show that GIRK-currents were transient stimulated by the produced H_2O_2 and / or $O_2^{\cdot-}$ (Mean Increase: 340 nA, Std. err: 38 nA, n = 9). This effect did not depend on coexpression of muscarinic m2-receptors, on their stimulation by acetylcholin nor did it occur in native (uninjected) oocytes. The presence of 0.5mM niflumic acid, a Cl^- channel blocker, did not abolish the effect. 100µM Ba^{++} on the other hand led to a nearly complete block of the effect. Application of 1000U/ml catalase dramatically decreased the free H_2O_2 concentration, whereas the effect measured electrophysiologically still persisted, indicating that free $O_2^{\cdot-}$ radicals mediated the transient increase in GIRK1/GIRK5 currents. Our results demonstrate that G-protein activated potassium channels are stimulatory modulated by mild oxidative stress. Currently we are investigating the pathophysiological role of redox modulation of GIRK1 channels.

Supported by the Austrian Research Foundation (SFB 007)

W-Pos38

VOLATILE ANESTHETIC DEPRESSION OF CA CHANNELS EXPRESSED IN OOCYTES
((**Kamatchi G**, Durieux M E, Chan C, Snutch T P, Lynch C III))
Dept of Anesthesiology, UVa Health Sciences Center, Charlottesville, VA, Biotechnology Laboratory, University of British Columbia, Vancouver, BC

The volatile anesthetics isoflurane (I) and halothane (H) inhibit Ca^{2+} currents of various types in various tissues. To compare anesthetic effects on different channels under similar conditions, the DNA encoding P/Q- (α_{1A}), L- (α_{1C}), R-type (α_{1E}) Ca channel subunits, with the β_{1b} ± α_2 subunit, were expressed in *Xenopus* oocytes.

Methods: The germinal vesicle of defolliculated oocytes was injected with 1 ng of α_1 DNA in a volume of 9.2 nl. For injection of 2 or 3 subunits, equimolar conc. not exceeding 2 and 3ng DNA were used respectively. Oocytes were incubated in Barth's solution at 18°C. Two electrode voltage clamp experiments were conducted on the 8th day after injection in solution containing (in mM) 40 Ba, 40 TEA(or 50 Na), 2 K, 5 HEPES with methanesulfonate as the anion to achieve pH 7.4. For the comparison in Table 1, oocytes were held at -80mV; depolarized for 0.8 s to 0 mV. Pipette solution had 3M CsCl. Signals were filtered at 1kHz, recorded at 10 kHz.

Results:

	Control inward		Anesthetic depression (% change from control)					
Subunits	Ba^{2+} current (nA)		1.5% halothane (0.9mM)			2.5% isoflurane (0.8mM)		
expressed	peak*	late†	peak	late	n	peak	late	n
$\alpha_{1A}+\beta_{1b}$	702±175	227±40	-22±2	-54±8	10	-21±4	-50± 9	10
$\alpha_{1A}+\beta_{1b}+\alpha_2$	234± 63	66±21	-25±6	-51±5	3	-27±3	-63± 9	3
$\alpha_{1C}+\beta_{1b}+\alpha_2$	151± 66	82±63	-21±9	-16	2	-14±2	-33± 2	3
$\alpha_{1E}+\beta_{1b}+\alpha_2$	662±113	119±42	-22±2	-52±4	6	-37±9	-80±10	3

* peak inward current for a depolarization to 0 mV was observed 30 - 60 ms after the begining of the depolarization. † the inward current seen at 830 ms was choosen arbitrarily as the late value.

The anesthetics typically caused greater depression of late I_{Ba} by enhancing the inactivation rate. I_{Ba} with anesthetic washout was usually within 8 % of control value.

Conclusion: I and H at equipotent concentrations caused similar depression of these three different Ca channel subtypes. (Support: NIH GM31144, GM52387).

W-Pos39

VOLATILE ANESTHETIC(VA) INHIBITION OF RECOMBINANT CARDIAC L-TYPE Ca^{2+} CHANNELS.
((**KJ Gingrich**[1], I Nikonorov[2], TJJ Blanck[2], RS Kass[3]))
Departments of Anesthesiology, U. of Rochester[1], Rochester, N.Y., Anesthesiology, Hospital for Special Surgery[2], N.Y.,N.Y., & Pharmacology, College of Physicians & Surgeons[3], Columbia U.,N.Y.,N.Y.

The VA halothane (HAL) is known to depress cardiac contractility. An important site of action of HAL is the L-type voltage gated calcium channel (LVGCC) but the mode of interaction of HAL with the LVGCC is poorly understood. We studied the effects of HAL on recombinant LVGCC expressed in transiently transfected HEK-293 cells in order to avoid contamination with native Ca^{2+} channels and to begin the process of identifying the molecular basis of HAL activity. Cells were co-transfected with cDNA encoding rabbit heart α_{1C-a} and β_2 and skeletal α_2/δ subunits as previously described (Bangalore at al. 1996). Voltage-gated Ba^{2+} currents (I_{Ba}) were measured using whole cell arrangements of the patch clamp procedure. The bath solution (mM) was 20 $BaCl_2$, 154 NMG-Cl, 10 Hepes (pH 7.4),and the pipette solution (mM) was 112 $CsCl_2$, 1 $MgCl_2$, 1 MgATP, 10 EGTA, and 5 Hepes (pH 7.4). Currents were measured in the absence and presence of HAL (0-0.8 mM) As previously reported, the voltage-dependent properties of I_{Ba} in the transfected HEK 293 cells strongly resembled those of native cardiac LVGCC (Bangalore et al. 1996), and HAL reversibly depressed peak I_{Ba} amplitude without changes in macroscopic gating. The depression of peak I_{Ba} amplitude was dose-dependent. The data were fit to the Hill equation yielding an IC_{50} of 0.19 mM and an n of 1.1. These data indicate that the recombinant channels are approximately twice as sensitive to HAL inhibition as native channels as previously reported (Eskinder et al.). Future experiments will be directed at determining the molecular basis for this difference in sensitivity.

W-Pos40

INTERACTION OF Ca^{2+} WITH DRUGS AND RECEPTORS. MODELING STUDIES ON A SYNTHETIC CALCIUM CHANNEL PEPTIDE.
((**V.S. Ananthanarayanan** and B.S. Zhorov)) Biochemistry Dept., McMaster Univ., Canada; Institute of Evolutionary Physiology and Biochemistry, St. Petersburg, Russia. (Spon. by H.P.Ghosh)

The mechanism by which various drugs modulate voltage-dependent Ca^{2+} channels is ill-understood. We have approached this problem by examining the interaction of verapamil (VP), with a 97-residue synthetic Ca^{2+} channel peptide (SCP) which functionally resembles authentic L-type Ca^{2+} channels (Grove et al. *Proc.Natl.Acad.Sci. USA*, 1991, **88**:6418), and comparing the data with those obtained earlier for dihydropyridine (DHP) drugs (Zhorov and Ananthanarayanan, *Biophys. J.* 1996, 70:22). Different possibilities of VP binding inside the Ca^{2+}-bound SCP were simulated using the Monte Carlo-with-energy-minimization method. In the optimal mode of the binding, VP fit snugly in the pore and interacts by its dimethoxyphenyl groups with two Ca^{2+} ions coordinated to the acidic residues of SCP. The isopropyl group of VP abutted a ring of four Ile residues which form the putative SCP gate. The occlusion of this gate by VP was strikingly similar to that accomplished by the methyl group of DHP blockers. This is compatible with the recent finding that DHPs and VP share a common binding site within the pore of the L-type channel (Schuster et al. *EMBO J.* 1996, 15:2365). Our studies suggest that, in general, the modulation of the L-type Ca^{2+} channel by structurally different drugs may require interaction of the ligand with the pore-bound Ca^{2+} and with the hydrophobic gate. (Supported by MRC, Canada and BRF, Russia).

W-Pos41

BLOCK OF CARDIAC CA^{2+} CHANNELS BY THE NEW CA^{2+} ANTAGONIST Ro 40-5967 : CONSEQUENCES ON HEART RATE AND CARDIAC OUTPUT.
M. Mangoni, V. Leuranguer, E. Bourinet, J. Nargeot, S. Richard. CNRS ERS155, BP 5051, Montpellier, Fr (spon. by S. Baudet).

Ca^{2+} antagonists of the dihydropyridine (DHP), benzothiazepine, and phenylalkylamine classes have selective effects for L- over T-type Ca^{2+} channels in the cardiovascular system. There is now compelling evidence that the recently developed Mibefradil (Ro 40-5967) is more selective for the T-type. Therefore, comprehension of the antihypertensive effect and the slowing of heart rate without reduced cardiac output observed for this drug in clinical trials, is of major interest. In the present study, using the whole-cell patch-clamp technique, we have evaluated the effects of Mibefradil on T- and L-type Ca^{2+} currents (ICa,T ; ICa,L) from both neonatal and adult rat ventricular myocytes in the presence of 2 mM external Ca^{2+} . Our data confirm that Mibefradil blocks ICa,T with more than 10-fold selectivity over ICa,L. ICa,L evoked from an holding potential (HP) of -80 mV was inhibited with an IC_{50} of 3 ±1 µM (n = 10). By comparison, the DHPs nifedipine and nicardipine produced an equivalent block at only 0.1 µM. At this concentration, Mibefradil had no effect but could, however, produce similar block when the HP was depolarized to -50 mV (IC_{50} : 0.09 ±0.3 µM ; n = 9). The effect of Mibefradil was also be much enhanced by increasing the rate or the duration of depolarizations which is consistent with a preferential effect on the inactivated state of the channels. In conclusion, both effects of Mibefradil on ICa,T and ICa,L could potentially contribute to the slowing of heart rhythm without concomitant negative inotropism. This will be evaluated by taking advantage of a numerical model of cardiac action potential. *(supported by Produits Roche).*

W-Pos42

EFFECTS OF ANESTHETICS ON THE CARDIAC L-TYPE CALCIUM CURRENT.
((**A.K. Camara**, G.C. Rehmert, W-M. Kwok, Z.J. Bosnjak))
Dept. of Anesthesiology Medical College of Wisconsin, Milwaukee, WI 53226.

We studied the effects of volatile anesthetics isoflurane and sevoflurane at two clinically relevant concentrations on the cardiac L-type calcium current ($I_{Ca,L}$). Utilizing the whole cell configuration of the patch clamp technique, standard protocols for obtaining I-V relationships and steady state-inactivation were used in freshly isolated guinea pig ventricular myocytes. At 0.41 mM (n=6), isoflurane had no effect on $I_{Ca,L}$. Isoflurane at 0.92 mM (n=9), attenuated the peak $I_{Ca,L}$ by 39±6% (p<0.05). This depression of peak calcium current was associated with an unexpected shift of -7.9±1 mV (p<0.05) in the reversal potential for calcium, $E_{Ca,L}$. Isoflurane also caused a shift in the steady-state inactivation curve by 7.8±0.9 mV in the hyperpolarizing direction with no effect on the activation curve. The rate of current inactivation, τ, determined by fitting traces with a single exponential function, was decreased by 9.8±3.8 msec. Sevoflurane, another ether-based volatile anesthetic, inhibited peak $I_{Ca,L}$ by 42.6±8.6%, shifted the steady-state inactivation curve by -9.7±1.8 mV, and decreased τ by 16.3±5.7 at the concentration of 0.9 mM. Unlike isoflurane, sevoflurane at the lower range of clinical concentration, 0.52 mM decreased peak $I_{Ca,L}$ by 28.8±5.4%, shifted the steady-state inactivation and decreased the rate of current inactivation by -12.1±2.6 and -10.0±2.1, respectively. In addition, both concentrations of sevoflurane caused a marked shift in the calcium reversal potential. The results for both anesthetics show large shifts in the steady-state inactivation curves and significant acceleration of current inactivation, indicating an interaction with the inactivation of $I_{Ca,L}$. The unusual shift in $E_{Ca,L}$ might indicate a change in the permeability characteristics of $I_{Ca,L}$ or a non-specific effect on the cell membrane by the anesthetic molecules.

W-Pos43

PROPOFOL MODIFIES CALCIUM CHANNELS EXPRESSED IN *XENOPUS* OOCYTES.
((E. Sullivan, R. Katayama and **A. M. Correa**)) Department of Anesthesiology, UCLA, Los Angeles, CA, 90095.

General anesthetics decrease the excitability of nerves and muscles by affecting the function of ligand-gated and voltage-gated ion channels. 2,6-Diisopropylphenol, Propofol, a potent, fast-acting intravenous anesthetic commonly used to induce anesthesia, is one such agent. The purpose of this study was to investigate the mechanism by which Propofol (Prop) modifies the function of voltage-gated Ca^{2+} channels. We used the cut-open oocyte technique to record from *Xenopus* oocytes expressing Ca^{2+} channels, and studied the changes induced by Prop on the kinetics and the conductance properties of the channels. *Xenopus* oocytes were injected with mRNA's encoding for the α1 main conducting subunits of the cardiac, α1C (ΔN60, an N-terminal truncation; L-type), the neuronal α1E (R-type) and α1A (Q/P-type) Ca^{2+} channels, combined with the $\alpha2\delta$ and the β1b or β3 auxiliary subunits. Currents were recorded in external 10 Ba. Prop, made fresh from a 0.1 M ethanol stock, was added to the external medium. At the concentrations assayed, 100 and 500 µM, Prop induced a concentration-dependent, voltage-independent decrease of the ionic currents and of the fractional conductance. The extent of reduction varied among experiments. In addition, Prop accelerated the rate of inactivation of the ionic currents. The depression of the currents was frequency dependent being more profound at higher pulse rates. The rate of decay of peak currents was increased 5 to 6-fold by 500 µM Prop pulsing at 1 Hz. Prepulse experiments revealed a decrease of the current by Prop in the potential range of -140 to -80 mV, hyperpolarized to the holding of -80 mV. The general features of these results apply to all three channel subtypes studied and are consistent with an anesthetic-induced decrease in excitability. Supported by NIH 1R29GM53781.

W-Pos44

ANALYSIS OF THE DIHYDROPYRIDINE RECEPTOR SITE OF L-TYPE CA2+ CHANNELS BY ALANINE-SCANNING MUTAGENESIS OF TRANSMEMBRANE SEGMENTS IIIS6 AND IVS6

((**Blaise Z. Peterson**, Gregory H. Hockerman, Michael R. Abbott, Barry D. Johnson, Todd Scheuer, and William A. Catterall)) Department of Pharmacology, University of Washington, Box 357280, Seattle, WA 98195-7280, USA.
(Spon. by Scot C. Kuo)

L-type Ca^{2+} channels are pharmacologically modulated by dihydropyridine (DHP) Ca^{2+} antagonists by an unknown mechanism. Experiments using photoreactive DHPs, chimeric Ca^{2+} channels, and chimeric single residue mutants indicate that the DHP receptor site resides largely within transmembrane segments IIIS6 and IVS6. The DHP receptor site is probably composed of both amino acid residues that are conserved between DHP-sensitive and -insensitive channel types as well as those that are not conserved. In order to identify both conserved and nonconserved molecular determinants of DHP binding within transmembrane segments IIIS6 and IVS6 of the Ca^{2+} channel α_{1CII} subunit, individual amino acids were systematically changed to alanine and analyzed by radioligand binding using the radiolabeled DHP antagonist . Expression of mutants with no detectable (+)-[^{3}H]PN200-110 binding was confirmed electrophysiologically. Mutation of several amino acid residues in transmembrane segments IIIS6 and IVS6, some of which had not been identified in previous studies, have large effects on DHP binding affinity. Mutants at each of four residues in IIIS6 have affinities for (+)-[^{3}H]PN200-110 binding more than 5-fold higher than wildtype. Mutants at each of three residues in IVS6 have binding affinities for (+)-[^{3}H]PN200-110 more than 3-fold higher than wildtype. In addition to these residues that disrupt DHP binding, mutation of one residue exhibits an IC50 for block by (+)-PN200-110 that is more than 100-fold lower than wildtype.

W-Pos45

TOWARDS A 1,4-DIHYDROPYRIDINE-SENSITIVE CALCIUM CHANNEL α_{1A} SUBUNIT

((**Martina J. Sinnegger**, Zhengyi Wang, Manfred Grabner, Steffen Hering, Jörg Striessnig, Hartmut Glossmann, and Jörg Mitterdorfer)) Institut für Biochemische Pharmakologie, Universität Innsbruck, Peter Mayrstr.1, A-6020 Innsbruck, Austria.

Pharmacological modulation by 1,4-dihydropyridines is a central feature of L-type calcium channels, which is therapeutically exploited to treat cardiovascular disorders. Identification of the structural requirements for 1,4-dihydropyridine (DHP) sensitivity is prerequisite to understand the molecular mechanism of calcium channel modulation by these drugs. Recently, amino acid residues in transmembrane segments IIIS5 (Mitterdorfer et al., 1996, J. Biol. Chem., *in press*), IIIS6 and IVS6 (Peterson et al., 1996, J. Biol. Chem 271: 5293-5296; Schuster et al., 1996, EMBO J. 15: 2365-2370) of the α_1 subunit were identified to substantially contribute to DHP sensitivity of L-type calcium channels. In a constructive approach we transfered these L-type residues to the DHP-insensitive α_{1A} subunit. When co-expressed with auxiliary α_2/δ and β_{1a} subunits in *Xenopus* oocytes, the resulting mutant AL35 exhibited sensitivity to the DHP antagonist isradipine. In contrast, agonist modulation by the DHP BayK 8644 was completely absent. This finding suggests, that a combination of L-type amino acid residues Thr-1066, Gln-1070, Ile-1180, Ile-1183, Tyr-1490, Met-1491, Ile-1497, and Ile-1498 (numbering according to $\alpha_{1C\text{-}a}$), embedded into α_{1A} sequence environment, is sufficient to support DHP antagonist sensitivity. Additional L-type residues are required for DHP agonist modulation of AL35. (Supported by FWF grants S6601 (H.G.). S6602 (J.S.), and S6603 (S.H.).)

W-Pos46

MOLECULAR DETERMINANTS OF HIGH AFFINITY PHENYLALKYLAMINE BLOCK OF L-TYPE CALCIUM CHANNELS IN TRANSMEMBRANE DOMAIN IIIS6 AND THE PORE REGION.

((G.H. Hockerman, B.D. Johnson, M.R. Abbott, T. Scheuer, and W.A. Catterall)) Dept. of Pharmacology, U. of Washington, Seattle, WA 98195-7280.

Recent studies of the phenylalkylamine binding site in the rat brain class C_{II} L-type calcium channel have found three amino acid residues in the transmembrane segment IVS6 to be critical for high affinity block. These three residues, Y1463, A1467, and I1470 are unique to L-type channels and mutation of these residues reduces the affinity for the phenylalkylamine -D888 to that of non-L-type channels. We have extended our analysis of the phenylalkylamine binding site to amino acids in transmembrane segment IIIS6 and the pore region. Eighteen consecutive amino acids in IIIS6 were mutated to alanine and the conserved glutamate residues in the pore region of each homologous domain to glutamine. Mutant channels were expressed in tSA 201 cells along with the β_{1b} and $\alpha_2\delta$ auxiliary subunits. Assay for block of Ba^{2+} current by -D888 at -60 mV revealed that mutation of five amino acids which are conserved between L-type and non-L-type channels and one non-conserved amino acid decreased affinity for -D888 from 10-20 fold. These results indicate that there are important determinants of phenylalkylamine binding in both transmembrane segments IIIS6 and IVS6 and the pore region, some of which are conserved across the different classes of calcium channels. Supported by NIH Grant POI HL44948 (WAC) and an NIH postdoctoral fellowship (GHH).

W-Pos47

INHIBITION OF RECOMBINANT L-TYPE CALCIUM CHANNELS BY (S)- AND (R)-ISRADIPINE REVEALS TWO DISTINCT MECHANISMS OF DHP CHANNEL BLOCK.

((**R. Handrock**, R. Rao-Schymanski, #F. Hofmann, S. Herzig)) Depts. Pharmacology, #TU München and Univ. Köln, 50924 Köln, FRG.

We examined the inhibitory effects of isradipine (PN 200-110) enantiomers on whole-cell Ba^{2+} currents in CHO cells stably expressing the $\alpha_{1C\text{-}b}$ subunit from rabbit lung. Block was evaluated at three different holding potentials (HP) of -80, -60 and -40 mV. Drug concentrations amounted to 0.1 - 30 nmol/l for (S)-isradipine and to 0.3 - 1000 nmol/l for (R)-isradipine. Calcium channel inhibition by both enantiomers was voltage-dependent, characteristic of the conventional "inactivated channel block" by dihydropyridines (DHP). Stereoselectivity was unaffected by the holding potential (see also Handrock and Herzig 1996, Eur J Pharmacol 309: 317-21).
Interestingly, concentration-dependent changes in the current decay during the test pulse became apparent, consistent with "open channel block". We compared stereoselectivity, HP-dependency and kinetics of block development of "open channel block" with the respective properties of "inactivated channel block":

	Kinetics	HP-dependency	Stereoselectivity
"Inactivated channel block"	slow (s)	present	marked
"Open channel block"	fast (ms)	absent	not detectable

In agreement with a previous study on channels mutated in IVS6 (Schuster et al. 1996, EMBO J 15: 2365-70), our results suggest that DHP-induced "open channel block" is mediated by a novel binding site.

W-Pos48

PHARMACOLOGICAL CHARACTERIZATION OF LOW-VOLTAGE-ACTIVATED (LVA) CALCIUM (Ca^{2+}) CURRENT IN ACUTELY DISSOCIATED ADULT RAT SENSORY NEURONS.

((**Slobodan M. Todorovic** and C. J. Lingle) Dept. of Anesthesiology, Washington University School of Medicine, St.Louis, Mo 63110(Spon. by S.Todorovic)

Our understanding of the pharmacological properties of different LVA currents remains limited, although LVA currents among different cell types exhibit important pharmacological differences. Here, we used whole cell patch-clamp to study pharmacological properties of LVA Ca^{2+} current in freshly dissociated neurons from dorsal root ganglia of adult (100-250 gm) rats. Inward barium current (in the presence of internal fluoride to reduce "L" type HVA and external 1 μM ω-conotoxin GVIA to block "N" type HVA current) was evoked from negative holding potentials of -90 mV to test potentials of -30 mV and showed complete inactivation during a 200 mS test pulse suggesting it was exclusively inactivating "T" type LVA current. Amiloride, a selective "T" type current blocker in these cells, blocked about 90% of current with K_d =75 μM and a Hill coefficient (n) of 0.99. LVA current was completely blocked by inorganic Ca^{2+} channel blockers: Lanthanum (K_d=0.53 μM) > zinc (K_d=13.6 μM) > cadmium (K_d=25 μM) > nickel (K_d=51 μM). The antiepileptics, phenytoin (K_d=7.3 μM; n=1.34) and ethosuximide (K_d=0.7 μM; n=1.6) blocked about 60% and 70% of LVA current, respectively. General anesthetics blocked LVA current completely and reversibly without affecting leak current in these cells: propofol (K_d=12.9 μM; n=1.04)> pentobarbital (K_d=93 μM; n=1.17) > octanol (K_d 156 μM; n=0.91). The steroid anesthetic, alphaxalone, was the most potent anesthetic blocker (K_d=0.79 μM, n=1.08), although blocking maximally only 60% of the peak LVA current. Coupled with results on other LVA currents, these data suggest that differences in pharmacological sensitivity of LVA Ca^{2+} currents among peripheral neurons, CNS, and neuroendocrine cells may contribute to the spectrum of effects of analgesic, anticonvulsant, and anesthetic drugs.

W-Pos49

EFFECTS OF TEMPERATURE ON HUMAN L-TYPE CARDIAC Ca^{2+} CHANNELS EXPRESSED IN *XENOPUS* OOCYTES.

((T.J.A. Allen, G. Mikala* & A.C. Dolphin)) Dept. Pharmacology, Royal Free Hospital School of Medicine, London, NW3 2PF UK & *Inst. Molecular Pharmacology & Biophysics, Univ. Cincinnati College of Medicine, Cincinnati, OH 45267-0828, USA

Calcium channels generally exhibit a high temperature sensitivity, but what mechanism underlies this observation? When examined in guinea pig cardiac myocytes, between 24-34°C, the temperature sensitivity of peak I_{Ca} is strongly dependent on ß-adrenergic activation: attenuating PKA activity increases the Q_{10} to ~3.2, whilst activating PKA lowers Q_{10} to ~2. Associated with this temperature range are distinct changes in the inactivation of the Ca current (*T.J.A. Allen, J. Card. Electrophys. (1996) 7 307-321*). The heteromeric nature of Ca channels lends to the notion that temperature may exert its effect, for example, on interaction of subunits or biochemical processes. We have therefore examined this further using cloned Ca channels. Peak Ca channel currents through expressed channels ($H\alpha_{1C}$ α_2/δ $H\beta_{1b}$) failed to respond to either forskolin or PKA inhibitors, but were strongly temperature dependent, with Q_{10} above 5 between 15-25°C. Inhibition of L-type channels with 20μM nifedipine revealed inward currents with a lower Q_{10} of close to 4, and currents through the carboxyl terminus deletion mutant, $H\alpha_{1C\text{-}\Delta1633}$, had Q_{10} above 6. These data are largely unexpected and differ significantly from intact cardiac myocytes, suggesting that temperature sensitivity may depend on the membrane environment, channel assembly and/or other regulatory factors.
Supported by the Peter Samuel Royal Free Fund and American Heart Association.

W-Pos50

EFFECTS OF PTHrp AND PTH ON L-TYPE CALCIUM CHANNEL CURRENTS IN UMR 106 CELLS

((**M. Lalonde**, B. Li, P.K.T. Pang, and E. Karpinski)) Department of Physiology, University of Alberta, Edmonton, Alberta, Canada T6G 2H7

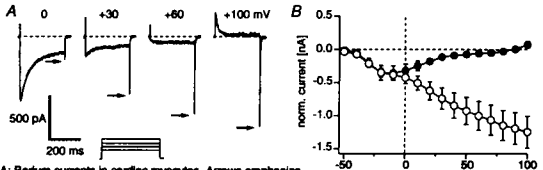
Parathyroid hormone (PTH) directly affects osteoblasts and is considered to stimulate bone resorption through its calcium mobilization effect. Parathyroid hormone related protein (PTHrp), which is responsible for humoral hypercalcemia of malignancy, is also important in bone development. The concentration of PTHrp in blood is lower than that of PTH; nevertheless, PTHrp is widely distributed in tissues and has paracrine, autocrine and endocrine mechanisms of action.

In the present study, the whole cell patch-clamp technique was used to compare the effects of bPTHrp and bPTH on L-type calcium channel currents in UMR 106 cells, an osteoblastic sarcoma cell line. bPTHrp(1-34) increased L-type calcium currents and the increase was concentration-dependent. bPTHrp produced a maximal increase of 152% at a concentration of 62.5 nM and the IC_{50} value was 3.4 nM. The maximum concentration of bPTHrp used (62.5 nM) did not shift the I-V relationship (fitted with a Boltzmann function) along the voltage axis. bPTHrp did not affect the inactivation rate of the calcium current. bPTH(1-34) had a less potent effect on L-type calcium currents, as it produced a maximal increase of 60% at a concentration of 7.5 μM and had an IC_{50} value of 40 nM. These results demonstrate differences in the potencies of bPTH and bPTHrp on L-type calcium channel currents in UMR-106 cells.

W-Pos51

THE APPARENT VOLTAGE-DEPENDENT INACTIVATION OF CARDIAC L-TYPE CALCIUM CHANNELS SEEMS TO BE A VOLTAGE-DEPENDENT BLOCK RELIEVED UPON REPOLARIZATION FOLLOWING STRONG DEPOLARIZATIONS ((**Andrea Fleig***, Friederike v.z. Mühlen** & Reinhold Penner*)) *Max-Planck-Institute for biophysical Chemistry and **Dept. of Cardiology& Pulmonology, University Göttingen, Germany.

Whole-cell patch-clamp recordings of Ca^{2+} and Ba^{2+} currents were made in freshly dissociated rat cardiac myocytes. Depolarization-induced Ca^{2+} (and Ba^{2+}) currents decayed rapidly to a small plateau phase of < 20% at the relevant potentials (0 mV to +130 mV). Nevertheless, large tail currents could be recorded following long and positive depolarizations. However, inactivated channels cannot contribute to instantaneous tail currents - irrespective of their individual gating mode [Pietrobon & Hess (1990) *Nature* 346:651]. This paradox may be resolved by a model where the apparent Ca^{2+} channel inactivation does not represent classical inactivation, but rather a block of conductance. This does not rule out some degree of voltage-dependent inactivation; but an additional block would simulate strong inactivation and cause an almost complete decay in Ca^{2+} current. Such a block could then only be reversed upon repolarizing the membrane following strong depolarizations, thus giving rise to large tail currents.



A: Barium currents in cardiac myocytes. Arrows emphasize tail currents. B: Mean I-V curve and activation curve of tail currents with barium as charge carrier.

W-Pos52

VOLTAGE-DEPENDENT FACILITATION OF CARDIAC L-TYPE CA^{2+} CURRENTS BY DEPOLARIZATION OF CELL RESTING MEMBRANE POTENTIAL. Lemaire[1], C. Piot[1], F. Leclercq[2], J.M. Frapier[2], J. Nargeot[1], S. Richard[1]. CNRS ERS155, BP5051 (1) and Hôpital A. de Villeneuve (2) Montpellier, Fr.

In addition to peak amplitude, decay kinetics of Ca^{2+} currents (ICa) are important in the control of voltage-gated Ca^{2+} influx, and thereby contraction, during cardiac action potentials. Using the whole-cell patch clamp technique in voltage-clamp conditions, we have found that moderate depolarization of the holding potential between -80 mV and -50 mV upregulate the activity of L-type Ca^{2+} currents (ICa) in both human and rat cardiomyocytes. There was both a moderate increase of ICa peak amplitude and a marked slowing of current decay which contributed to a significant increase of Ca^{2+} influx. This process is primarily voltage-dependent because ICa does not begin to activate until ~-30 mV. No Ca^{2+} entry would occur when potential changes from -80 mV to -50 mV and no change in the degree of Ca^{2+}-dependent inactivation of ICa is expected. It was related to changes in the gating properties rather than to an increase in the number of activable Ca^{2+} channels. This type of facilitation could be brought about (when absent) or enhanced (when present) by β-adrenergic and 5HT4-serotoninergic (in human atrial myocytes) receptors stimulation or extracellular application of permeable dibutyryl-cAMP ; i.e. by agents promoting cAMP-dependent phosphorylation of Ca^{2+} channels. In conclusion, the resting membrane potential can be an important regulator of L-type Ca^{2+} currents kinetics and, thereby, of cardiac performance of the beating heart. This mechanism may play an important role during physiological stress associated with elevated sympathetic tone and levels of circulating catecholamines

W-Pos53

GONADOTROPIN RELEASING HORMONE INDUCED Ca^{2+} INFLUX IN NON-SECRETING PITUITARY ADENOMA CELLS : ROLE OF VOLTAGE DEPENDENT Ca^{2+} CHANNELS AND PROTEIN KINASE C

((**N. Prevarskaya§**,R. Skryma§ , L. Dufy-Barbe* and Bernard Dufy*)) §Laboratory of Cells Physiology, University of Lille 1 and *Laboratory of Neurophysiology,CNRS UMR 5543,University of Bordeaux II , France.

The action mechanism of GnRH on the cytosolic free calcium concentration ($[Ca^{2+}]i$) and high-threshold voltage-dependent Ca^{2+} channel activity was studied in human non-secreting (NS) pituitary adenoma cells. $[Ca^{2+}]i$ was monitored in individual cells by dual emission microspectrofluorimetry using indo 1. The whole-cell recording patch-clamp technique was used to study Ca^{2+} channels. A short application of GnRH (1 to 100 nM) induced an increase in $[Ca^{2+}]i$ due to Ca^{2+} entry through plasma membrane voltage-sensitive L-type Ca^{2+} channels. Protein kinase C (PKC) depletion induced by a pretreatement with 1 µM PMA for 24 hrs abolished spontaneous Ca^{2+} transients and the action of GnRH on $[Ca^{2+}]i$ and Ca^{2+} channels. Phloretin (250 µM) and staurosporine (20 nM), two protein kinase C inhibitors, inhibited Ca^{2+} channel activity, thereby suppressing the effect of GnRH. On the other hand, activation of PKC by a short application of PMA (10 nM) stimulated Ca^{2+} influx through Ca^{2+} channels. These findings demonstrate that, in human NS adenoma cells, GnRH induces an increase in $[Ca^{2+}]i$, principally due to Ca^{2+} entry through L-type voltage-activated Ca^{2+} channels. PKC regulates this mechanism as well as basal ion channel activity, thus exerting both positive and negative control of $[Ca^{2+}]i$ in stimulated and unstimulated NS adenoma cells.

W-Pos54

NORADRENALINE AUGMENTS INWARD BA2+ CURRENTS IN ISOLATED GUINEA-PIG HEPATOCYTES. ((T, Nagano, *R. Sato, H, Matsuda, A. Kitamura, T. Aramaki)) Nippon Medical School, Tokyo, Japan, *Kinki University, Osaka, Japan

Applying the patch clamp technique, we reported in the last meeting (1996), that Ca^{2+} permeable channels, which is different from voltage operated Ca^{2+} channels, exist in the plasma membrane of guinea-pig hepatocytes and it may be a pathway for Ca^{2+} inflow in hormone unstimulated states. In this study, we investigated whether this channel could be a pathway for Ca^{2+} inflow also when a rise of intracellular Ca^{2+} concentration is induced by noradrenaline. The patch clamp technique was applied to isolated guinea-pig hepatocytes obtained by collagenase perfusion method. $BaCl_2$ (110 mM) was used as pipette internal solution. After G Ω seal formation, sporadic inward Ba^{2+} currents were observed in a cell attached patch at resting membrane potential. The opening amplitude was approximately 0.6 pA and open probability was 0.004. External application of 20 μM noradrenaline activated inward Ba^{2+} currents. The opening amplitude of single channel currents was not changed, and the open probability was increased. Noradrenaline in the internal pipette solution did not affect the channel activity. These results indicate that this channel introduces Ca^{2+} into the cytoplasm not only in the basal state but also in the hormone stimulated state, and its activation occurs via intracellular second messenger, not by direct interaction with hormone receptor.

W-Pos55

FACILITATION OF THE T-TYPE CA CURRENT IS ENHANCED BY NA IONS. ((J.L. Alvarez, A. Artiles and **G. Vassort**)) Cardiology, La Habana, Cuba; INSERM U-390, Montpellier, France.

The properties of the low-threshold Ca current, I_{CaT}, were investigated in bullfrog isolated atrial cardiomyocytes using the whole-cell patch-clamp technique under control conditions and during β-adrenergic stimulation. In a previous work (Alvarez et al., 1996), we suggested that the double-pulse-induced facilitation of I_{CaT} results from a voltage-dependent relief of the Gi protein inhibitory tone. Facilitation itself was not due to protein kinase A-dependent phosphorylation but its amplitude was increased presumably following phosphorylation of the Gi protein. The present results demonstrate that facilitation was not dependent upon Ca ions nor upon a Ca-calmodulin kinase-dependent phosphorylation. T-type current amplitude under control conditions and during β-adrenergic stimulation was similar when the charge carrier was Sr ion, or when the intracellular Ca buffer was 20 mM EGTA or 10 mM BAPTA; facilitation was similarly unaffected. Facilitation was also unchanged when the Ca-calmodulin peptide inhibitor CaMKII (290-309) was added to the pipette solution. However, a low-Na solution (50% Na) enhanced facilitation whatever the current carrier were Ca or Sr ions while I_{CaT} was slightly reduced. Both effects are in agreement with an increased inhibitory tone attributable to a PTX-sensitive Gi protein since in the presence of pertussis toxin I_{CaT} amplitude was slightly increased and facilitation suppressed. During β-adrenergic stimulation facilitation was markedly reduced; an effect consistent with a reduction in agonist binding to the receptor (Limbird et al.,1982).

W-Pos56

HIPPOCAMPAL CA1 NEURONS CA^{++} CURRENT INACTIVATION AFFECTED BY NA^{+} BASED INTERNAL SOLUTION.

((**Sergey Purnyn**, Sheryl G. Beck)) Department of Pharmacology and Experimental Therapeutics, Loyola University Medical Center, Maywood, Illinois 60153

Ca^{++} currents in hippocampal CA1 neurons of the rat were recorded in whole cell configuration with patch electrodes filled with Na+ based internal solution to block K^{+} currents. Na current was blocked by 1 μM TTX applied extracellularly or by 2 mM QX-314 in the pipette. Internal Ca^{++} was buffered by 10 mM of EGTA. Calcium L-current, induced by positive voltage steps from a holding potential of -60 mV, had the same threshold and V-I dependence when recorded with either Na^{+} or Cs^{+} based internal solutions, however the Ca^{++} current showed almost complete inactivation with a time constant of about 120 ms at room temperature when recorded with Na^{+} based internal solution. Complete removal of inactivation took up to 30 s as shown with paired pulse protocol. There was no trace of such inactivation when Cs^{+} was used as the main internal cation. Based on these preliminary findings, we suggest that calcium channel inactivation may be present in CA1 hippocampal pyramidal cells when physiological concentrations of Na^{+} are present. Supported by PHS NS 28512.

W-Pos57

INHIBITION OF N-TYPE CALCIUM CHANNEL CURRENTS BY EXTERNAL SODIUM.

((**L. Polo-Parada** and S. J. Korn)) Physiology and Neurobiology, University of Connecticut, Storrs, CT 06269

Selectivity for Ca^{2+} over Na^{+} and other cations in L-type Ca^{2+} channels is believed to result from a competitive mechanism, whereby the very much higher affinity of Ca^{2+} for an intrapore binding site (Kd=~1μM) compared with other cations prevents entry and permeation of other cations through the pore. Current models suggest that binding of a second Ca^{2+} in the pore changes the Kd for Ca^{2+} to ~14 mM, and Ca^{2+} currents result. Kuo and Hess (*J. Physiol., 1993, 466:657*) found that Li^{+} (and Na^{+}) could compete with Ca^{2+} for an intrapore binding site, with a Kd for Li^{+} of about 75 mM. This suggests that at physiological concentrations, external Na^{+} should compete with Ca^{2+}. We have been studying N-type Ca^{2+} channels in chick dorsal root ganglion neurons. Removal of Ca^{2+} and Mg^{2+} resulted in a large Na^{+} conductance that was blocked 95% by 10 μM Ca^{2+} and 81% by 1 mM Mg^{2+}. With 2 mM Ba^{2+}, external Na^{+} blocked Ba^{2+} currents with an IC50 of 119 mM. To test for competition between Na^{+} and Ba^{2+}, we examined Na^{+} block of Ba^{2+} currents at different [Ba^{2+}]. Reduction of [Ba^{2+}] increased Na^{+} block. Elevation of Ba^{2+} prevented block by Na^{+} with an IC50 of 2 mM. Similar results were found when using Li^{+} instead of Na^{+}, and Ca^{2+} instead of Ba^{2+}. Na^{+} did not affect the voltage-dependence of Ba^{2+} current activation or inactivation. These results suggest that the permeation mechanism in N-type Ca^{2+} channels is similar to that of L-type Ca^{2+} channels. Furthermore, at physiological concentrations of Na^{+} and Ca^{2+}, external Na^{+} significantly influences Ca^{2+} current magnitude. *Supported by NSF and the UC Res. Found.*

W-Pos58

BIOPHYSICAL PROPERTIES AND cAMP-DEPENDENT REGULATION OF SINGLE L-TYPE CALCIUM CHANNELS FROM HUMAN MYOCARDIUM.

((S. **Herzig**, F. Schröder, R. Handrock, A. Haverich[#], S. Hirt[#])) Department of [#]Cardiovascular Surgery, University Kiel, and Department of Pharmacology, University Köln, 50924 Köln, FRG. (Spon. E. Wettwer)

The properties of cardiac L-type calcium channels from human ventricular myocardium have not yet been described at the single channel level. We obtained isolated cardiomyocytes by enzymatic digestion of left ventricular tissue samples from explanted hearts (transplant recipients with terminal heart failure). In cell-attached recordings (70 mmol/l $BaCl_2$), the availability of the channels to open upon depolarization was maximal at a test potential of +20 mV and a holding potential of -100 mV (frequency 1.6 Hz). We therefore characterized single channel gating using this protocol. Open times (mean value 0.59±0.07 ms, n=9) were fitted by a simple exponential function, while closed time (mean value 5.5±1.1 ms, n=5) histograms clearly contained two components. Gating can therefore be described by a C ⇔ C ⇔ O scheme, as shown for other mammalian L-type channels. Notably, the first latency (mean value 23±2 ms, n=5) distribution contained a fast and a slow component. Active and blank sweeps were clustered in runs, suggesting that slow modulatory events, e.g. phosphorylation, govern channel behavior. Indeed, stimulation of the channel activity with 8-Br-cAMP enhanced the fraction of active sweeps. Thus, in qualitative terms, single L-type channel properties of human heart cells resemble those of other mammals. It remains to be elucidated in detail whether heart failure causes alterations, as compared with cells from healthy myocardium.

SODIUM CHANNELS I

W-Pos59

MODULATION OF CARDIAC Na^{+} CHANNEL GATING BY EXTERNAL CATIONS

((**C. Townsend**, H.A. Hartmann and R. Horn)) Dept of Physiol, Institute of Hyperexcitability, Jefferson Med College, Philadelphia, PA 19107; Dept of Molec Physiol & Biophys, Baylor College of Med, Houston, TX,77030.

Human heart Na^{+} channels were expressed transiently in mammalian cells and *Xenopus* oocytes and Na^{+} currents measured using 150 mM intracellular Na^{+}. Raising $[Na^{+}]_o$ or $[Li^{+}]_o$ increases outward Na^{+} current at positive voltages while decreasing the driving force for the current. This anomalous effect of permeant ion concentration, especially obvious in a mutant (F1485Q) in which fast inactivation is partially abolished, is due to an alteration of peak open probability during brief depolarizations. Substituting external Na^{+} for N-methylglucamine also affected slow inactivation (SI), increasing the rate of recovery from SI at -140 mV in F1485Q channels, decreasing the rate of SI at depolarized voltages, and shifting steady-state SI in a depolarized direction. Single channel recordings of F1485Q show a decrease in the number of blank records when $[Na^{+}]_o$ is increased. Clustering of blank records when depolarizing at a frequency of 0.5 Hz suggests that these periods of inactivity represent the sojourn of a channel in a slow-inactivated state. Neither open time, closed time, nor first latency is affected by $[Na^{+}]_o$ at +60 mV. However raising $[Na^{+}]_o$ decreases the duration of the last closed interval terminated by the end of 90 ms depolarizations, leading to an increased number of openings. A single rate constant for entry into a slow-inactivated state is reduced in high $[Na^{+}]_o$ at +60 mV.

W-Pos60

FUNCTIONAL CHARACTERIZATION OF S804F, A MYOTONIA-ASSOCIATED MUTATION IN IIS6 OF THE SKELETAL MUSCLE SODIUM CHANNEL.

((**D.S. Green***, L.J. Hayward*, A.L. George[†], and S.C. Cannon*)) *Dept. of Neurobiology Harvard Med. Sch. Boston, MA 02114; [†]Dept. Medicine and Pharmacology Vanderbilt Univ. Med. Center Nashville TN 37232

Twenty different point mutations of the skeletal muscle Na channel α subunit have been identified in the heritable muscle disorders hyperkalemic periodic paralysis, paramyotonia congenita, and the sodium channel myotonias (SCM). We have tested the functional consequences of a novel mutation, S804F, located at the cytoplasmic face of transmembrane segment 6 of domain II and associated with a potassium-aggravated form of SCM. Whole-cell Na currents were recorded from HEK cells transfected with cDNAs encoding the human α subunit (either WT or S804F) and the human β_1 subunit.

S804F disrupted fast inactivation as evidenced by a 3-fold increase in the persistent Na current after a 50 msec step to -10 mV, and a 2-fold slowing for the decay rate of I_{Na} (τ_h) at depolarized potentials. The voltage dependence of steady-state inactivation (h_∞) was not significantly altered, nor was the rate of recovery from inactivation after a 30 msec depolarization to -10 mV. The peak conductance - voltage relation was identical for WT and S804F. These data are consistent with previous observations of disease-associated α subunit mutants: the predominant alteration in function is an impairment of inactivation and the defects are milder for myotonia-associated mutants compared to those causing paralysis.

Supported by NIH Grants: GM15605 (DSG), RO1-AR42703 (SCC), RO1-NS32387 (ALG), a HHMI postdoctoral fellowship (LJH), the Klingenstein Foundation (SCC), the Lefler Fund (SCC), and the Freudenberger Fund (DSG).

W-Pos61

EFFECTS OF TEMPERATURE AND MEXILETINE ON THE MYOTONIA CAUSATIVE SODIUM CHANNEL MUTATION F1473S

((R. Fleischhauer, H. Lerche, N. Mitrovic, A.L. George*, F. Lehmann-Horn)) Dept. of Applied Physiology, University of Ulm, Germany and *Depts. of Medicine and Pharmacology, Vanderbilt University, Nashville, TN, USA. (Spons. by H.Bader)

The point mutation F1473S replacing Phe by Ser within IV/S4-S5 of the α-subunit of the adult human muscle sodium channel was studied by heterologous expression in HEK293 cells using the whole cell and single channel patch clamp technique. F1473S causes Paramyotonia congenita (PC), a disease characterized by muscle stiffness sometimes followed by weakness in a cold environment. The symptoms are alleviated by the local anesthetic mexiletine, a lidocaine analogue. Compared to wild type (WT), F1473S exhibits a 2-fold slowing of inactivation, a 20-mV depolarizing shift of steady-state inactivation and a 5-fold acceleration of recovery from inactivation (N.Mitrovic et al. / Neuroscience Letters 214 (1996) 9-12). Single channel recordings revealed prolonged mean open times (WT vs .F1473S; 0.28±0.01 vs. 0.39±0.03) and an increased number of late channel openings that increased further with cooling (for 15°; 22°. 30°C WT vs. F1473S [% of peak current]: 0.55±0.20%; 0.20±0.03%; 0.05±0.01% and 1.13±0.11%; 0.96±0.15%; 0.82±0.24%). Thus, it seems that the late sodium current has to exceed a certain threshold for clinical symptoms to occur. The temperature dependence of the fast inactivation time constant was slightly decreased for F1473S in the range from 15 to 30°C as evaluated by an arrhenius plot (E, 21.8 vs. 18.5 kcal/mol). So far, phasic or use-dependent block by mexiletine (100μM) was examined by repetitive 10ms-depolarisations to 0 mV from -120mV holding potential. At 10Hz, phasic block was little but significantly reduced for mutant vs. WT (25.65 ±0.15% vs. 31.56±0.17% reduction in peak amplitude). According to this result we found that recovery from mexiletine block was faster in the mutant (501±78 vs. 620±48ms). The results suggest that IV/S4-S5 might be involved in local anesthetic interaction.

W-Pos62

BIOPHYSICAL CHARACTERISTICS OF THE HUMAN SKELETAL MUSCLE SODIUM CHANNEL MUTATION I1160V UNDERLYING CONGENITAL MYOTONIA. ((**J.E. Richmond**, A.L. George, P.C. Ruben)) Dept. Biology, Utah State University, Logan, Utah 84322-5305.

The missense mutation I1160V in human skeletal muscle (HskM) sodium channels is associated with a disorder causing myotonia without muscle weakness. To understand the biophysical basis for this disorder we examined I1160V and wild type HskM sodium channels (WT) expressed in Xenopus oocytes using the macropatch voltage clamp technique. The conductance curves, rates of fast inactivation and steady-state fast inactivation curves for I1160V were identical to WT. There was, however, a significant increase in the recovery rates from fast inactivation in I1160V compared to WT over a physiologically relevant range of potentials (-60 to -100mV). This may explain the muscle hyperexcitability. In addition, steady-state slow inactivation in I1160V was found to be left shifted by ~-20mV compared to WT. This, and the absence of a persistent current may explain the lack of muscle weakness.

W-Pos63

FUNCTIONAL ANALYSIS OF MUTATIONS IN THE NA^+ CHANNEL INACTIVATION GATE ((**S. Kellenberger**, J.W. West, T. Scheuer and W.A. Catterall)) Dept. of Pharmacology, U. of Washington, Seattle WA 98195 (Spon. by F. Vincenzi)

F1489 in the intracellular linker connecting domains III and IV (L_{III-IV}) of the rat brain IIA Na^+ channel α subunit is required for stable inactivation and several gly and pro residues in L_{III-IV} play a role in the closing of L_{III-IV} during inactivation. We have investigated the role of other hydrophobic clusters in L_{III-IV} and of the residues adjacent to IFM1488-90, and analyzed selected gly and pro mutations on the single channel level. Mutation of the hydrophobic clusters YY1497/8 and MVF1523-5 did not detectably destabilize the inactivated state at depolarized potentials. Mutation YY1497/8QQ, however, slowed macroscopic inactivation and accelerated recovery from inactivation. Among the residues immediately surrounding IFM1488-90, mutation of T1491, but not D1487, strongly disrupted inactivation. Thus, F1489 and T1491 are unique in contributing to the stability of the inactivated state at depolarized potentials. The exact position of F1489 is also critical, as substitution of a phe residue at position 1488 or 1490 in the F1489Q mutant background did not restore normal inactivation. Single channel analysis of G1484A, G1485A and P1512A mutant channels showed that slowed macroscopic inactivation in these mutants is partially due to increases in open duration and latency to first opening. These mutations also increase the probability that Na^+ channels will enter a mode in which inactivation is further impaired. Several mutations in L_{III-IV} alter gating transitions in the pathway leading to Na^+ channel opening, indicating conformational coupling between channel activation and inactivation.

W-Pos64

ROLE IN FAST INACTIVATION OF THE IV/S4-S5 LOOP OF THE HUMAN MUSCLE SODIUM CHANNEL PROBED BY CYSTEINE MUTAGENESIS ((H. Lerche, W. Peter, N. Mitrovic, R. Fleischhauer, U. Pika-Hartlaub, M. Schiebe, F. Lehmann-Horn)) Dept. of Applied Physiology, University of Ulm, Germany

The cytoplasmic loop linking segments S4 and S5 has been proposed to form a receptor for the inactivation ball of the shaker potassium channel (Isakoff et al. 1991, Nature 353, 86-90). To investigate the role of this structure in sodium channel inactivation, we scanned the S4-S5 loop of the fourth repeat (IV/S4-S5) of the adult human muscle sodium channel (hSkM1) by cysteine mutagenesis. Every single amino acid (aa) from R1469 to N1484 was mutated into a cysteine and studied by functional expression in the mammalian cell line TSA201 using the whole cell patch clamp technique. Intracellular effects of the sulfhydrylreagents MTSET (1mM) and MTSES (2.5mM) on the mutations were tested by adding them to the pipette solution. In general, MTSET and MTSES had similar effects, but MTSET reacted more rapidly. All effects of the reagents were independent of the membrane potential. For one mutation we could not detect sodium currents in transfected cells (N1484C). Without reagents, only two of the 15 cysteine mutations studied exhibited signficant differences compared to WT in the time course of inactivation or the ratio steady-state to peak current (I_{ss}/I_{peak}): F1473C slowed inactivation 2-fold and L1482C induced an I_{ss} of 3% of I_{peak} (1% for WT). M1477C and S1478C induced a 10mV hyperpolarizing shift of steady-state inactivation and slowed recovery from inactivation 3-fold. The sulfhydrylreagents slowed inactivation of R1469C, F1473C, M1476C and M1477C by 2- to 3-fold. If IV/S4-S5 formed an α-helix, these four aa's would all face the same side of the helical wheel. Steady-state inactivation of F1473C, M1477C and S1478C was shifted by 10-15mV in the depolarizing direction indicating destabilization of the inactivated state. In summary, IV/S4-S5 plays an important role in sodium channel inactivation, but further experiments have to be performed to define a receptor for the inactivation gate.

W-Pos65

SECONDARY STRUCTURE OF THE D4/S4-5 LINKER PROBED BY SYSTEMATIC CYSTEINE ACCESSIBILITY METHOD. ((G.N.Filatov, S.D.Kraner, R.L.Barchi)) Univ.of Pennsylvania, Philadelphia, PA 19104. (Spon. by G.Filatov)

Each of the four repeat domains (D1-D4) in voltage-gated sodium channels contains six putative transmembrane helices (S1-S6). The intersegmental S4-5 loop may be part of the binding site for the inactivation particle, formed by ID3-4 loop. We utilized the cysteine scanning and accessibility method (SCAM) to examine structural features of the S4-5 linker. Seventeen residues (1461-77 RSkM1 numbering) were individually substituted with cysteines. All, except P1473 and N1477, resulted in the formation of functional channels. F1466C significantly slowed inactivation; minor effects on steady-state inactivation and recovery from inactivation were observed for A1467C, M1469C, A1474C, and L1475C, while the rest of the mutations exhibited wild type phenotype.

Sulphydryl reagents [2-(trimethylammonium)ethyl]methanethiosulfonate bromide (MTSET) and sodium (2-sulfonatoethyl)methanethiosulfonate (MTSES) were used to probe the accessibility of each position. Mutations F1466C, M1469C, and M1470C showed dramatic slowing in inactivation after intracellular exposure to 2 mM MTSES at resting membrane potential. Residues A1467 and A1474 were modified only at depolarizing potentials, implying that conformational changes in this region occur during activation. Substitutions R1462C, L1465C, and F1476C showed a minor slowing of inactivation. The results support an alpha-helical secondary structure for residues preceding P1473 and a *random* coil thereafter. The hydrophobic surface of the helix is not accessible to modifying reagents. The opposite face of the helix, which contained charged and polar residues, is accessible in the resting state. A functional model of inactivation will be presented.

W-Pos66

MUTATIONS IN CYTOPLASMIC PORTIONS OF SODIUM CHANNELS AFFECTING INACTIVATION: S4-5 SEGMENTS. ((**L.Tang** and R. G. Kallen)) Department of Biochemistry and Biophysics, U of Penn Sch of Med, Mahoney Institute for Neuroscience, Philadelphia, PA 19104-6059.

The primary sequence of the S4-5 segment of Domain 4 of the human heart sodium channel (hH1) is: L^{1645}LFALSM^{1651}M^{1652}LPALF^{1657}. We have mutated 11 out of 12 of these amino acids singly or in pairs, generally to glutamine (Q) but in some cases to glutamate (E) or alanine (A). There is a suggestion of a gradient of effects from the N-ter of this segment (adjacent to the voltage-sensing S4 segment) to the C-ter (close to S5) since L1645Q/L1646Q, F1647Q/A1648Q, F1647E/A1648E, L1649Q, M1651Q/M1652Q, and M1551A/M1652A mutations slow inactivation kinetics while the phenotype of the L1653Q and P1654Q/A1655Q channels are unchanged compared with that of wild-type. The unusual phenotype of the F1647Q/A1648Q mutation, in which the voltage-dependence of the time constants for current decay has been lost, is virtually identical to that previously reported for Y1494Q/Y1495Q (located in the ID3-4 loop) [O'Leary *et al.*, J. Gen. Physiol., 641-658, 1995]. This result suggests that the linkage between activation voltage sensors and the inactivation gates can be disrupted comparably by a subset of specific mutations in ID3-4 or D4S4-5. Further experiments deal with whether these specific mutation sites form an actual physical linkage between the core of the channel and the ID3-4 segment that is widely believed to contain the inactivation particle.

W-Pos67

INTERACTION BETWEEN THE PUTATIVE SODIUM CHANNEL INACTIVATION PARTICLE AND DOMAIN III S4-S5. ((**M.R. Smith**, E.J. Yu, and A.L. Goldin)) Department of Physiology and Biophysics, University of California, Irvine, CA 92697.

The F1489 residue in the III-IV linker of the rat brain IIA sodium channel has been shown to be important for fast inactivation, possibly forming the nucleus of a fast inactivation particle (West et al., 1992, PNAS 89:10910-10914). Inactivation is thought to occur through hydrophobic interactions between this inactivation particle and a docking site that has not been identified. Substitution of glutamine for alanine at position 1329 in the S4-S5 linker of domain III significantly slows inactivation compared to that of the wild type channel. We have constructed compensatory charge mutations at A1329 and F1489 in an attempt to show an interaction between the putative inactivation particle and the S4-S5 region. The mutant channel F1489D demonstrates almost no fast inactivation, whereas combining F1489D with A1329K partially restores fast inactivation. The F1489D/A1329K channel has a single channel mean open time that is similar to that of the wild type channel and is approximately 50 times shorter than a channel containing only the F1489 mutation. Substituting a positive charge in the DIII S4-S5 linker at positions 1331 or 1327 in combination with F1489D also partially restores inactivation, though to a lesser extent than A1329K/F1489D. We are currently examining the effects of increased ionic strength to demonstrate that the interaction between F1489D and the S4-S5 region is electrostatic.

W-Pos68

PROBING THE ROLE OF PHENYLALANINE RESIDUE IN THE INACTIVATION GATE OF hH1 Na CHANNELS BY CYSTEINE MODIFYING REAGENTS. ((I. Deschênes*, E. Trottier*, L.-Q. Chen*, R.G. Kallen# and **M. Chahine***)) *Laval Hospital Research Center, Laval University, Quebec, Canada. #Department of Biochemistry and Biophysics,University of Pennsylvania School of Medicine, Philadelphia, PA 19104-6059. (Spon. by A. Sculptoreanu)

It has been suggested that the region linking the domain III and the domain IV of the voltage-gated sodium channels forms the inactivation gate. The IFM group is proposed to play an essential role in the fast inactivation of sodium channels with phenylalanine residue of the IFM group being most important. In order to elucidate the role of the phenylalanine, this residue was replaced with a cysteine on hH1. Expression of this mutation in tsA201 cells is associated with a residual current of 7.49 ± 0.77% of maximum current, a loss of the voltage-dependence of the time constants of inactivation compared to hH1, a shift of the steady-state inactivation to more depolarized voltages, and the recovery from inactivation that is faster than for the wild-type hH1. Sulfhydryl reagents (MTS) reacting with cysteine residues were used as probes, intracellularly at 1 mM. When we applied MTSEA and MTSES reagents, the residual currents recorded were 19.13 ± 2.66% and 20.50 ± 2.84% respectively. In presence of MTSET the residual current was more important: 66.74 ± 6.18%. We tested the possibility that a benzene group might suppress the residual current du to the nature of the phenylalanine, we used a product that transfer a benzene group (p-chloromercuriphenylsulfonic acid). Contrary to our expectations, the residual current was found to be larger then what obtained with MTS-reagents with a percentage of 81.77 ± 3.06% of residual current. We conclude that the phenylalanine is accessible from the intracellular side and plays an important role in fast inactivation.

W-Pos69

ULTRA-SLOW INACTIVATION IN THE SKELETAL MUSCLE SODIUM CHANNEL IS INFLUENCED BY PORE RESIDUES.
((**H. Todt**, S. Dudley and H. Fozzard)) Cardiac Electrophysiology Laboratories. The University of Chicago, Chicago, IL 60637. (Spon. by S.Dudley)

A slow component of recovery from inactivation has been observed in rat skeletal muscle (μ1-) sodium channels when held at depolarized potentials for 40 ms-10 s.[1,2] External pore residues have been implicated in the mechanism of slow recovery from inactivation.[1] Whereas the time constant of slow recovery is in the order of several s, ultra-slow inactivation with time constants in the order of minutes can be induced by even longer periods of depolarization[2]: μ1 sodium channels were heterologously expressed in *Xenopus* oocytes and recovery from inactivation at -120 mV after a 300 s prepulse to -30 mV was monitored by repetitive 50 ms test pulses to -10 mV at 20 s intervals. In addition to native channels, five constructs carrying mutations in the region of the putative selectivity filter were investigated for the time constants (t1, t2 in s) and the magnitude of contribution (A1, A2) of slow and ultra-slow inactivation, respectively.
t-dev= time constant of development of ultra-slow inactivation (s) at -30 mV.

Construct	n	t1	t2	A1	A2	t-dev
wild type	5	8.80 ± 1.20	55.80 ± 10.40	0.68 ± 0.05	0.29 ± 0.05	221.30 ± 8.90
DI-D400A	4	6.30 ± 1.20	23.00 ± 2.10	0.42 ± 0.05	0.56 ± 0.04	129.00 ± 6.40
DII-E755A	2	6.00 ± 0.30	71.90 ± 13.20	0.93 ± 0.03	0.03 ± 0.03	
DIII-K1237E	4	11.80 ± 3.30	141.40 ± 10.40	0.18 ± 0.02	0.78 ± 0.04	126.20 ± 27.50
DIII-K1237S	3	9.20 ± 0.70	98.70 ± 6.30	0.14 ± 0.05	0.85 ± 0.04	115.20 ± 8.10
DIV-A1529D	3	7.00 ± 1.30	121.70 ± 17.60	0.28 ± 0.05	0.73 ± 0.03	103.50 ± 35.50

Mutations in domains III and IV exhibited a substantially greater component of ultra-slow inactivation than WT. We conclude that residues in the putative selectivity filter of the sodium channel influence ultra-slow inactivation.
1. Balser et al, *J Physiol* 494.2:431-442, 1996; 2. Wang and Wang, *Eur J Physiol* 432:692-699

W-Pos70

EXTERNAL PORE RESIDUE MODULATES SLOW INACTIVATION AND GATING COUPLING IN μ1 NA CHANNELS ((J.R.Balser, N.G. Kambouris, S. Stepanovic, E. Marban, G Tomaselli)) The Johns Hopkins University School of Medicine, Baltimore, MD 21287 (Spon. by J.R. Balser)
When expressed in *Xenopus* oocytes and depolarized for brief (50 msec) periods, μ1 Na channel α subunits inactivate through two different mechanisms. While 30% of the channels enter a fast-inactivated state from which recovery is rapid (< 50 msec), the remainder assume a more stable slow-inactivated conformation from which recovery is slow (> 2 sec). In earlier work we showed that substitution of the aromatic tryptophan in the first domain P segment with cysteine (W402C) markedly reduced the fraction of slow-inactivating channels and delayed recovery from fast inactivation, suggesting the chemical character of the 402 residue influences inactivation gating. We therefore substituted amino acids of different size, charge, and hydrophobicity at the 402 position. Replacement with a polar serine residue (W402S) slowed the rate of recovery from slow inactivation, but did not modify the fast recovery process or the relative percentage of channels entering the two inactivated states. In contrast, replacement with neutral alanine (W402A) increased the fraction of fast-inactivated channels, but did not alter the rate of recovery from either inactivated state. Replacement with an aromatic residue (W402F) produced recovery kinetics indistinguishable from wild-type. These results suggest that the aromatic character of the 402 residue is critical to the wild-type gating phenotype, that the 402 residue mediates coupling between fast and slow inactivation gating, and that cysteine produces a unique recovery phenotype.

W-Pos71

MOLECULAR DETERMINANTS OF MODULATION OF THE RAT BRAIN SODIUM (Na^+) CHANNEL BY THE β1SUBUNIT. ((**K.A. McCormick**, T. Scheuer, and W.A. Catterall)) Dept. of Pharmacology, U. of Washington, Seattle, WA 98195-7280

The rat brain Na^+ channel is composed of three glycoprotein subunits, α, β1 and β2. When expressed in *Xenopus* oocytes, β1 modulates the gating properties of the channel-forming α subunit. Deletion analyses from our laboratory and others have shown that an intact extracellular domain is essential for β1 function. The β1 extracellular domain shows sequence similarities with members of the Ig-fold protein superfamily. The Ig-fold is a sandwich of two β-sheets, stabilized by a hydrophobic core. Alanine scanning mutagenesis was used to test the Ig-fold hypothesis. Mutant β1 and wild type α RIIa cRNAs were co-injected into *Xenopus* oocytes, and Na^+ currents were recorded using two-electrode voltage clamp. Mutation of residues in the predicted hydrophobic core to alanine prevented β1 modulation of α RIIa. A phenotypic map of the results is in good agreement with the proposed location of core and non core strands within the Ig-fold. In a second set of experiments, chimeras were created in which portions of β1 were replaced with myelin P0, an Ig-fold protein. Six β1-P0 chimeras behaved like wild type β1. The null mutations lie in positions predicted to form either surface-exposed loops, or non-core β-strands. The data support our structural model of the β1 subunit, and have helped to narrow our search for molecular determinants of β1 function. Further site-directed mutagenesis has identified discrete regions of the β1 subunit which are good candidates for effectors of α subunit function.
Supported by NIH Grant NS25704 (WAC) and a NIH Postdoctoral Fellowship (KAM).

W-Pos72

THE NA CHANNEL β1 SUBUNIT DESTABILIZES THE INACTIVATED STATE(S). ((**J.W. Kyle**, T. Sadanaga, D.A. Hanck, G.S. Tonkovich and M. Sheets)) University of Chicago, Chicago IL 60637 and Northwestern University, Chicago, IL 60611

We studied the coexpression of β1 with heart (hH1a) or skeletal muscle (μ1) Na channel isoforms in transfected tsA201 and HEK 293 cells. Cells were transiently transfected with either the α1 subunit cDNA alone or co-transfected with an excess of β1 cDNA. Sodium currents were recorded 2-4 days posttransfection at 12°C using the whole cell patch clamp configuration. To compensate for time-dependent shifts in kinetics, all protocols were begun 15 min following seal formation. Similar to previous reports, β1 increased current density. Coexpression of β1 with either hH1a or μ1 had no significant effect on either the time to peak amplitude of I_{Na}, the $V_{1/2}$ of conductance (activation), or $V_{1/2}$ of voltage dependent availability. Coexpression of β1 and hH1a altered the slope of the steady-state availability relationship from 6.63±1.04 mV (n=18) without β1 to 5.37±0.64 mV (n=21) with β1 (Students t-test, $p<0.05$). A similar, significant change in slope was detected for μ1 without β1 (6.22±0.90 mV, n=19) compared to with β1 (4.91±0.54 mV, n=25). At negative potentials where the C→I transition should dominate, β1 coexpression increased the rate of recovery from and decreased the rate of development of inactivation for both hH1a and μ1. Voltage dependences of these effects were consistent with β1 acceleration of inactivated to closed state transitions (I→C) in both heart and skeletal muscle isoforms. These data can explain our previous demonstration that β1 subunit decreases the sensitivity of hH1a to block by lidocaine by increasing the recovery from lidocaine block.

W-Pos73

PROBING THE OUTER MOUTH OF THE 'S4 CHANNEL' OF A VOLTAGE-GATED SODIUM CHANNEL. ((**N. Yang**, A.L. George and R. Horn)) Dept of Physiol, Jefferson Med College, Philadelphia, PA 19107; Dept of Medicine, Vanderbilt School of Medicine, Nashville, TN 37232.

The 2nd and 3rd basic residues of the S4 segment of domain 4 of the human skeletal muscle Na^+ channel are known to be translocated from a cytoplasmic to an extracellular position during depolarization. These two arginine residues, which we denote as D4:R2 and D4:R3, are separated by two hydrophobic residues, a leucine (L1452) and an alanine (A1453). We examined the voltage-dependent accessibility of a cysteine residue substituted for D4:R3 and for each of these hydrophobic residues. Accessibility was assayed by a marked slowing of inactivation kinetics during modification by extracellular and/or intracellular methanethiosulfonate reagent. Modification of D4:R3C by the cationic reagent MTSET caused a 1.2-fold increase in the time constant of recovery from inactivation at -120 mV, whereas the anionic reagent MTSES caused a 35-fold increase in this time constant. The voltage dependence of the reaction rates are identical for extracellular application of MTSET and MTSES, suggesting that D4:R3C is not only accessible to hydrophilic reagents, but also situated outside the membrane electric field, at depolarized voltages. Although both D4:R2C and D4:R3C are exposed externally at depolarized voltages, L1452C and A1453C are not exposed externally at any voltage. However, L1452C and A1453C are modified by internal application of MTSES at hyperpolarized voltages only. We propose that all 4 of these residues enter and cross the S4 channel upon depolarization, and that the hydrophobic face of this alpha-helical portion of D4/S4 remains in contact with a hydrophobic region at the extracellular mouth of the S4 channel.

W-Pos74

MOLECULAR MOTIONS WITHIN THE PORE OF VOLTAGE-DEPENDENT SODIUM CHANNELS REVEALED BY ENGINEERED DISULFIDE TRAPPING. ((**Jean-Pierre Bénitah**, Ravi Ranjan, Maria Janecki, Gordon F. Tomaselli, Eduardo Marban)). The Johns Hopkins University, Baltimore, MD.

The pores of ion channel proteins are often modeled as static structures. In this view, the high throughput rate and selectivity reflect rigidly-constrained backbone and side-chain orientations. Such a picture is at variance with the generalization that biological proteins are flexible, capable of major internal motions on biologically-relevant time scales. We tested for motions in the pore of the μ1 sodium channel by introducing pairs of cysteine residues throughout the pore-lining segments. Two distinct pairs of residues (Y401C+E758C and D400C+E755C) spontaneously formed disulfide bonds bridging domains I and II. Eight other permutations, involving all four domains, were capable of disulfide bonding in the presence of the redox catalyst copper phenanthroline. Each of three fulcrum position (D400C, Y401C and K1237C) exhibited a distinctive, specific pattern of disulfide trapping. Analysis of the disulfide bond formation rates enabled estimation of the amplitude of translational motions (Careaga & Falke, J. Mol. Biol. 226:1219, 1992). Some pairings apparently require backbone motions as large as 15 Å. While the results are inconsistent with a single fixed structure for the pore, a novel molecular model can account for all the data. In this model, each of the P segments is an extended loop structure. The contribution of each P segment to the pore is highly asymmetrical. The relaxed structure has an aperture of approximately 4x6 Å, which constricts when constrained to the dimensions necessary for disulfide bond formation. Our findings provide direct evidence for sizable, rapid motions of the pore-lining segments.

W-Pos75

ACCESSIBILITY OF P-LOOP CYSTEINE RESIDUES ON THE AMINO-TERMINAL SIDE OF THE PUTATIVE SELECTIVITY FILTER OF THE SODIUM CHANNEL ((**T. Yamagishi**, R. Ranjan, E. Marban, G.F. Tomaselli)) Johns Hopkins Univ., Baltimore MD 21205

The P-segments of the voltage-dependent Na^+ channel form asymmetric loop structures that line the outer mouth of the ion-selective pore (Chiamvimonvat et al., *Neuron* 16:1037-1047, 1996). These loops are comprised of amino acids immediately C-terminal to the putative selectivity filter. The residues which form the cytoplasmic mouth of the pore of the channel have not been identified. Three amino acids immediately N-terminal to the putative selectivity filter (D400, E755, K1237, and A1529, DEKA) in each of the four domains of the rat skeletal muscle Na^+ channel (μ1) were individually mutated to cysteine and expressed in HEK 293 cells. Inward and outward Na^+ currents were measured with the whole-cell configuration of the patch clamp technique under a variety of ionic conditions. Side-chain accessibility was probed by sensitivity to the thiol-avid blocker Cd^{2+} and by reactivity with methanethiosulfonate (MTS) reagents applied to both the inside and the outside of the cell. The outward current through the wild-type and all of the mutant channels was unaffected by internal Cd^{2+} (100 μM); however, internal QX-314 blocked the channels in a voltage-dependent fashion. Similarly, 1 mM MTSEA applied to the inside of the membrane did not affect wild-type or mutant outward currents. Surprisingly, two mutants in domain III (F1236C and T1235C) and one in domain IV (S1528C) are blocked with high affinity by *external* Cd^{2+}. The inward current through F1236C and S1528C is also inhibited by saturating concentrations of MTSEA applied *externally*. Unlike the analogous region of the K^+ channels, residues immediately N-terminal to the DEKA selectivity residues are not accessible from the inside of the channel; instead these residues in domains III and IV contribute to formation of the external pore lining.

W-Pos76

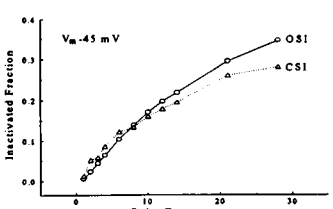
RAT BRAIN SODIUM CHANNEL CURRENT POTENTIATION BY PKA INDUCTION IN *XENOPUS* OOCYTES. ((**R.D. Smith** and A.L. Goldin)) Department of Microbiology & Molecular Genetics, University of California, Irvine, CA 92697.

PKA phosphorylation normally causes brain sodium channel current amplitude to be attenuated (Li et al., 1992, Neuron 8:1151-1159; Gershon et al.,1992, J. Neurosci. 12:3743-3752). This effect is mediated by phosphorylation of specific PKA sites in the cytoplasmic linker between domains I and II of the channel α subunit (Smith and Goldin, 1996, J. Neurosci. 16:1965-1974). If these I-II linker PKA sites are mutated or deleted, PKA induction in *Xenopus* oocytes produces a reversible potentiation of current amplitude. This effect is strikingly similar to the response to PKA induction observed for the rat cardiac sodium channel (Schreibmayer et al.,1994, Receptors and Channels, 2:339-350). A chimeric channel consisting of the brain sodium channel (rBIIA) with the muscle sodium channel (rSkM1) I-II linker did not show an increase in current following PKA induction, indicating that the I-II linker has a role in the enhancement of current. Additional chimeras and deletions were constructed to probe specific regions of the brain channel linker. Substitution or elimination of various regions of the linker significantly diminished the response to PKA induction, further implicating the linker in the mechanism for current increase. The mutations that eliminated the response are widely distributed across the linker, suggesting that the increase is not due to phosphorylation of a specific residue in the linker.

W-Pos77

OCCUPANCY OF Na CHANNEL INTERMEDIATE STATES (*(Clay M Armstrong, & Kamran Khodakhah)*) Department of Physiology, University of Pennsylvania, Philadelphia, PA 19104.

Sodium channels inactivate predominantly from the Open state, but for small depolarizations there is closed state inactivation, or CSI (Bean, 1981). We measured the rate of CSI (in squid axons) over a range of voltages and compared it to the rate of open state inactivation (OSI). OSI was determined from the ∫ Open fraction × dt, assuming i) all inactivation is OSI at +20 mV, and ii) that OSI rate = κ × Open fraction, where κ is V independent. CSI = measured inactivation - OSI. CSI is maximal at -45 mV (in 100Na 100Ca externally), and decreases sharply as V increases, and is zero at -25 mV. CSI rate at -45 mV exceeds OSI rate, judged from slopes (see graph) for a few milliseconds after depolarization, then is slightly slower. This presumably means that occupancy of the intermediate state(s) develops more quickly than that of Open; and then occupancies are almost equal. At -35 mV CSI rate is almost inappreciable after a short burst, reflecting favored occupancy of Open. Physical models for these phenomena will be considered.



W-Pos78

CYSTEINE LABELING & CHARGE IMMOBILIZATION IN SQUID Na CHANNELS. (*(Kamran Khodakhah, Alexey I Melishchuk, and Clay M Armstrong)*) Department of Physiology, University of Pennsylvania, Philadelphia, PA 19104. (Spon. Robert E Forester)

We investigated the effects of labeling the native cysteines in Na channels of squid giant axons with a newly designed cysteine labeling reagent methane-thiosulfonate-propyl-triethyammonium (MTS-PTrEA). In voltage clamped squid giant axons intracellular, but not extracellular perfusion of MTS-PTrEA irreversibly abolished the Na current (I_{Na}). The disappearance of I_{Na} was concurrent with a large hyperpolarizing shift in the steady state inactivation of the Na channels. MTS-PTrEA had no effect on I_{Na} after removal of Na channel inactivation with pronase. Study of the Na channel gating currents revealed that modification of the channels with MTS-PTrEA resulted in immobilization of the gating charge. Gating currents from modified channels were similar to those recorded from fully inactivated channels in the absence of the compound. The rate of labeling of Na channels by 500 μM MTS-PTrEA was voltage dependent, and few channels were modified when the axon was clamped at -110 mV. The labeling rate could be increased by changing the holding potential to more depolarized values (>-80 mV) or by opening Na channels with repeated depolarizing pulses. Interestingly, low concentrations of MMTS (a hydrophobic cysteine labeling reagent) did not significantly affect I_{Na}; but it protected the channels from modification by MTS-PTrEA. After MMTS, MTS-PTrEA reversibly blocked Na channels (K_i≈10 mM). This was similar to the block of Na channels with TEA. Thus modification of a cysteine in the intracellular part of squid giant axon Na channels by MTS-PTrEA, but not MMTS, results in charge immobilization.

W-Pos79

CONDUCTANCE AND SELECTIVITY PROPERTIES OF αβγ-rENaC PORE IN PLANAR LIPID BILAYERS. ((**I.I.Ismailov**, V.Gh.Shlyonsky, and D.J.Benos)) The Univ. of Ala. at Birmingham, Dept. of Physiology & Biophysics, Birmingham, Al 35294.

We have previously shown that the selectivity of cloned heterotrimeric epithelial sodium channel (αβγ-rENaC) for IA group cations in planar lipid bilayers, corresponds to Eisenmann's sequence XI (*Ismailov et al., FASEB J., 10, A77, 1996*). Here we report that the relative permeability coefficients, calculated using reversal potentials measured under 1 M bi-ionic conditions, differed from the ratios of single channel conductances in symmetrical 1 M Li^+, Na^+ and K^+ solutions ($G_{Li}/G_{Na}=1.63\pm0.21$, $P_{Li}/P_{Na}=2.05\pm0.24$, $G_K/G_{Na}=0.04\pm0.01$, $P_K/P_{Na}=0.07\pm0.01$). We also found that conductance of the channel saturated with increasing ion concentration, with a K_m of 51.3 ± 3.8 mM and 48.0 ± 4.5 mM and G_{max} (substate conductance) of 31.9 ± 3.5 pS and 19.5 ± 2.3 pS for Li^+ and Na^+, respectively. The Li^+ and Na^+ concentration dependence of αβγ-rENaC unitary conductance deviated from first order Michaelis-Menten kinetics. Furthermore, the dependence of unitary current on the mole fraction of Na^+ in symmetrical mixtures of Na^+-Li^+ (1 M) exhibits an anomalous mole fraction effect. These characteristics, together with the finding that channel Na^+/K^+ selectivity was strongly dependent on ion concentration (P_K/P_{Na} decreased from 0.11 ± 0.01 to 0.04 ± 0.01 as measured from reversal potentials at 50 and 3000 mM biionic conditions, respectively), suggested multiple occupancy of the αβγ-rENaC pore. On the other hand, an anomalous mole fraction effect was not evident when the channel was bathed with 100 mM salt solutions, suggesting that at physiological ion concentrations the channel may be single occupied or empty. Supported by NIH Grant DK 37206.

REGULATION OF ION CHANNELS II

W-Pos80

INHIBITION OF MAXI K CHANNELS FROM CORONARY SMOOTH MUSCLE BY TETRANDRINE, A HYPOTENSIVE ALKALOID. ((**Guillermo Pérez**§, Bruce Cassels*, and Ignacio L. Reisin‡)) §Cát. Fisiol., ‡Cát. Qca. Gral. e Inorg., Fac. Farm. y Bioq. U.B.A. & PROSIVAD-CONICET. Junín 956, 1113 Buenos Aires, R. Argentina; *Dpto. de Quimica, Fac. Ciencias, U. Chile, Chile.

Tetrandine (TET) is a vasoactive compound extracted from the Chinese medicinal herb *Radix stephania tetrandrae*. It is known as a calcium channel antagonist on smooth muscle cells and other preparations. It has also been reported that a particular type, CTX insensitive, of Maxi K channels from neurohypofisial nerve terminals are inhibited by TET (Wang & Lemos Pflüegers Arch. **421:** 558-565, 1992). We report here the action of TET on single coronary smooth muscle Maxi K channels. TET caused, (μM range), a dose dependent inhibition of Maxi K channels by inducing a flickering behavior. TET decreases mean open time (36 ms to 4 ms, control, and 12.5 μM internal TET, respectively, 40 mV) and increases mean closed time (3 ms to 14 ms, control and TET, respectively, 40 mV). TET inhibition is dose dependent and acts when applied on either side of the membrane. K_D was 23.3 ± 3 μM (n = 3, external, 0 mV) and 9.9 ± 2 μM (n = 2, internal, 40 mV). TET inhibition was stronger at depolarized potentials, (Po_{Ctrl} 80 mV = 0.96, Po_{Ctrl} 40 mV = 0.96 and Po_{TET} 80 mV = 0.52, Po_{TET} 40 mV = 0.60). These results demonstrates that coronary Maxi K channels -known as CTX sensitive channels- are readily inhibited by TET in a dose and voltage dependent manner.

W-Pos81

EFFECTS OF NIFEDIPINE ON THE CARDIAC TRANSIENT OUTWARD K^+ CHANNEL EXPRESSED IN *XENOPUS* OOCYTES.

((L. Genous, **T.L. Yarbrough**, B.J. Padanilam, H. Lee, and E.F. Shibata)) Depts. of Physiology & Biophysics and Internal Medicine. University of Iowa, Iowa City, IA 52242.

The human transient outward K^+ current (Kv1.4) is responsible for the rapidly repolarizing phase of the cardiac action potential. Within the past few years, the α and β subunits of the channel, designated HK1 and Kvβ3, respectively, have been cloned. Little is known about how Kvβ3 modulates the activity of HK1, and how it alters the drug response of HK1. mRNA from HK1 and Kvβ3 cDNA was injected into *Xenopus laevis* oocytes and two electrode voltage clamp techniques were used to measure current. Co-expression of HK1 and Kvβ3 resulted in a substantial decrease of the time constant of current decay as compared to expression of HK1 alone. The dihydropyridine, nifedipine (100 μM), produced a significant decrease in the peak current amplitude and time constant of current decay in HK1 oocytes. The effects of Nifedipine were not altered in oocytes co-expressing HK1 + Kvβ. This suggests that Kvβ3 has little contribution to the effects of nifedipine on HK1.

W-Pos82

CYTOPLASMIC FACTORS REGULATING THE INACTIVATION KINETICS OF THE HUMAN Kv 1.4 CHANNEL.

((**B.J. Padanilam**, E.F. Shibata, T. Hoshi and H. Lee)) Depts. of Internal Medicine and Physiology & Biophysics. University of Iowa, Iowa City, IA 52242.

Regulation of inactivation kinetics of human Kv 1.4 (HK1) channel expressed in *Xenopus laevis* oocytes by cytoplasmic pH, redox state and ATP was examined using the patch clamp technique. Cytoplasmic alkaline pH reversibly slowed the inactivation time constant (τ) and increased the peak current amplitude (I_p). Acidic pH decreased I_p without changing τ. Proton titration curve showed that the pK of τ was close to the reported value of cysteine (8.5-9). The non-charged cysteine modifier, MMTS, blocked the pH effects on τ. Cytoplasmic application of reduced glutathione accelerated τ whereas H_2O_2 slowed τ. MMTS blocked the slowing of τ by H_2O_2 indicating that redox state and cysteine cross-linking modulate channel gating. In addition, ATP and the non-hydrolyzable ATP analogue, AMP-PNP, accelerated τ suggesting a direct nucleotide regulation of channel inactivation. These results show that multiple cytoplasmic factors compete to modulate HK1 channel gating.

W-Pos83

SULFHYDRYL MODIFICATION OF DELAYED RECTIFIER K^+ CURRENTS IN CANINE PULMONARY SMOOTH MUSCLE CELLS.

((D.-S. Ahn and J.R. Hume)) Dept Physiology & Cell Biology, Univ. Nevada School of Medicine, Reno, NV 89557

It has been suggested that hypoxic modulation of 4-AP sensitive, voltage dependent K^+ currents (I_{KV}) may play an essential role in pulmonary hypoxic vasoconstriction (HPV). However, redox modulation of I_{KV} in pulmonary smooth muscle cells has not yet been examined. We examined the effect of oxidizing and reducing agents on I_{KV} in freshly isolated canine pulmonary smooth muscle cells by using whole cell and single channel recording techniques. I_{KCa} was minimized by performing all experiments in Ca^{2+}-free solutions with intracellular Ca^{2+} buffering. DTNB(5,5'-dithio-bis(2-nitrobenzoic acid), 50μM)), a membrane permeable oxidizing agent, significantly increased I_{KV} (288.6±50% of control) which was completely reversed by 1mM dithiothreitol(DTT) at +20mV (105.7±6.7% of control). DTNB produced a small negative shift in half-maximal steady-state activation and the time constants for activation at -10mV(25.2±1.7ms vs 16.0±0.9ms) and deactivation at -50mV (98.6±13.7ms vs 53.7±5.6ms) were significantly decreased. Inactivation of I_{KV} at +20mV was accelerated by oxidation (τ_{fast}=680±87 vs 514±79ms, τ_{slow}=6.2±1.7 vs 3.9±0.3sec). The recovery time constant at -80mV was not significantly affected by DTNB (2.73±0.28 vs 2.74±0.31sec). In inside-out patches, the activity of 4-AP sensitive, 13 pS K^+ channels was increased by DTNB (% change of NP_o=198.7±29.6, n=3) and this DTNB-induced increase in channel activity was reversed by 1mM DTT. These results suggest that I_{KV} channel activity is modulated by redox state and this effect may contribute to inhibition of I_{KV} channel activity during hypoxia (supported by NIH HL 49254).

W-Pos84

INTRACELLULAR SPERMINE UNDERLIES THE INWARD RECTIFICATION OF $\alpha_4\beta_2$ NEURONAL NICOTINIC ACETYLCHOLINE RECEPTORS EXPRESSED IN *XENOPUS* OOCYTES.

((A. **Haghighi** and E. Cooper)) Dept. of Physiology, McGill University, Montreal, Quebec, Canada H3G 1Y6

To study the rectification properties of neuronal nicotinic acetylcholine receptors (nAChR), we expressed cDNA transcripts coding for α_4 and β_2 neuronal nAChR subunits in *Xenopus* oocytes and recorded whole-cell ACh-evoked currents from these oocytes using a two electrode voltage-clamp. As shown previously, the current-voltage relationship for the macroscopic ACh-evoked currents exhibits strong inward rectification, indicating that under these conditions $\alpha_4\beta_2$ receptors conduct little current at positive membrane potentials (0 to +60mV). However, when recording from single $\alpha_4\beta_2$ receptors in outside-out patches from oocytes, we observed no significant rectification: these receptors conduct current equally well at both positive and negative membrane potentials; the open channel probability at +60mV is at least 75% of that at -60mV. Our results suggest that the rectification observed for the macroscopic ACh-evoked currents from oocytes is due to channel blockade by intracellular constituents. Since intracellular polyamines have been implicated in the rectification of certain ion channels, we investigated the effects of spermine on $\alpha_4\beta_2$ receptors. For these experiments, we recorded single $\alpha_4\beta_2$ receptors in outside-out patches in the presence or absence of spermine (0.05-100μM) in the recording electrode. Spermine significantly decreases the open channel probability of $\alpha_4\beta_2$ receptors between 0 and +60mV, suggesting the involvement of intracellular polyamines in rectification of neuronal nAChRs. *Supported by a grant to EC from MRC Canada and a doctoral studentship to AH from NSERC Canada.*

W-Pos85

EXTRACELLULAR ATP INCREASES THE ACTIVITY OF A FATTY ACID- AND STRETCH-ACTIVATED K^+ CHANNEL: POSSIBLE ROLE FOR FATTY ACIDS AS SECOND MESSENGERS ((**Hui Zou**, Mehmet Ugur, John V. Walsh Jr., and Joshua J. Singer)) Dept. of Physiology, Univ. of Massachusetts Medical Center, Worcester, MA 01655.

We previously reported that external ATP immediately activates a P2X-7 or P2Z like, non-selective cation channel (Biophys. J. 70:A201, 1996) in toad stomach smooth muscle cells. Recently, we found that application of ATP to these cells, after a few seconds, also activates the K^+ channel which we have shown to be activated by fatty acids and membrane stretch (J. Physiol. 484:331, 1995). The effect of ATP on the K^+ channel may be mediated by the generation of fatty acids, because in cell-attached patches ATP application to the extra-patch membrane activated a channel that resembled the fatty acid-activated K^+ channel and its activity was decreased by albumin which binds fatty acids and removes them from cell membranes. Moreover, in the whole-cell configuration ATP induced an outward current at 0 mV, presumably carried by K^+, which was also suppressed by albumin. Although the P2X response ceased soon after ATP application was terminated, the whole cell K^+ current, as well as the single channel K^+ currents seen in cell-attached patches, persisted for minutes. Trace amounts of Ca^{2+} were required in order to see the ATP-induced K^+ channel activity since chelating Ca^{2+} with EGTA or BAPTA reduced the response. However, higher concentrations of divalent cations reduced both P2X and the K^+ channel responses. Activation of this K^+ channel showed some other similarities to the P2X response in theses cells: agents that induced K^+ channel activity (e.g. ATP and BzATP) were also P2X receptor agonists; while agents that showed little or no effect on the P2X receptor (e.g. ADP and UTP) did not activate the fatty acid-activated K^+ channel. This study suggests that stimulation of a P2 receptor in toad stomach smooth muscle cells may be associated with the activation of certain phospholipases which release fatty acids that activate the K^+ channel. (Supported by NIH)

W-Pos86

DESENSITIZATION AND TACHYPHYLAXIS OF K^+ CURRENTS ACTIVATED BY ANGIOTENSIN II (AII) IN INTESTINAL MYOCYTES OF THE GUINEA-PIG ILEUM.

((Silva B.A., Romero F., Nouailhetas V.L.A. and **Aboulafia J.**)) Dept. of Biophysics/UNIFESP, EPM, São Paulo, Brazil. (spon. by G. Malnic)

Prolonged treatment of the isolated guinea-pig ileum (GPI) with 10^{-7} M AII causes a slow fading of the tonic component of the contractile response (desensitization), while repeated administrations of this peptide at short intervals induce a decrease of both the phasic and tonic components (tachyphylaxis). We compared the effects of AII and its tachyphylactic (Sar^1-AII) or non-tachyphylactic analogs (Lys^2-II and Suc^1-AII) on the unitary currents through the Ca^{2+}-activated K^+-channels ($I_{K,Ca}$), and its modulation by Na^+ ions. The experiments were performed in the cell-attached configuration of the patch-clamp technique in myocytes from the longitudinal layer of GPI, bathed in high-K^+ solution. Desensitization experiments were done by exposing the myocytes with the peptide to be tested for 10 min. Tachyphylaxis protocol consisted of repeated additions of the peptide to the perfusion solution for 90 s at 5-min interval between two consecutive treatments. At Vm 40 mV, the prolonged administration of AII or any of its analogs caused an increase of NPo values of the $I_{K,Ca}$. The activation of $I_{K,Ca}$ in the first treatment with AII or Sar^1-AII was significantly reduced during the two subsequent treatments (tachyphylactic state of the channel), in contrast to the repeated activation of $I_{K,Ca}$ induced by each treatment with the non-tachyphylactic analogs. Surprisingly there was no cross-tachyphylaxis between AII and Lys^2-AII. Simultaneous addition of 10 mM NaCl and AII in the perfusion solution avoid the tachyphylactic state of the channel. If the behavior of the $I_{K,Ca}$ population at physiological conditions were similar to that here described, the tachyphylactic state of the channel would retard the repolarization of the tissue, thus impairing the pharmaco-mechanical coupling to further administrations of AII. Concerning the desensitization phenomenon, these results suggest that the slow fading of the tonic component of the contractile response might be due to a persistent $I_{K,Ca}$ activation leading to a partial repolarization of the cell membrane. This assumption was corroborated by the absence of the desensitization of the contractile response to AII in the presence of cesium cloride, a known K^+-channel blocker.
Financial support: FAPESP, CNPq and CAPES

W-Pos87

NITRIC OXIDE (NO) DIRECTLY ACTIVATES Ca^{2+}-ACTIVATED K^+ CHANNELS FROM RAT BRAIN RECONSTITUTED INTO PLANAR LIPID BILAYER.

((**Sungkwon Chung**, Jung Hoon Shin,* **Chang Kook Suh**,* and Dae-Yong Uhm)) Dept. of Physiology, Chung-Ang Univ. College of Medicine, Seoul 156-756 and *Dept. of Physiology and Biophysics, Inha Univ. College of Medicine, Inchon 403-752, KOREA.

Nitric oxide (NO) has been reported to have many roles *in vivo* ranging from the neurotransmitter in brain to the relaxant in smooth muscles. Recently, using inside-out patches, Bolotina et al. (1) showed that relaxing effect of NO in aortic smooth muscle is through direct activation of Ca^{2+}-activated K^+ channels (maxi-K), resulting in hyper-polarization.

Since the cellular function of NO in brain is unknown, we have investigated whether NO affects the activity of maxi-K channel from rat brain using the lipid bilayer reconstitution method, which would provide well defined environment for channels free from many modulators and effectors. Maxi-K channels were identified by their calcium-dependency and large conductance (220 pS). In order to apply NO, we utilized an antibiotic, streptozotocin (STZ), which releases NO upon illumination (2). When the light was turned on, the channel activity increased about 2-fold within several tens of sec. The effect was reversed several seconds after the light was turned off. Considering the half-life of NO in solution, the effect of STZ can be explained to be due to the released NO. The kinetic analysis of single channel revealed that the effect of NO is to shorten the mean closed time, while leaving the mean open time unchanged. These results suggest that the maxi-K channels might be one of the targets of NO in brain.

1. Bolotina et al. (1994) Nature 368: 850-853
2. Kwon et al. (1994) FASEB J. 8: 529-533

W-Pos88

TWO Ca-DEPENDENT CURRENTS ACTIVATED BY MUSCARINIC AGONISTS IN A PANCREATIC ß CELL LINE. ((**A. Kozak** and D. E. Logothetis)). Dept. Physiol. & Biophys., Mt Sinai Sch. of Med., CUNY, New York, NY 10029. (Spon. by J. Sui)

The cytosolic calcium elevation which brings about insulin release is thought to originate both from Ca^{2+} influx via the voltage gated calcium channels and release from internal calcium stores. We have used a β cell line (βTC-3) to describe calcium-dependent currents which can be activated by cytosolic calcium elevations under physiological conditions. To this end we have employed the perforated-patch recording, a technique which leaves the internal calcium environment unaltered. When Cs^+ was used as the internal cation (to block the outward potassium channels), long depolarizing voltage steps activated slow outward currents followed by slow tail currents. We concluded that this was a Cl^- current based on its sensitivity to niflumic acid and DIDS (100 μM), changes in external Cl^- ion concentrations and permeability to other monovalent anions (I^-, Br^-, NO_3^-). The removal of external calcium, 100 μM Cd^{2+} or 100 nM nifedipine abolished the current reversibly, whereas application of 1 μM Bay K increased it. When K^+ was the internal cation, the depolarizing voltage steps elicited and additional current, which was K^+ selective and permeable to Rb^+ as well. This K^+ current was recorded in the presence of 10 mM TEA, to eliminate the Maxi K^+ conductance. Like the chloride current, this potassium current was abolished by Cd^{2+} and nifedipine application and increased by Bay K. The current showed slow tail currents and was sensitive to block by charybdotoxin and quinine but not apamin or tubocurarine. Carbachol or muscarine (50-100 μM) could also elicit both $I_{Cl(Ca)}$ and $I_{K(Ca)}$ reproducibly in presence or absence of external cadmium. Muscarinic agonists failed to activate, however, an inwardly rectifying current present in these cells. It is likely that the muscarinic agonists act by releasing calcium from internal stores. The calcium-dependent K^+ and Cl^- currents may play a role in the electrical activity of the β cells in response to various secretagogues, which increase cytosolic Ca^{2+} content.

W-Pos89

INHIBITION OF DELAYED RECTIFIER K^+ CURRENT BY ENDOTHELIN-1 (ET-1) IS MEDIATED BY PHOSPHOLIPASE C AND PROTEIN KINASE C IN RAT PULMONARY ARTERIAL MYOCYTES

((**L.A. Shimoda**, J.T. Sylvester and J.S.K. Sham)) Division of Pulmonary and Critical Care Medicine, Johns Hopkins School of Medicine, Baltimore, MD, 21224.(Spon. By A. Davidoff)

Previously, we found that ET-1 causes membrane depolarization and pronounced inhibition of delayed rectifier K^+ (K_{DR}) current in rat intrapulmonary arterial smooth muscle cells (PASMCs) (Shimoda et al., AJRCCM 153: A581, 1996). The mechanism by which ET-1 inhibits K_{DR} is unclear; however, it is known that ET-1 activates the phospholipase C (PLC) signal transduction cascade in smooth muscle. Therefore, we used whole cell patch-clamp techniques to determine if the PLC/protein kinase C (PKC) pathway was involved in ET-1-induced inhibition of K_{DR} current in freshly isolated rat PASMCs. Cells were superfused with modified Tyrode solution and dialysed with pipette solution containing (in mM): 35 KCl, 90 K-gluconate, 10 NaCl, 0.5 GTP, 10 BAPTA, 3 $CaCl_2$, 5 MgATP and 10 HEPES (pH 7.2; $[Ca^{2+}]_i \approx 75$ nM). Whole cell outward K^+ currents were activated by depolarizing pulses from a holding potential of -60 mV to test potentials of -30 to +40 mV. Ca^{2+}-activated K^+ currents were inhibited by charybdotoxin (100 nM) and the remaining outward K^+ current completely inhibited by 10 mM 4-aminopyridine, suggesting K_{DR} current. At a test potential of +20 mV, ET-1 (10^{-11} to 10^{-7} M) caused significant concentration-dependent inhibition of K_{DR} current, with maximum inhibition (-56.2 ± 3.8%) occurring at 10^{-7}. Exposure to U73122, a PLC inhibitor, abolished the response to ET-1. In the presence of staurosporine (1 nM), a nonselective PKC inhibitor, or GF 102893X (30 nM), a putative inhibitor of the Ca^{2+}-dependent isoforms of PKC, the inhibitory effect of ET-1 (10^{-9} M) was abolished or significantly reduced from -32.8 ± 6.7 to -7.1 ± 3.1%, respectively. Furthermore, ET-1-induced inhibition of K_{DR} was significantly reduced when intracellular Ca^{2+} was eliminated with 10 BAPTA/0 Ca^{2+}. Our results, consistent with recent evidence suggesting PKC activation inhibits K_{DR} in systemic smooth muscle, indicate that ET-1-induced inhibition of K_{DR} current in PASMCs is mediated by the PLC/PKC pathway, primarily via activation of Ca^{2+}-dependent isoforms of PKC, although Ca^{2+}-independent isoforms may also be involved.

W-Pos90

DUAL MODULATION OF K_{ATP} CURRENTS BY PROTEIN KINASE A AND PROTEIN KINASE C IN PIG CORONARY VASCULAR MYOCYTES.
((**G.C. Wellman**, J.M. Quayle and N.B. Standen)) Dept. Cell Physiology & Pharmacology, University of Leicester, LE1 9HN, UK

We have examined the actions of two vasoactive stimuli, calcitonin gene-related peptide (CGRP) and carbamylcholine (CCh) on K_{ATP} currents in freshly dissociated pig coronary arterial myocytes using the conventional whole-cell patch clamp technique. The potent vasodilator, CGRP (50 nM) produced a membrane current of -91 ± 26 pA (n=11) in cells dialyzed with 0.1 mM ATP at a holding potential of -60 mV in symmetrical 140 mM K^+. When intracellular ATP levels were increased to 3 mM, CGRP-induced currents were reduced by greater than 50 %. CGRP responses under both conditions were completely abolished by the blocker of K_{ATP} channels, glibenclamide (10 μM) and reduced in the presence of H-89 (1 μM), an inhibitor of protein kinase A. Furthermore, a glibenclamide-sensitive current was produced in response to an activator of adenylyl cyclase (forskolin, 10 μM). In contrast to the stimulatory influence of the PKA pathway on K_{ATP} currents, 1-Oleoyl-2-acetyl-sn-glycerol (OAG, 10 μM), a membrane permeant activator of protein kinase C decreased pinacidil-induced K_{ATP} currents. Carbamylcholine (20 μM), a muscarinic receptor agonist, was also found to inhibit pinacidil-stimulated K_{ATP} currents. These data suggest that protein kinase A and protein kinase C produce opposing actions on coronary smooth muscle K_{ATP} channels. Both of these mechanisms may be important in the regulation of coronary blood flow by vasoactive substances.

This work has been supported by a grant from the MRC.

W-Pos91

DEACTIVATION KINETICS OF GIRK CURRENTS ARE ACCELERATED BY RGS PROTEINS
((**P. Kofuji**, H.A. Lester, C. A. Doupnik)) Caltech, Div. Biology 156-29, Pasadena CA 91125.

One well described aspect of native G protein-activated inwardly rectifying K^+ channels (GIRK) is their fast activation and deactivation kinetics with step applications of agonists for G protein-coupled seven helix receptors. In atrial cells, acetylcholine (ACh)-evoked GIRK currents deactivate in < 1s with rapid removal of ACh, presumably due to the rapid dissociation or removal of Gβγ dimers from the channel. The off rate of the muscarinic response is much faster than the intrinsic GTP hydrolysis rate of Gαi or Gαo subunits measured *in vitro* (2-5/min), suggesting GIRK channels may possess an intrinsic GTPase activating protein (GAP) activity similar to other G protein effector molecules. We investigated the molecular determinants of GIRK deactivation by coexpression of various components of this signalling pathway in oocytes and CHO cells. In CHO cells transfected with plasmids encoding GIRK1, GIRK2 and the m2 type muscarinic receptor, application of 1 μM ACh induced GIRK currents that activated rapidly (τ_{act} ~ 500 ms), but deactivated slowly (τ_{deac} = 5-13 s) compared to atrial cells (though this rate is similar to the GTPase rate of Gα subunits). The ACh-evoked currents were abolished by pertussis toxin pretreatment, indicating the involvement of Gi or Go proteins. Similar slow deactivation of GIRK currents is seen in oocytes. We investigated the effect of the newly described class of proteins, "regulators of G protein signalling" (RGS), which accelerate the *in vitro* GTPase activity of Gαi and Gαo proteins via a direct interaction with the Gα proteins (Berman et al, Cell 86: 445, 1996). Coexpression of the RGS4 isoform with GIRKs and the m2R in CHO cells, led to expression of GIRK currents that now deactivated with a time constant (τ_{deac} = 0.4 to 1.5 s) comparable to that measured for atrial cells (τ_{deac} = 0.5 to 1 s). We conclude that GIRK channels do not act as GAPs as previously suggested; instead, RGS proteins exert this role *in vivo*. Support: NIH, NIMH, AHA, Guenther Foundation.

W-Pos92

G PROTEIN ALPHA SUBUNIT SPECIFICITY IN THE REGULATION OF THE MUSCARINIC POTASSIUM CHANNEL.
((**Y. Ni**, S. Graber, A. S. Otero and G. Szabo)) Dept. of Mol. Physiol. and Biol. Phys., U. of Virginia, Charlottesville, VA 22906-0011 and Dept. of Pharmacology and Toxicology, West Virginia U., Morgantown, WV 26506-9223.

GTP-binding protein α subunits expressed in Sf9 insect cells were applied to excised patches of frog atrial cell membranes in which the K_{ACh} channels were previously activated in a receptor-independent manner. While application of G protein βγ subunits did not further enhance channel activity in these patches, application of non-activated, GDP-bound inhibitory subunits (α_i) altered channel activity in a subunit specific manner. Five nM α_{i2} slightly enhanced channel activity (3.0 fold, ±1.1 (n=4) but 10 nM α_{i2} essentially abolished it (2.0 ±0.8 % of control). In contrast, α_{i1} and α_{i3} subunits at these concentrations had no effect on channel activity although at higher concentrations (25 nM) a small but non specific reduction was observed. Application of βγ subunits restored channel activity following the application of α_{i2}. These data suggest that it is the α_{i2} subunit that is involved in the coupling of receptor activation to channel opening. The data also implies a novel paradigm for this signal transduction system in which the α_i subunit is responsible for the inhibition of the constitutively active channel formed presumably by the GIRK1-CIR polypeptides. The data suggests that channel activation occurs through release of the α subunit from a pre-formed complex with the channel. This relief from inhibition takes place upon interaction with activated receptor, and thereby results in channel opening.

Supported by NIH Grant HL 37127 (GS) and PhRMA Research Rrant (SG).

W-Pos93

HISTIDINE 118: PART OF THE pH SENSOR IN THE PLANT KAT1 K^+ CHANNEL
((**X. D. Tang**, I. Marten and T. Hoshi)) Dept of Physiology & Biophysics, The University of Iowa, Iowa City, IA 52242

The KAT1 K^+ channel cloned from *Arabidopsis* has been found to be regulated by internal and external pH. We have postulated that the histidine residues may act as the pH sensor of the KAT1 channel.

Macropatch recordings show that lower internal pH (6.2) has two effects on the KAT1 channel expressed in *Xenopus* oocytes. We found that lower internal pH (6.2) shifts the G(V) curve to the more positive direction and decreases the channel conductance. Single-channel recordings showed that internal pH 6.2 decreased the single-channel current amplitude by ~20% compared with that at pH of 7.2 at the clamping voltage of -160 mV. Lower external pH (5.6) also shifts the G(V) curve to the more positive direction but single-channel conductance is not altered by different external pH.

We have mutated all histidine residues in the KAT1 channel and identified that the single-channel current amplitudes of the H118D and H118E channels are less sensitive to lower internal pH (6.2). However, the external pH (5.6 and 7.2) effects are markedly reduced in the H118E mutant channel. We conclude that histidine 118 acts as a part of the pH sensor in the KAT1 K^+ channel. (Supported by NIH, HFSP)

W-Pos94

CARDIAC MYOCYTE Kv1.5 K+ CHANNEL mRNA EXPRESSION IS INFLUENCED BY CELL-CELL INTERACTIONS.
((**K.M. Hershman** and E.S. Levitan)) Dept. of Pharmacology, University of Pittsburgh, Pittsburgh, PA 15261. (Spon. by L. Huang)

A dramatic increase in Kv1.5 mRNA expression is found in adult rat ventricular myocytes isolated 1 day after administration of dexamethasone. Here we report that culturing these myocytes for 3 hours results in a rapid downregulation of Kv1.5 mRNA. No loss of Kv4.2, KvLQT1 or cyclophilin messages is seen in this period. Furthermore, the change in Kv1.5 mRNA occurs in the absence or presence of dexamethasone, serum, laminin, or cyclic AMP analogs. In contrast, a number of experimental paradigms indicate that Kv1.5 mRNA expression is proportional to cell density. Thus, we find that Kv1.5 mRNA expression is maintained for up to 8 hours when myocytes are cultured in conical tubes. Under these conditions, the cells spontaneously pellet. Furthermore, with the same number of myocytes, plating in a 60 mm dish results in higher levels of Kv1.5 mRNA than in a 100 mm dish. Also, as fewer and fewer cells are plated in 60 mm dishes, the Kv1.5 mRNA signal (normalized to total RNA) decreases dramatically. In contrast, reducing cell number does not have a large effect when cells are allowed to pellet. These results indicate that the absolute number of myocytes cultured is not critical. Rather, even small numbers of myocytes are able to maintain elevated Kv1.5 levels as long as myocytes are in close proximity to one another. The loss of Kv1.5 message in low density cultures is not prevented by conditioned medium from high density cultures. However, in some experiments, coculturing with paraformaldehyde-fixed cells attenuated the loss of Kv1.5 mRNA. These findings seem to rule against a role for a stable paracrine factor and in favor of the hypothesis that Kv1.5 gene expression is affected by cell-cell interactions dependent on direct cell contact between myocytes.

W-Pos95

LARVAL NEURONS FROM dunce (*dnc*) LEARNING AND MEMORY *DROSOPHILA* MUTANTS LACK AN OUTWARD CURRENT COMPONENT DOWN-REGULATED BY CAMP.
Ricardo Delgado and Pedro Labarca. Centro de Estudios Cientificos de Santiago, and Departamento de Biologia, Fac. de Ciencias, Univ. de Chile. Casilla 16443, Santiago 9, Chile.

Whole-cell currents were investigated in wild type neurons (wt, n=351) and in neurons derived from *dnc* (n=192). Four outward currents could be identified, based on electrophysiological properties, sensitivity to cAMP, and K^+ channel blockers. In *dnc*, a cAMP-regulated, mantained outward current component could not be detected. In addition, the scrutiny evidenced differences between wt and *dnc*neuron populations. For example, class I neurons (11% of total), exhibiting cAMP-regulated outward currents were absent in *dnc*. In turn, class III neurons, exhibiting a cAMP-insensitive, inactivating current (τ_{inact}= 16.0 ± 0.5 ms) as the major outward component, were significantly more abundant in *dnc* (28 vs 6.3%). No differences in voltage-gated inward currents was evident, the most conspicuous inward current being a TTX-sensitive, inactivating Na^+ current. To the best of our knowledge, this work offers the first evidence that neurons from *dnc*, a learning and memory *Drosophila* mutant deficient in a form of phosphodiesterase, might lack a specific cAMP-down regulated outward current; and that, population wise, wt and *dnc* neurons differ in outward current profiles. (Supported by FONDECYT, HFSP. and a Catedra Presidencial en Ciencias , to PL).

W-Pos96

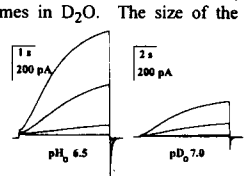
pH REGULATION OF THE PROTON CONDUCTANCE OF CHANNELS FORMED BY M2 PROTEINS OF INFLUENZA A VIRUSES.((**I.Chizhmakov**, F.M.Geraghty, A.Skinner, T.Betakova, **D.C. Ogden**, and A.J.Hay)) National Institute for Medical Research,The Ridgeway,Mill Hill,London NW7 1AA,UK.

M2 proteins of three influenza A viruses A/Weybridge/7 (H7N7) (W), A/Rostock/54(H7N1) (R) and A/Part Chalmers/1H3(H3N) (PC) were expressed in MEL cells and ion channel conductance investigated with whole cell patch-clamp. The channels formed by W,PC and R M2 show similar high proton selectivity but differ in regulation of permeability by extracellular (pHo) and intracellular (pHi). W and PC M2 channels are regulated by pHo while the R M2 is regulated by pHi with decreased permeability at pHo>7.0 and pHi>7.0, respectively. In both cases there was also a decrease in voltage sensitivity of the proton current between -60 mV and +60 mV at high pH. Analysis of these results in terms of a three barrier model shows that the decrease in proton permeability and the change in voltage dependence can be described by changes in location of the central energy barrier, the rate limiting step in proton flux. In the case of W and PC M2, at pHo>7 the barrier shifts towards an external access step so that exit from the pore for outward proton flux becomes rate limiting. In the case of R M2, it shifts to an internal access step at pHi>7 and exit from the pore for inward proton flux becomes rate limiting. Mutagenesis of M2 proteins showed that the difference in properties between W and R M2 is due to three amino acids different in the transmembrane domain.

W-Pos97

DEUTERIUM REGULATION OF VOLTAGE-GATED PROTON CHANNELS IN ALVEOLAR EPITHELIAL CELLS ((**V. V. Cherny** and T. E. DeCoursey)) Dept. of Molecular Biophysics & Physiology, Rush Medical Center, Chicago, IL

Voltage-activated H^+ currents in rat alveolar epithelial cells were studied using voltage-clamp techniques. The effects of substituting deuterium, D_2O, for water on the pH dependence of gating were explored. D^+ permeated proton channels. The voltage-dependence of H^+ channel gating is sensitive to pH_o and pH_i, and was regulated by pD_o and pD_i in an analogous manner. The time constant of H^+ current activation was 2-3 times slower in D_2O than in H_2O. This is a larger deuterium isotope effect than reported for other channels, but is consistent with primary kinetic isotope effects for proton abstraction reactions. In contrast, deactivation (τ_{tail}) was slowed at most ~1.5 times in D_2O. The size of the isotope effect indicates that a protonation/deprotonation event is a rate-limiting step in channel opening. This conclusion is consistent with a proposed gating mechanism in which the first step in activation is a deprotonation reaction (Cherny *et al*, 1995. *J. Gen. Physiol.* 105:861-896). The greater D_2O isotope effect on activation than deactivation suggests that either the external and internal regulatory sites are chemically different, or the first step in channel closing occurs before deprotonation at the internal site.



Support: NIH grant HL52671, American Heart Association Grant-in-Aid.

W-Pos98

THE EFFECTS OF CALCIUM BUFFERS ON MECHANOELECTRICAL TRANSDUCTION IN TURTLE HAIR CELLS ((**A.J. Ricci** & R. Fettiplace)) Department of Neurophysiology, University of Wisconsin, Madison, WI 53706.

Adaptation of the hair cells' mechanoelectrical transducer channels, triggered by calcium influx, is thought to optimize the transducer's sensitivity near the resting position of the hair bundle. Paradoxically, the hair cells are arranged with the transducing apparatus in the hair bundles enveloped in a very low (20 -100 μM) calcium solution. To address this paradox, we have examined the effects of varying the concentration of intracellular calcium buffer, BAPTA, on transduction in an intact cochlear epithelium. In this preparation, the endo-lymphatic and peri-lymphatic surfaces could be perfused with different calcium concentrations. The speed and extent of transducer adaptation increased on reducing the concentration of intracellular BAPTA, and with 0.1 mM or less BAPTA, the transducer channels adapted almost completely in less than 1 ms. For a fixed [BAPTA], the time constant of adaptation was found to vary systematically along the cochlea in parallel with the size of the transducer conductance. We propose that adaptation may contribute a variable high-pass filter to cochlear frequency selecitivity. The effects of perfusing 50 μM calcium endolymph depended on the electrode's [BAPTA]: with 3 mM BAPTA, adaptation was abolished, but in most recordings with 0.01 or 0.1 mM BAPTA, rapid adaptation persisted; the current-displacement curve was also shifted less negative the lower the [BAPTA]. We suggest that for adaptation to function in vivo the stereocilia must contain a very low concentration of native calcium buffer, lower than in the cell body, where the buffer is equivalent to approximately 1 mM BAPTA (Tucker & Fettiplace, 1996, J. Physiol. 494: 613).

W-Pos99

F-ACTIN NETWORK REGULATES THE ACTIVITY OF Na^+-SELECTIVE CHANNELS IN HUMAN MYELOID LEUKEMIA CELLS. THE ROLE OF PLASMA GELSOLIN AND INTRACELLULAR CALCIUM. ((**A.V. Maximov**, E.A. Vedernikova & Yu.A. Negulyaev)) Inst. of Cytology, RAS, St.Petersburg, 194064, Russia. (Spon. by I.I.Ismailov).

The non-voltage-gated amiloride insensitive Na-selective (P_{Na}/P_K =3) channels were studied in human leukemic K562 cell plasma membranes using the patch clamp technique. These channels have unitary conductance of 12 pS and display a low level of background activity. F-actin disrupters cytochalasin-D and plasma gelsolin, but not colchicine (which affects microtubules), induced a significant increase in channel open probability (P_o). Elevating of free intracellular calcium by the ionophore 4Br-A23187 also increased channel activity in cell-attached patches, although no direct effect of Ca^{2+} on channels was found in inside-out patches. In contrast, polymerization of G-actin monomers at the cytoplasmic side reduced channel P_o to background level. The analysis of dwell-time distributions and current-voltage curves showed that modification in the cellular microfilament system resulted in a dramatic change in channel kinetics with no change in selectivity or conductance. Our data suggest that: 1) actin filaments interact with Na channels in K562 cells (channels activate with F-actin disassembly and inactivate with F-actin assembly); 2) under physiological conditions, Ca^{2+}-induced actin depolymerization may be one mechanism regulating sodium influx into K562 cells. Supported by Russian Basic Research Foundation Grant #95-04-1142a.

W-Pos100

REGULATION OF EPITHELIAL SODIUM CHANNELS BY SHORT ACTIN FILAMENTS. ((B.K.Berdiev[1], A.G.Prat[2], H.F.Cantiello[2], D.A.Ausiello[2], C.M.Fuller[1], B.Jovov[1], D.J.Benos[1], and **I.I. Ismailov**[1])) [1]Univ. AL. at Birmingham, Dept. Physiol. & Biophys., Birmingham, AL 35294; [2]Harvard Med. School, MA Gen. Hosp. E Boston, MA 02129. (Spon. by J.K.Bubien)

Cytoskeletal elements play an important role in the regulation of ion transport in epithelia. We have studied the effects of actin filaments of different length on α,β,γ-rENaC (rat epithelial Na^+ channel) in planar lipid bilayers. We found that 1) short actin filaments caused a two-fold decrease in unitary conductance and a two-fold increase in open probability (P_O) of α,β,γ-rENaC; 2) α,β,γ-rENaC could be transiently activated by protein kinase A (PKA) plus ATP in the presence, but not in the absence, of actin; 3) ATP in the presence of actin could also induce a transitory activation of α,β,γ-rENaC, although with a shortened time course and with a lower magnitude of change in P_o; 4) DNAse-I, an agent known to prohibit elongation of actin filaments, prevented activation of α,β,γ-rENaC by ATP or PKA plus ATP; 5) cytochalasin D, added after rundown of α,β,γ-rENaC activity following ATP or PKA plus ATP treatment, produced a second transient activation of α,β,γ-rENaC; 6) gelsolin, a protein that stabilizes polymerization of actin filaments at certain lengths, evoked a sustained activation of α, β, γ-rENaC at actin:gelsolin ratios <32:1, with a maximal effect at an actin:gelsolin ratio of 2:1. These results suggest that short actin filaments activate α,β,γ-rENaC. PKA mediated phosphorylation augments activation of this channel by decreasing the rate of elongation of actin filaments. These results are consistent with the hypothesis that cloned α,β,γ-rENaCs form a core conduction unit of epithelial Na^+ channels, and that interaction of these channels with other associated proteins, such as short actin filaments,confers regulation to channel activity. Supported by NIH Grants DK 37206 and DK 19406.

W-Pos101

pH SENSITIVITY OF PEG-INDUCED EFFECTS ON *S. AUREUS* α-HEMOLYSIN SINGLE CHANNEL CURRENT

((J.J. Kasianowicz† and S.M. Bezrukov‡)) † NIST, ‡ NIH

Nonelectrolyte polymers are commonly used to size ion channels[1]. However, we recently showed that PEG (poly(ethylene glycol)) can interact with channels as judged by the effect of differently-sized polymers on the single channel conductance and current noise[2]. We show here that PEG partitioning into the pore and the polymer-induced current noise are altered by changes in pH. Specifically, for the channel formed by *Staphylococcus aureus* α-hemolysin, decreasing the pH from 7.5 to 4.5 causes the PEG-induced conductance reduction and the peak current noise to occur at a lower characteristic polymer molecular weight. Note that if one assumed PEG did not interact with this channel, the latter result would be disturbing because the conductance at pH 4.5 is *greater* than that at pH 7.5[3]. Our results suggest that this pore might contain hydrophobic residues because PEG tends to associate with such regions in proteins[4]. They may also underscore the oft-neglected fact that pore size does not always correlate with channel conductance[5]. **References**: (1) Krasilnikov, O.V., et al. 1992. *FEMS Microbiol. Immun.* **105**: 90; Bezrukov, S.M. & I. Vodyanoy. 1993 *Biophys. J.* **64**: 16; (2) Bezrukov, S.M., I. Vodyanoy, R.A. Brutyan & J.J. Kasianowicz. 1996. *Macromolecules, accepted*; (3) Kasianowicz, J.J. & S.M. Bezrukov. 1995. *Biophys. J.* **69**: 94; (4) Arakawa, T. & S.N. Timasheff. 1985. *Biochemistry* **24**: 6756; (5) Finkelstein, A. 1985. *Ann. NY Acad. Sci.* **456**: 26. Supported by the NAS/NRC (JJK) and the ONR (V.A. Parsegian).

W-Pos102

STOCHASTIC RESONANCE IN NON-DYNAMICAL SYSTEMS WITHOUT RESPONSE THRESHOLDS

((**S.M.Bezrukov**, M.A.Pustovoit, and I.Vodyanoy)) LSB/DCRT, NIH, Bethesda, MD; St.Petersburg Nuclear Physics Inst., Russia; Office of Naval Research, London, UK.

Only a few years ago it was generally accepted that the noise-facilitated signal transduction or 'stochastic resonance' (SR) phenomenon can occur exclusively in dynamical systems subjected to random forcing. Recently however, K.Wiesenfeld et al. hypothesized (*Phys.Rev.Lett.* **72**, 2125 (1994)), and F.Moss and colleagues have shown (*Int.J.Bifurc.Chaos* **4**, 1383 (1994) and *Europhys.Lett.* **29**, 191 (1995)) both experimentally and theoretically, that the simplest 'stochastic resonator' consists only of signal, noise, and a threshold device. In this paper we introduce yet another class of systems where a noise-induced increase in the output signal-to-noise ratio can be observed. These systems are both non-dynamical and *threshold-free*. We find SR in a very general model -- a random pulse train where the probability of pulse generation is exponentially dependent on an input which is composed of a sine-wave signal plus random noise. Thus, we demonstrate that SR is a fundamental property of a broad variety of 'kT-driven' systems ranging from semiconductor p-n junctions to voltage-dependent ion channels (*Nature* **378**, 362 (1995)). The general theory is supported by computer simulations showing the existence of SR in a remarkably simple model of the time-dependent doubly stochastic Poisson process.

W-Pos103

WATER IN A CHANNEL, MOBILE PROTONS, AND GATING.

((J. Lu and M. E. Green)) Chemistry Department, City College of CUNY, New York NY 10031

Our earlier model for gating of ion channels is extended to show the effect of charge motion in the channel and its protein wall. This wall is taken to be nearly rigid. We now show that the charges, if taken to be protons in a series of pairs of energy wells, can be localized by a field comparable to a few mV across a membrane. The protons would be on amino acid side chains, here simplified to potential wells. When the proton energies in the wells are matched, as might occur as the membrane depolarizes, the protons can tunnel on a time scale comparable to gating. Tunneling calculations will be described, including some realistic three dimensional wells. The potential seen by ions in the channel is largely due to these protons, and changes as they move. Water in the channel is important, and Monte Carlo simulation is used to obtain its behavior in the presence of ion(s) and charges. The mobility of the water also depends on the potentials. Our calculated fields from local charges can exceed 10^9 V m^{-1}, a plausible range for short distances, and at least two orders of magnitude greater than the membrane potential; it strongly orients the water and affects its density in a section of the model which is tapered, as in the pore lining in a channel. We show the energy as a function of the position of a mobile ion and of the charge configuration in the wall. In certain configurations there is a force pushing the ion down the channel, while in others the ion would experience no net force, and be trapped, closing the channel. Gating, in this model, is the motion of the local charges under the influence of the membrane potential, causing the transition from closed to open. Reasons for not allowing parts of the protein to "pop up" will also be given.

W-Pos104

ENERGETIC PARAMETERS FOR GATING OF THE *E. COLI* LARGE CONDUCTANCE MECHANOSENSITIVE CHANNEL (MSCL).

((Wade J. Sigurdson[1], **Sergei I. Sukharev**[2], Ching Kung[2,3] and Frederick Sachs[1])) [1]Dept. Biophysics, SUNY, Buffalo, NY 14214; [2]Laboratory of Molecular. Biology. and [3]Dept. of Genetics, U. of Wisconsin, Madison, WI 53706. (Spon. by Feng Qin)

We measured the actual tension acting on purified, reconstituted large conductance mechanosensitive channels (MscL) and assessed the energetic parameters for the gating. Previous *in vitro* expression experiments (Sukharev et al., Nature 368:265) and functional reconstitution of highly purified MscL indicated that this channel is gated directly by tension transmitted via the lipid bilayer. The channel complex is assumed to respond to tension (T) and open state transition-related expansion (ΔA) in the membrane plane. A two-state model predicts a Boltzmann-type partitioning of channels between the closed and open states with the exponential factor of (ΔG-TΔA)/kT, where ΔG is the free energy of channel transition in unperturbed membrane. We recorded activities of purified MscL in liposome patches evoked by various pressure gradients. Simultaneous video imaging of the patch curvature permitted direct assessment of the membrane tension. The $P_o(T)$-dependence was a steep sigmoidal curve with a mid point at 18 dyne/cm, well fitted with parameters ΔG=15.8 kT and ΔA=3.5 nm^2. MscLs posses at least 5 conducting states. Kinetic analysis of a 7-state (2 closed, 4 substate, 1 full) linear model, suggests that the rate limiting step to opening is passage from a closed state to a short-lived sub-state. Forward transition rates increase sequentially as the next largest conductance state is reached, suggesting a cooperative relation between gating of the 6 channel subunits and channel conductance. (Supported by NASA, NIH)

W-Pos105

MECHANOELECTRICAL TRANSDUCTION AND ADAPTATION IN HAIR CELLS OF THE MOUSE UTRICLE.

((**J.R. Holt**[1], A. Rüsch[2], D.P. Corey[1] and R.A. Eatock[2])) [1]Harvard Medical School, Boston, MA 02114; [2]Baylor College of Medicine, Houston, TX 77030.

In inner ear organs tuned to stimulus frequencies >10 Hz, the hair cell transduction current adapts with time constants (τ) of 10-100 ms. Do transduction currents in mammalian vestibular organs, sensitive to much lower frequency head movements, adapt, and does variation in the adaptation rate contribute to the known variation in response dynamics of vestibular afferents?

Whole-cell transduction currents were recorded from hair cells in utricular epithelia excised from young mice, maintained in artificial perilymph with 1.3 mM Ca^{2+}. Stimuli were displacements of the hair bundle, effected by a fluid jet. Recordings were obtained from neonatal hair cells and from both mature hair cell types (I and II) found in mammalian vestibular organs.

Displacement-current functions were fitted by first- or second-order Boltzmanns. The operating range was ~1-2 μm, measured at the tip of the tallest stereocilia. Peak currents exceeded 300 pA. Most but not all cells adapted to 500-ms steps. Time courses were usually double-exponential, with fast and slow τ's ranging from 3 to 60 ms and from 20 to 200 ms, respectively. Adaptation to positive steps was accompanied by a shift and broadening of the displacement-current function.

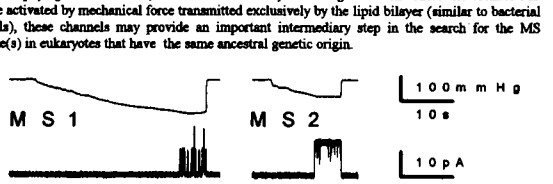
Supported by NIDCD grant DC02290 (RAE) and HHMI (DPC).

W-Pos106

CHARACTERISATION OF MECHANOSENSITIVE CHANNELS FROM THE ARCHEON *HALOFERAX VOLCANII*.

((**A.C. Le Dain**, A. Kloda, A. Ghazi* and B. Martinac)) Dept. of Pharmacology, Univ. Western Australia, Nedlands WA 6907, Australia; *Laboratoire des Biomembranes, Univ. Paris-Sud, 91405 Orsay, France.

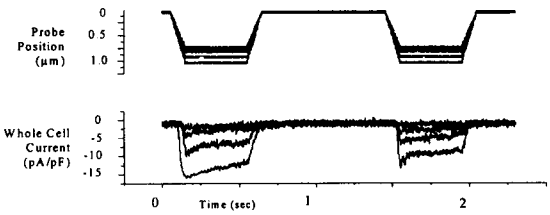
Mechanosensitive (MS) ion channels have been documented in many cell-types from *Eukarya* and *Eubacteria*. The third domain of the phylogenetic tree of life encompasses the *Archaea* (formerly archaebacteria), that are neither closer to eukaryotes or eubacteria. We describe the characteristics of the first MS channels observed from the archeon *Haloferax volcanii*. Membranes were reconstituted in artificial liposomes and examined with the patch-clamp technique. Two types of MS channels gated by application of suction to the patch pipette were observed (MS1 and MS2), that were distinguished by conductance and gating properties but had similar activation pressures (see Figure, +20mV holding potential). MS1 had a conductance of 580pS (±20mV, n=3 patches) and an activation pressure of -108±9mmHg (n=3). MS2 had a conductance of 720pS (±20mV, n=5 patches) and was activated by -103±12mmHg (n=3). In addition, MS2 rectified at negative potentials, with a 53% reduction in current amplitude at -60mV (-29.3±0.5pA, n=3) compared to +60mV (62.6±4.2pA, n=3). Similar to MS channels from the other phylogenetic domains, MS2 was blocked by 0.2mM gadolinium. Since the archael MS channels are activated by mechanical force transmitted exclusively by the lipid bilayer (similar to bacterial MS channels), these channels may provide an important intermediary step in the search for the MS channel gene(s) in eukaryotes that have the same ancestral genetic origin.



W-Pos107

MECHANOSENSITIVE CURRENTS IN VENTRICULAR MYOCYTES.
((**G. C. L. Bett** & F. Sachs)) Biophysical Sciences, 120 Cary Hall, SUNY, Buffalo, NY 14214. (Spon. By R. A. Spangler)

Mechanical deformation of ventricular cells leads to activation of mechanosensitive ion channels (MSCs). Glass pipettes and AFM cantilevers were used to press on ventricular cells isolated from 17 day old chick embryos. The position of the mechanical stimulation probe was driven by a computer-controlled piezo electric manipulator.



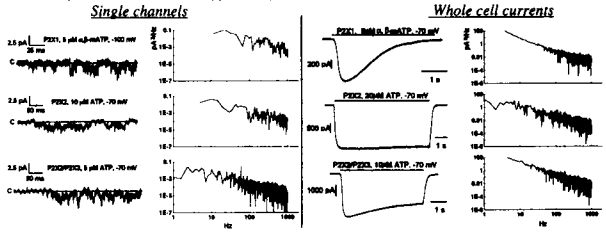
Dose-response curves of pressing on a cell voltage-clamped at -70 mV indicate that the MSC current inactivates with time, at a rate inversely related to the degree of mechanical deformation, i.e., tension in the cytoskeleton/membrane complex. This rate of inactivation is not voltage-dependent, so may be associated with some cytoskeletal property. Recovery from inactivation is time-dependent.

GCLB is an AHA NY State Affiliate Fellow. This work also supported by an NIH grant to FS.

W-Pos108

STUDIES ON THE ATP-GATED CHANNELS STABLY TRANSFECTED IN HEK CELLS. ((**S. Ding**[1], F. Sachs[2])) [1]Dept of Chemical Engineering, [2]Dept of Biophysical Science, SUNY at Buffalo, Buffalo, NY 14214

We studied the single channel and whole cell currents of P2X1, P2X2 and P2X2/P2X3 receptors* stably transfected in HEK cells. Single channel currents recorded from outside-out patches didn't show a binary conductance. The P2X1 receptor exhibited pronounced desensitization as compared with P2X2 and P2X2/P2X3. Neither the differential power spectra of whole cell currents nor the differential power spectra of single channels show a distinct relaxation. These results suggest that P2X family receptors have multiple conductances.(*Courtesy by Dr. A. Surprenant. The work is supported by MDA)



W-Pos109

STRUCTURE-FUNCTION STUDIES ON THE PUTATIVE P-DOMAIN OF AN ATP-GATED CATION CHANNEL. ((**K.E. Parker** and A. Scarpa) Case Western Reserve University School of Medicine, Department of Physiology and Biophysics, Cleveland, OH 44106)

ATP is used as an extracellular signaling molecule by a wide variety of cell types, including smooth and cardiac muscle, macrophages and neurons in the central nervous system. The ATP signal is sensed through ATP-gated nonselective cation channels (P2x receptors), among other receptor types. P2x receptors contain within their second putative transmembrane domain predicted amino acid sequences that resemble the pore-lining domain (P-domain) of other cation channels. This is particularly true for the P2x1 receptor expressed in rat vas deferens smooth muscle. We have constructed point mutants within the P-like-domain of the P2x1 receptor (kindly supplied by Dr. Gary Buell) for the purpose of investigating whether it indeed forms the pore of this ATP-gated nonselective cation channel. The changes we have made fall into three classes: mutants in residues conserved between the Shaker potassium channel and the P2x receptor that form internal and external TEA-binding sites in the potassium channel; mutants in residues that correspond to potassium channel residues that define the selectivity of the channel; and mutants in fixed charges on the putative internal and external sides of the P2x1 P-like-domain. Using standard patch-clamp methods in the whole-cell and cell-attached configurations, we will be conducting experiments evaluating the effects of these mutations on the selectivity, unitary conductance and possible TEA block of the mutant P2x receptors expressed in HEK 293 cells.

W-Pos110

MEMBRANE INTERACTION OF THE TH8 HELIX OF THE ISOLATED TRANSMEMBRANE DOMAIN OF DIPHTHERIA TOXIN: A SITE-DIRECTED SPIN LABELING STUDY. ((**Kyoung Joon Oh**[1], Can Cui[2], Hangjun Zhan[2], R. John Collier[2], and Wayne L. Hubbell[1])) [1]Jules Stein Eye Institute and Department of Chemistry & Biochemistry, University of California, Los Angeles, CA 90095; [2]Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, MA 02115.

A series of single cysteine substitution mutants of the diphtheria toxin transmembrane (T) domain were prepared and selectively spin-labeled at consecutive residues on the TH8 helix. The mobility of the spin labels inferred from the electron paramagnetic resonance spectra of the mutants in solution at pH 8 are consistent with the locations of the labels in the X-ray structure. Upon membrane binding at pH 4.6, a major reorganization of the tertiary structure is detected. For example, residue 336 which is immobilized in the interior of the T domain in the solution structure becomes more mobile and exposed to the interior of the bilayer at an immersion depth of about 14 Angstroms. On the other hand, residues such as 335 and 338 become more immobilized in the membrane bound state, which indicates that these residues are placed at newly formed tertiary or quaternary contact sites. Interestingly, residue 348 which is located at the beginning of the TH8-TH9 interconnecting loop becomes immobilized in the membrane-bound state. A similar change was previously observed at the residue 351 in the loop. These results indicate that the interhelical loop is not flexible in the membrane-bound state but involved tertiary ot quaternary interactions. The topology of the TH8 helix in the membrane will be presented based on the accessibilities of the nitroxide side chains to collision with polar (NiEDDA) and nonpolar (O_2) reagents.

W-Pos111

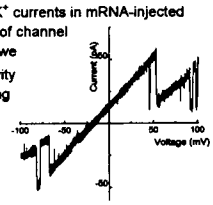
INVESTIGATION OF THE CELLULAR TOXIC MECHANISMS OF CARIBBEAN MAITOTOXIN ((Xin-zhong Lu, Lin Wang, Arin Bhattacharjee and **Ming Li**)) Department of Pharmacology, University of South Alabama, Mobile, AL 36688

Maitotoxin (MTX) is a potent water-soluble marine dinoflagellate toxin. Caribbean-MTX (C-MTX) is a novel type of MTX isolated from tropical fish, however the cellular toxic mechanisms have not been investigated. In the present study, we used whole-cell patch-clamp techniques and fluorescence calcium imaging to determine the cellular toxic mechanisms of C-MTX in mammalian cells. Pancreatic ß-cells are a system where the effects of MTX have been previously reported, and HIT-T15 is an insulin-secreting cell line that stably expresses L-type calcium current, making it a suitable model for this study. Using the fluorescence calcium indicator Indo-1 AM, we found that there is a profound increase in intracellular free calcium 3 minutes after application of 200 nM C-MTX to HIT-T15 cells. On the other hand, application of C-MTX did not increase the L-type calcium current in these same cells but large voltage-independent cationic currents appeared after applying C-MTX into the extracellular bath solution. These results indicate that C-MTX induced a voltage-independent inward current which by itself or through a secondary mechanism results in a large amount of cationic influx. We conclude that C-MTX causes the opening of non-selective, voltage-independent ion channels which permits abnormal calcium influx. The elevated level of intracellular calcium concentration resulting from this calcium influx would lead to cellular toxicities.

W-Pos112

CONSTRUCTION AND CHANNEL ACTIVITY OF A PLM-IsK CHIMERA
((**Zhenhui Chen**, J. Randall Moorman, Larry R. Jones)),
Indiana University and University of Virginia.

Phospholemman (PLM) and I_{sK} belong to the same super family of proteins which have only one transmembrane domain and can induce Cl^- or K^+ currents in mRNA-injected oocytes. Affinity purified PLM exhibits a complex pattern of channel activity in lipid bilayers. However, I_{sK} fails to do so. Here we report the construction, purification,and the channel activity of a PLM-I_{sK} chimera. Three cDNA constructs, ---encoding the N-terminus PLM (PLM residues 1-17), the transmembrane region of I_{sK} (I_{sK} residues 46-67), and the C-terminus PLM (PLM residues 37-72), were PCR generated using primers containing proper restriction sites. The three protein coding regions were then ligated together to form the intact construct. The chimeric protein was expressed in sf21 insect cells, and purified using monoclonal antibody affinity chromatography. The identity of the PLM-I_{sK} chimera was verified by amino acid sequencing. As shown above, when reconstituted into bilayers using 200:50mM KCl *cis:trans*, the chimeric protein exhibited similar channel activity as wildtype PLM, including cation- and anion-selective switchings, and a cation-selective conductance state with voltage-dependent closings. Since there is no sequence homology between the transmembrane regions of PLM and I_{sK}, these results suggest that other unique regions of PLM are responsible for the voltage-dependent selectivity changes and closings. To explore this possibility, we are currently purifying PLM molecules with mutations in the N- and C-terminal domains.



W-Pos113

MOLECULAR CLONING OF MTRP-4 FROM MOUSE βTC3 CELLS.

((**Feng Qian**, Sarmila Dasgupta, Andrey Kuznetsov and Louis H. Philipson)) Dept. Of Medicine MC1027, The University of Chicago, Chicago, IL 60637

The transient receptor potential (trp) gene and trp-like (trpl) gene of *Drosophila* encode a capacitative calcium influx channel and a non-selective cation current activated by IP_3, respectively. Previous studies have suggested that a similar non-selective cation current may play a role in regulating membrane potential in pancreatic β cells, which are excitable cells. We therefore sought to determine if trp or trpl related genes were expressed in neuroendocrine cells by screening a mouse insulinoma cell (βTC3) library with a mouse cDNA probe related to trp and trpl. 5 overlapping clones with inserts up to 3.3kb were isolated. The single open reading frame encodes a protein of 974 amino acids termed mTrp-4, with an overall 40% amino acid identity and 60% amino acid similarity to other published trp sequences. RNA blotting studies showed that mTrp-4 message was highly expressed in brain but not in spleen or liver. The in situ hybridization of mouse brain showed widerspread expression in the CNS and mTrp-4 message was also detected in glial cells in culture. The presence of possibly alternatively spliced transcripts was suggested by a variant in one of the cDNA clones, and this variant transcript was detected in normal brain and a pituitary tumor cell line. The heterologous expression of mTrp-4 cDNA tagged with a human proinsulin c-peptide at the carboxy end was detected by immunohistochemistry in CHO and βTC3 cells. These studies suggest that mTrp4 is a member of the mammalian trp-related gene family, and that is highly expressed in brain and endocrine tissue-derived cells.

W-Pos114

MOLECULAR CLONING OF A PUTATIVE CYCLIC NUCLEOTIDE GATED ION CHANNEL FROM LIMULUS POLYPHEMUS

((F.H. Chen*, M. Ukhanova*, D. Thomas*, G. Afshar*, B-A. Battelle*, S. Tanda*, R. Payne*)) *Dept. of Zoology, Univ. of Maryland, College Park, MD 20742; *Whitney Lab, Univ. of Florida.

We have cloned a putative cyclic nucleotide gated channel from the horseshoe crab *Limulus polyphemus*. Cyclic GMP-gated channels have been proposed to mediate the electrical response to light in *Limulus* ventral photoreceptor cells (Johnson et al. Nature, 324:468. 1986). We applied the RT-PCR method to amplify mRNA isolated from *Limulus* ventral nerves which contain photoreceptors and amplified a 540 bp cDNA fragment with homology to the cGMP binding domain of known cyclic nucleotide gated channels. The *Limulus* ventral eye cDNA fragment was then used as a hybridization probe to screen cDNA libraries constructed from *Limulus* brain. We have obtained one putative cGMP gated channel from two overlapping partial cDNA clones. This 6 Kb cDNA clone appears to be full length. It has an ORF encoding 905 amino acids and untranslated regions of 472 bp at the 5' and ~3 Kb at the 3' end. Primer extension experiments suggest that the 5' end of the transcript is 60 bp upstream from the beginning of the cDNA clone. The typical eukaryotic polyadenylation signal (AAUAAA) is present 14 bp upstream of the poly A tail. This clone shares 41% identical amino acids with the alpha subunit of the bovine rod cGMP gated channel over the region containing the transmembrane domains, the voltage sensor S4, the pore and the cGMP binding domain. It has a novel C-terminal of about 200 amino acids. This domain is proline rich and contains a consensus sequence (TPLPPSP) for a SH3 binding protein. Since cDNA fragments of the clone can be amplified from ventral nerve RNA, the clone is a possible candidate for the channel detected in excised patches from the photoreceptive membrane of *Limulus* ventral photoreceptors (Bacigalupo et al. PNAS, 88:7938. 1991).

W-Pos115

P2X7 RECEPTORS EXPRESSED IN XENOPUS OOCYTES DO NOT FORM NON-SELECTIVE PORES.

((Mehmet Ugur, **Steven Petrou**, Joshua J. Singer and John V. Walsh, Jr.)) Department of Physiology, University of Massachusetts Medical School, Worcester, MA 01655. (Spon. by S. N. Treistman)

We investigated the properties of P2X7 receptors expressed in Xenopus oocytes. Oocytes were injected with approximately 13 ng of mRNA (plasmid kindly supplied by Dr. Gary Buell) and allowed to incubate for two days. Responses to Benzoyl benzoyl ATP (BzATP; 100 μM) were measured in BAPTA-injected (~5 mM) oocytes using standard two electrode voltage clamp procedures. A single application of BzATP (1-30 s duration) in low divalent cation solution (in mM: 96 Na^+; 2 K^+; 5 HEPES; 0.1 Ba^{2+}; pH 7.5) produced a large inward current that reversed between -15 and -10 mV. This current slowly decayed over the course of several minutes. Upon complete recovery (10-15 min), further application of BzATP could not elicit inward current, suggesting that in oocytes BzATP responses completely desensitize. We then investigated "pore" formation in response to brief (1 s), long (30 s) or repeated (1s every 30 s or every 12 min) applications of BzATP by monitoring conductance and reversal potentials of the BzATP-activated current in solutions containing Na^+ or the large organic cation N-Methyl-D-Glucamine (NMDG). Following the exchange of Na^+ solution with NMDG solution, BzATP-activated inward current fell dramatically and reversal potentials shifted from -15 mV to around -50 mV. Thus, NMDG did not appear to serve as an effective current carrier. In earlier work, HEK 293 cells transfected with plasmids encoding P2X7 receptors expressed a BzATP-activated cation current as well as a BzATP-activated, non-selective conductance or "pore" which was permeant to NMDG and other large cations (Surprenant *et. al. Science*, **272**: 735, 1996). In the present study, the failure of NMDG to carry BzATP-activated currents suggests that, in Xenopus oocytes, P2X7 receptors do not form non-selective conductance pathways or "pores." One interpretation of these results is that pore formation may be controlled by factors in addition to P2X7 receptors. (Supported by NIH)

W-Pos116

P2X4 CHANNELS CLONED FROM A *XENOPUS* OOCYTE cDNA LIBRARY.

((**P. F. Juranka**, A. Haghighi*, E.Cooper* and C.E. Morris))
Neuroscience, Loeb Institute, Ottawa Civic Hospital, Ottawa, Ontario K1Y 4E9; *Department of Physiology, McGill University, Montreal, Quebec H3G 1Y6 Canada

In an attempt to identify genes that code for mechanosensitive cation (MSC) channels like those in oocytes, we screened a *Xenopus* oocyte cDNA library for homologs to purinergic cation channels (P2x1 and P2x2). We isolated several cDNAs, each having a 67% predicted amino acid identity to rat P2x4 channels. Insofar as *Xenopus* oocytes have been used by us (i.e. AH, EC) and by others for expressing rat P2x channels and show no endogenous ATP currents, the presence of these P2X cDNAs in a *Xenopus* oocyte library is surprising. But in terms of our motivation for doing the screen - the hypothesis that P2X and MSC channels are members of a common family - the outcome is encouraging.

Using 2 electrode voltage clamp, we tested oocytes for endogenous functional ATP channels, recording from >100 oocytes while rapidly perfusing ATP at up to 300μM. None yielded detectable ATP-evoked currents. However, when we "overexpressed" the cloned *Xenopus* cDNAs (P2x4Xa ...P2x4Xe) individually in *Xenopus* oocytes by nuclear injection, we obtained ATP-evoked currents for all but P2x4Xb. The currents, evoked by ATP concentrations from 1-100 μM, were up to 1μA, exhibited slow desensitization in the continuous presence of agonist, reversed near 0 mV and rectified slightly at positive potentials. P2x4Xb, the cDNA which did NOT give rise to ATP-evoked currents when overexpressed in oocytes (n=54), differs from the other *Xenopus* P2x4 cDNAs by 3 amino acids in the coding region. This suggests a crucial role for these residues in gating the channel by ATP and makes the P2x4Xb clone an interesting candidate for a stretch channel component.

Supported by grants to CEM and to EC from MRC, Canada

W-Pos117

PATCH CLAMPING OF SUB-MICRON DIAMETER LIPOSOMES CONTAINING SYNAPTIC VESICLE MEMBRANE FRAGMENTS.

((**Marie L. Kelly**, Dennis A. Przywara, and Dixon J. Woodbury)) Departments of Physiology and Pharmacology, Wayne State University School of Medicine, Detroit, MI 48201.

Many subcellular organelles are too small to patch clamp. Cholinergic synaptic vesicles from *Torpedo californica* fall into this category with a diameter of only 90 nm. Previously, Yakir and Rahamimoff [J. Physiol. (Lond) 485:683-97, 1995] reported patching enlarged (20 μm) synaptic vesicles made by fusing many synaptic vesicles together. We have tried an alternate approach using liposomes. The method follows the same procedure we have used for making joined vesicles, by fusing synaptic vesicle membrane fragments with liposomes [Kelly and Woodbury, Biophys. J. 70:2593-99, 1996]. The diameter of these joined vesicles is 250-1000 nm. The tip of our patch pipettes are less than 1 μm. Thus, it is possible to patch a joined vesicle. We report the patching of joined vesicles that contain ion channels. Observed channels are compared to synaptic vesicle channels observed with the bilayer technique. Supported by NIH grant MH50003 to DJW.

W-Pos118

PROPERTIES OF THE HYPERPOLARISATION-ACTIVATED CATION CURRENT (I_H) IN TRIGEMINAL MESENCEPHALIC NUCLEUS (MNV) NEURONES.

((**B. Khakh** & G. Henderson)) Pharmacology Dept, University of Bristol, BS8 1TD, Bristol, UK. (Spon by S. Tripathi)

I_H is the neuronal equivalent of the hyperpolarisation-activated mixed cation current, I_f, found in the heart. In neurones I_H is known to be important in the regulation of firing rate and in nociceptive neurones modulation of I_H is thought to be the mechanism by which prostaglandins can cause and opioids can reduce hyperalgesia. In the present study we have made patch-clamp recordings from MNV neurones in brain slices and characterised I_H in these cells.

Application of hyperpolarising membrane potential steps activated non-inactivating time-dependent inward currents. Steady state and tail current analysis showed that the I_H activation curve had a V_{50} of around -93mV. Activation and deactivation kinetics of I_H were dependent on the prepulse potential and could best be described by two exponentials, for maximal activation τ_1 and τ_2 were 100 and 750ms, and for maximal de-activation τ_1 and τ_2 were 50 and 300ms. The extrapolated reversal potential of I_H was -55mV. Extracellular Cs^+ (IC_{50} 400μM) rapidly and reversibly blocked I_H in a use-independent manner. The I_f inhibitor ZD7288 (1μM), blocked I_H with maximal effect occurring after 10min of drug exposure; this effect did not reverse with washout periods of 10min. Bath application of forskolin (10μM) to elevate $[cAMP]_{ic}$ caused an increase in the maximum amplitude of I_H by ~ 10% and a ~ 5mV +ve shift in the V_{50} for activation.

Thus we have demonstrated the existence of I_H in proprioceptive neurones. Modulation of I_H may be functionally important in the processing of proprioceptive information.

W-Pos119

EFFECT OF INHIBITORS OF MITOCHONDRIAL AND SARCOPLASMIC RETICULUM CA^{2+} UPTAKE ON THE TIME COURSE OF CA^{2+}-ACTIVATED Cl^- CURRENTS IN SMOOTH MUSCLE CELLS. ((I. A. Greenwood, R. M. Helliwell and W. A. Large)) Dept. Pharmacology and Clin. Pharmacology, St George's Hospital Medical School, London.

In rabbit portal vein smooth muscle cells the decay of Ca^{2+}-activated Cl^- currents recorded as either a spontaneous transient inward current (STIC) or tail current (I_{tail}) following depolarization -induced Ca^{2+} influx can be fitted by a single exponential with a time constant (τ) of 70-90 ms at -50 mV. The decay has been proposed to reflect slow gating of the chloride channel and not the kinetics of the activating Ca^{2+} pulse. We have investigated the effect of two blockers of the sarcoplasmic reticulum Ca-ATPase, thapsigargin and cyclopiazonic acid (CPA) as well as the mitochondrial protonophore carbonylcyanide m-chlorophenylhydrazone (CCCP) on the kinetics of STICs and I_{tail} in rabbit portal vein smooth muscle cells. Currents were recorded from cells held at -50 mV and bathed in a K^+-free solution containing 10 mM Ca^{2+} with a pipette solution which contained 126 mM CsCl and 200 $\mu g\ ml^{-1}$ amphotericin. Thapsigargin (100 nM) and CPA (10 μM) inhibited STICs gradually over a 4 min period with a small decrease in the STIC τ before they were abolished. CPA applied for 6 min reduced the amplitude of I_{tail} evoked by a 100 ms depolarization to +10 mV from 85±29 to 41±14 pA (n=10) but had no significant effect on the τ value. 2 μM CCCP evoked a transient inward current of 11±0.5 pAand inhibited STICs within 30 s. CCCP (0.5 - 2 μM) did not affect τ (n=6). 2 μM CCCP initially prolonged the decay of I_{tail} with a maximal increase from 102±9 ms to 320±50 ms (n=5) followed by a rapid reduction in the amplitude of I_{tail} and I_{Ca}. All effects were reversible within 5-8 min. These data suggest that the decay of the STIC is not determined by the kinetics of Ca^{2+} uptake into either the sarcoplasmic reticulum or the mitochondria. However, the decay of I_{tail} is influenced by the uptake of Ca^{2+} into the mitocondria which probably refects the greater increment in $[Ca^{2+}]_i$ obtained in the sub-sarcolemmal space following depolarization-evoked Ca^{2+} influx. This work was supported by The Wellcome Trust.

W-Pos120

α_1-ADRENOCEPTOR ACTIVATION OF A NON-SELECTIVE CATION CURRENT IN VASCULAR SMOOTH MUSCLE BY 1,2,-DIACYL-*SN*-GLYCEROL (DAG) VIA A PROTEIN KINASE C INDEPENDENT MECHANISM.
((Helliwell, R.M. and Large, W.A.)) Department of Pharmacology and Clinical Pharmacology, St George's Hospital Medical School, London, SW17 ORE, U.K.

In the present work we have investigated the transduction mechanisms linking the α-adrenoceptor to the cation channel. We have previously shown that a G-protein coupled to phospholipase C (PLC)is involved[1]. This study examined the possibility that DAG, one of the products of PLC hydrolysis, was responsible for the activation of I_{cat}. Membrane currents were measured in freshly dispersed smooth muscle cells using the whole-cell recording technique.
The intracellular solution contained (mM) CsCl 18; CsAsp 108; $MgCl_2$ 1.2; HEPES 10; glucose 11; BAPTA 10 (Ca^{2+} concentration was buffered to 14nM), pH 7.2 and the extracellular solution contained NaCl 126; $CaCl_2$ 1.5; HEPES 10; glucose 11, pH 7.2, nicardipine (3μM) and niflumic acid (100μM).
Bath application of 10μM of the membrane permeable DAG analogue, 1-oleoyl-2-acetyl-*sn*-glycerol (OAG), evoked a noisy inward current with a mean amplitude of 11 ±1pA (n=27). The I/V relationship of this current was identical to noradrenaline-activated I_{cat} and had a similar reversal potential (E_r). When 126mM Na^+ was reduced to 63mM, substituting with Tris, the mean E_r for noradrenaline-activated current was shifted from +11 ±2mV to -8 ±1mV(n=4) and the OAG-activated current was shifted from +9 ±0.7mV to -7 ±0.9mV (n=3). Bath application of phorbol 12, 13 dibutyrate (100nM -5μM) did not evoke I_{cat} in 35 out of 35 cells whereas subsequent addition of OAG evoked I_{cat} with a mean amplitude of 10 ±2pA (n=5). Furthermore chelerythrine (5μM) had no effect on the subsequent activation of I_{cat} by OAG (mean amplitude= 10±1pA, n=5). Bath application of a DAG lipase inhibitor (10μM, RHC80267) also activated I_{cat} (5 out of 11 cells, mean amplitude = 10 ±3pA). These data indicate that DAG activates I_{cat} via a novel protein kinase C independent mechanism.
1) Helliwell, R.M. and Large, W.A. *J. Physiol.* 494, 53-54P

W-Pos121

LOSS OF FENAMATE-ACTIVATED K^+ CURRENT IN EPITHELIAL CELLS FROM FREEZE-WOUNDED RABBIT CORNEAS ((**M.A. Watsky**)) Dept. of Physiology and Biophysics, University of Tennessee, Memphis, TN, 38163.

The corneal epithelium provides a barrier between the external environment and the cornea. It is frequently injured through physical or chemical insult. In previous work we characterized a nonselective cation current in corneal endothelial cells dissociated from corneas injured by scraping or freezing that was not present in cells from uninjured corneas. We have also reported the loss of a delayed-rectifying K^+ current in keratocytes dissociated from freeze-wounded corneas. The purpose of the current study was to determine if the whole-cell currents of corneal epithelial cells also change during corneal wound healing. Rabbit corneas were wounded using three different procedures: epithelial scraping, heptanol exposure, or freezing with a liquid N_2 cooled probe (all rabbits were anesthetized). The epithelium was allowed to heal for 12-48 hours, at which time the animals were sacrificed and the epithelial cells dissociated from the cornea. Whole-cell currents were examined using the amphotericin-perforated patch technique. As has been reported previously, the primary whole-cell current in epithelial cells from uninjured corneas was a fenamate-activated K^+ current. In epithelial cells from scrape and heptanol wounded corneas, this conductance was generally unchanged. In all cells examined from freeze-wounded corneas, however, the fenamate-activated current was absent or significantly attenuated. The wound-selective nature of the K^+ current loss in epithelial cells may be the result of the death of the underlying keratocytes and endothelial cells that results only from the freeze-wounding procedure.

Supported by NIH Grant #R29EY10178

W-Pos122

DIVERSITY OF CHANNELS GENERATED BY DIFFERENT COMBINATIONS OF EPITHELIAL SODIUM CHANNEL (ENaC) SUBUNITS. ((C. M. McNicholas and **C. Canessa**)). Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, CT 06520-8026. (Spon. by W. Boron). ENaC is a multimeric protein formed by three homologous subunits: α, β and γ, that are expressed at different levels in various epithelia. We have examined the functional properties of channels formed by association of α with β; and of α with γ in injected oocytes using two-electrode voltage clamp and patch-clamp techniques. We found that $\alpha\beta$ channels differ from $\alpha\gamma$ in the following properties: a) the Na^+/Li^+ permeability ratio of $\alpha\beta$ is less than one, while of $\alpha\gamma$ is 1.7; b) the apparent affinity for external Na^+ and Li^+ of $\alpha\beta$ is lower (K_m 350 mM and 360 mM respectively) than of $\alpha\gamma$ (K_m 35 mM and 50 mM respectively); c) the single channel conductance of $\alpha\beta$ (4.4 pS for Na^+ and 4.2 for Li^+) is smaller than $\alpha\gamma$ (5.1 pS for Na^+ and 10.8 for Li^+); d) the half-inhibition constant (K_i) of amiloride is 20-fold higher for $\alpha\beta$ than $\alpha\gamma$, while the guanidinium K_i is equal for both types of channels. To identify the protein domains involved in amiloride binding, we constructed chimeras that contained the N-terminus of γ and the C-terminus of β. Channels formed by co-expression of α with the various chimeras were examined. We identified a stretch of 15 amino acids, immediately precedent the second transmembrane domain of the β subunit, as the domain conferring lower amiloride affinity to $\alpha\beta$ channels.

W-Pos123

THE CHARACTERISTICS OF THE Na/Ca EXCHANGE IN MOUSE PANCREATIC B-CELLS AND ITS POSSIBLE INFLUENCE ON B-CELL ELECTRICAL ACTIVITY.
((**K. Bokvist,** D. Gall and A. Herchuelz)) Islet Cell Physiology, NN7-Symbion, Fruebjergvej 3, Copenhagen, Denmark (Spon. by Sören Peter Olesen)

We have used a combination of the patch-clamp technique and microfluorometry to characterize the Na/Ca exchange of the pancreatic β-cell. Intracellular Ca^{2+} ($[Ca^{2+}]_i$) transients were generated by voltage-clamp depolarizations and the removal of extracellular Na ($[Na^+]_o$) led to a reduced rate of Ca-extrusion at $[Ca^{2+}]_i$ levels above 1 μM. At peak $[Ca^{2+}]_i$ levels the $[Na^+]_o$-dependent component accounted for 35% of the Ca^{2+} removal. The replacement of $[Na^+]_o$ by choline or sucrose led to the disappearance of a small (3-4 pA) inward tail current following the depolarization. The $[Na^+]_o$-dependency of this tail current indicate that it reflects the Na/Ca exchange and that the exchanger carries a net charge. This $[Na^+]_o$ dependent current was further studied in whole-cell experiments with $[Ca^{2+}]_i$ buffered to 3.5 or 14 μM and other ion permeabilities blocked. The exchange current had a linear whole-cell current-voltage relationship with a conductance of 0.03 or 0.14 nS and reversal potentials of -55 or -22 mV, respectively. Removal of $[Na^+]_o$ or the inclusion of 10 μM XIP (exchanger inhibitory protein) in the pipette solution blocked this current. Given the Na and Ca concentrations used and the reversal potentials found our data suggest that the β-cell Na/Ca exchange has a 3Na:1Ca stoichiometry and is electrogenic. Furthermore, the β-cell Na/Ca exchange contribute to Ca extrusion at high $[Ca^{2+}]_i$ levels and generate exchange currents that may modulate β-cell electrical activity.

W-Pos124

EFFECT OF ANTISENSE INHIBITION OF Na/Ca EXCHANGE ON CYTOSOLIC Ca^{2+} OSCILLATIONS IN PANCREATIC ß CELLS. ((F. Van Eylen, J. Albuquerque and A. Herchuelz)) Department of Pharmacology, Université Libre de Bruxelles, Brussels, Belgium.

In the pancreatic ß cell, Na/Ca exchange (NCX) displays a quite high capacity but has remained difficult to investigate because of the absence of specific inhibitors. In order to better characterize the role of NCX in the ß cell, phosphorotioated antisense oligonucleotides (AS-oligos) targeted against the Na/Ca exchanger were used to repress the expression of the exchanger in pancreatic islet cells. NCX activity was evaluated by measuring cytosolic free Ca^{2+} concentration $[Ca^{2+}]_i$ in single ß cell using fura 2. Exposure of pancreatic ß cells to 500 nM of the AS-oligos for 20 to 24 hrs inhibited NCX activity by about 55 % as measured by the inhibition of the increase in $[Ca^{2+}]_i$ provoked by extracellular Na^+ removal. In contrast, control oligos (non-sense and mismatched) did not affect NCX activity. In the absence of AS-oligos pretreatment, 82 % of islet cells exposed to 11.1 mM glucose displayed increases in $[Ca^{2+}]_i$, the increases being either oscillatory (55 %) or sustained (45 %). After AS-oligos pretreatment, only 31 % of islet cells responded to glucose and 33 % of the responding cells displayed $[Ca^{2+}]_i$ oscillations that, in addition, were altered quantitatively and qualitatively. The present study shows that AS-oligos may specifically inhibit Na/Ca exchange in pancreatic ß cells. Inhibition of Na/Ca exchange expression markedly alters glucose-induced $[Ca^{2+}]_i$ increases and oscillations.

W-Pos125

INVESTIGATION OF A CALCIUM PERMEABLE NONNMDA RECEPTOR IN CORTICAL NEURONS. ((**J.M. Mulvaney** and L.M. Nowak)). Dept. of Pharmacology, Cornell University, Ithaca, NY 14850.

Excitatory amino acid receptors are the predominant receptors mediating fast excitatory neurotransmission in the vertebrate central nervous system. In the present study, single channel recording techniques were used to examine ionotropic nonNMDA receptors activated by kainate (100-250 μM). Kainate activated multiple channels with different conductances in outside-out patches excised from cultured mouse cortical neurons (ED 14.5) maintained in a standard sodium-containing solution (in mM: 150 NaCl, 2.8 KCl, 1.0 $CaCl_2$, 10 HEPES-NaOH and 0.3 μM TTX; pH 7.3). To ensure that the currents under investigation are due to activation of nonNMDA receptor-channels all experiments were done in the presence of 20 μM bicuculline and an NMDA antagonist (200 μM APV or 500 μM αAA). In addition, the composition of the pipette solution (in mM: 75 CsAc, 75 CsCl, 10 HEPES-KOH, 10 EGTA-Ca^{2+}; pH 7.2) was such that the estimated E_{Cl} was -22 mV while the estimated E_{Cation} was approximately 0 mV. One channel activated by kainate had an unusually large conductance (~60 pS). The permeability of nonNMDA receptor/channels to calcium ions was investigated due to the importance of calcium influx in developmental cellular processes such as synaptogenesis and gene expression. Kainate-activated channels were recorded in outside-out patches bathed initially in the standard sodium-containing solution and then in a monovalent free/high calcium solution (in mM: 115 mM $CaCl_2$, 10 HEPES-$Ca(OH)_2$ and 0.3 μM TTX; pH 7.3). The PCa:PNa for the kainate-activated channel with the largest conductance in the high calcium solution was ~ 3.0. NMDA channels recorded under the same conditions verified that the kainate-activated channels observed in the high calcium solution were definitely not NMDA channels.

W-Pos126

ACTIVATION OF THE IONIC CURRENT BY APAMIN.
((A.V. Povstyan,* V.V. Rekalov,* and M.F. Shuba#))
*Bogomoletz Inst. of Physiology, Kiev, Ukraine,
#Dept. of Biophysics, T.Shevchenko Kiev University, Kiev, Ukraine.

Apamin is a widely known blocker of small conductance Ca^{2+}-activated K^+ channels (SK). But our previous investigations showed that it also has an influence on holding current, and purpose of our study was to investigate the nature and mechanism of this effect. We used whole-cell mode of the patch-clamp technique for the investigation of apamin action on holding current in single smooth muscle cells isolated from guinea pig taenia coli. The intracellular concentration of EGTA was 0.3 mM. Application of apamin (0.5 μM) led to deflection of holding current into inward direction and increased membrane conductance. This effect was observed in normal physiological salt solution (PSS) as well as in PSS, contained Cs^+ and Co^{2+} instead of K^+ and Ca^{2+} respectively. At the same time substitution of Na^+ in PSS by choline or *N*-methyl-D-glucamine prevented this effect. Effect of apamin reappeared upon Na^+ returning into PSS. The obtained results suggested that apamin activate Na^+ or some Na^+-dependent ionic (except K^+ and Ca^{2+}) conductance of membrane and that depolarization of the cell, caused by apamin could be explained by the activation of ionic current, but not inhibition of SK conductance.

W-Pos127

FUNCTIONAL EXPRESSION OF TRPC1, A HUMAN HOMOLOG OF THE *DROSOPHILA* TRP PROTEIN. ((**W.G. Sinkins** and W.P. Schilling)) Rammelkamp Center for Education and Research, MetroHealth Campus, Case Western Reserve University, Cleveland, OH 44109-1998.

Previous studies in our laboratory have shown that the Drosophila TRP protein forms functional, cation-permeable channels when expressed in Sf9 insect cells. Electrophysiological measurements and studies with the fluorescent Ca^{2+} indicator fura-2 have shown that TRP channels are activated by thapsigargin (TG), are blocked by low concentrations of La^{3+} or Gd^{3+}, and are more permeable to Ca^{2+} than to Ba^{2+}. Sensitivity to TG appears to reside in the proposed cytoplasmic COOH-terminal domain of TRP. TRPC1 is a human homolog of TRP that, in terms of amino acid composition, is 61% similar, 38% identical to its insect counterpart. The human protein is shorter than Drosophila TRP, 759 aa *versus* 1275 aa; TRPC1 lacks a large part of the TRP COOH-terminal domain. Contrary to expectations, reports from other labs suggest that when expressed in mammalian cells, TRPC1 forms cation channels that are activated by depletion of the internal Ca^{2+} store [X. Zhu *et al.*, Cell 85:661-671, 1996; C. Zitt *et al.*, Neuron 16:1189-1196, 1996]. We have functionally expressed TRPC1 using the Sf9/baculovirus system. Fura-2 experiments reveal that TRPC1 appears to form constitutively active cation channels. As compared to control cells, in TRPC1-expressing cells $[Ca^{2+}]_i$ is increased from 106 ± 6 nM to 186 ± 15 nM at 28 hr postinfection, and from 123 ± 10 nM to 240 ± 31 nM at 42 hr postinfection. In control cells, Ba^{2+} permeability is low under basal, unstimulated conditions and is markedly increased following application of 200 nM TG; the stimulated Ba^{2+} influx is completely blocked by 0.5 uM La^{3+}. In TRPC1 cells, basal Ba^{2+} influx is higher than that of control cells and is relatively insensitive to 0.5 uM La^{3+}. Furthermore, TG-stimulated Ba^{2+} influx in TRPC1-expressing cells actually is decreased in comparison to control. In Sf9 cells TRPC1 channels thus appear to be unaffected by depletion of internal Ca^{2+} stores, but might act as a dominant negative mutant to inhibit endogenous store-operated channels (SOCs). Therefore TRPC1 may not be a SOC *per se*, but might be a subunit which alone can form channels in Sf9 cells; mammalian cells may provide additional subunits that confer TG sensitivity. (Supported by GM 52019; WGS is the recipient of an AHA-Ohio Affiliate Fellowship).

W-Pos128

MOLECULAR DETERMINANTS OF Ca^{2+}-BLOCKAGE AND Ca^{2+}-PERMEATION IN CYCLIC NUCLEOTIDE-GATED (CNG) CHANNELS.
((**R. Seifert**, E. Eismann, U.B. Kaupp)) Institut für Biologische Informationsverarbeitung, Forschungszentrum Jülich, 52425 Jülich, Germany.
(Spon. by K.W. Koch)

CNG channels from rod and cone photoreceptor cells and from olfactory sensory neurons are permeable to both monovalent and divalent cations. While passing through CNG channels external Ca^{2+} blocks the current of the more permeant Na^+- and K^+-ions. Analysis of the voltage-dependence of this blockage reveals distinct binding efficiencies and Ca^{2+}-permeation properties for the different CNG channel types. Since the amino acid sequences within the pore region of CNG channels are highly conserved, other parts of the protein must have an influence on the permeation properties.To determine the molecular basis of the differences we are currently investigating chimeric channel proteins and point mutants by patch clamp methods. We are using blockage of monovalent current by external Ca^{2+} as a probe for the interaction of Ca^{2+}-ions with CNG channels. Our results suggest that the "inner core"-region including S5, pore and S6 determines Ca^{2+}-blockage and -permeation in CNG channels. The transmembrane regions S5 and S6 appear to modulate Ca^{2+}-blockage and -permeation by imposing different protonation patterns onto glutamate residues within the pore. Protonation of these glutamates thus controls the interaction of distinct CNG channels with Ca^{2+}-ions.

W-Pos129

Ca^{2+} PERMEABLE CHANNEL ACTIVATED DURING APOPTOSIS OF A PROSTATIC CANCER CELL LINE. ((**Andrés Gutiérrez**, Lucía García, Jaime Mas-Oliva and Agustín Guerrero)). Dept. of Bioenergetics, IFC, UNAM, México city and Dept. of Biochemistry, CINVESTAV-IPN, Mexico city. (Spon. by Sergio Sánchez-Armass)

Apoptosis depends on Ca^{2+} influx in some cell types like thymocytes or prostatic cells. However, it is has not been identified those Ca^{2+} permeable channels of the plasma membrane responsible for this influx. We have combined microfluorometry of fura-2 and patch clamp to study the activation of Ca^{2+} permeable channels during ionomycin induced apoptosis in a prostatic cancer cell line (LNCaP). Ionomycin at concentrations that induced apoptosis, first depleted internal stores and later activated an endogenous and electrophoretic Ca^{2+} influx pathway. Using the cell attached recording of patch-clamp technique we have identified a Ca^{2+} independent, Ca^{2+} permeable, non-selective, cation channel of 20 pS conductance activated during the Ca^{2+} influx stage and strictly associated to conditions that induced cell death. Membrane depolarization reduced both apoptosis and Ca^{2+} influx suggesting that the 20 pS channel is responsible for the Ca^{2+} influx required to trigger cell death in this type of cells. This is the first report of the activation of a Ca^{2+} permeable channel that seems to be involved in triggering apoptosis in prostatic cells.

W-Pos130

A NOVEL LARGE CONDUCTANCE, NONSELECTIVE CATION CHANNEL IN UNDIFFERENTIATED PC12 CELLS. ((**Alejandro M. Dopico** and Steven N. Treistman)) Dept. Pharmacol. & Mol. Toxicol., and Program in Neurosci., Univ. Mass. Med. Sch., Worcester, MA 01655.

In PC12 cells, a model system widely used in neurobiology, several ion channels have been identified: L- and N-type of voltage-gated Ca^{++}-channels, purely voltage-gated K^+ channels and Ca^{++}-dependent K^+ channels, both tetrodotoxin-sensitive and insensitive Na^+ channels, nicotinic acetylcholine receptor channels, and ATP-gated cation channels. Nonselective cation channels are a heterogenous group, in which nicotinic acetylcholine receptors, NMDA-receptors, cyclic nucleotide gated-channels, gap junction channels, bacterial porins, the subgroup of Ca^{++}-dependent nonselective cation channels, and mechano-gated cationic channels are included. In the present work, using single channel recordings from inside-out patches, we characterize a new type of nonselective cation channel in undifferentiated PC12 cells. This channel shows unique features: similar permeability for K^+, Na^+, and Li^+, and a large unitary conductance (~300 pS in symmetric 145 mM K^+). Channel activity is not sensitive to $[Na]^+$ but to $[Ca^{++}]_e$, and is not modified by applying suction to the patch (up to 75 mmHg). The pharmacology of this channel is currently under study. Although its physiological role remains unknown, the combination of Na^+ permeability and high conductance might provide an ionic pathway for membrane depolarization in the PC12 cell, even if the total number of channels in the cell is low. Support: NIH grant AA05512 (SNT).

W-Pos131

ION SELECTIVITY OF A MEMBRANE CONDUCTANCE ACTIVATED BY REMOVAL OF EXTRACELLULAR CALCIUM IN *XENOPUS* OOCYTES

((Y. Zhang, D.W. McBride, O.P. Hamill)) Physiology and Biophysics, UTMB, Galveston, TX 77555.

In *Xenopus* oocytes, a large inward current (~ 10 uA at -60 mV) develops when external Ca^{++} is removed. This current has often been interpreted as reflecting a non-specific membrane breakdown. However, in 1995 two groups (J. Physiol. 484, 953; Pflug. Arch. 429, 820) independently reported that it was a reversibly activated current (Ic) which was inhibited by external Ca^{++}. Despite some consistent results, the two groups reached opposite conclusions regarding the ion selectivity of Ic. While one group concluded it was cation selective, the other concluded it was anion selective. To resolve this controversy we measured the ion selectivity of Ic under conditions when external cation and anion concentrations were changed simultaneously. Shifts in reversal potential following both increase and decrease in external ion concentration are consistent with Ic being predominantly cation selective but with a significant permeability to Cl^- (P_K:P_{Na}:P_{Cl} of 1:1:0.2).

W-Pos132

AN INWARD AND OUTWARD RECTIFIER CONDUCTANCE IN MOUSE OSTEOCLASTLIKE CELLS, GROWN FROM SPLEEN CELLS IN COCULTURE WITH OSTEOBLASTIC CELLS.

((D.L. Ypey[1], R. DeGrooth, P.J. Nijweide)) Depts. of Physiology[1] and Cell Biology, Leiden University, Leiden, The Netherlands.

A recently developed osteoclast-osteoblast (OCL-OBL) coculture model for the in vitro study of osteoclast differentiation combines mouse spleen cells with fetal mouse calvarial OBL. OCL precursor cells in the spleen cell suspension, when seeded on a 3-day OBL culture, proliferate, fuse and differentiate to OCL in 7-12 days. Here we report on the expression of voltage activated ion channels in these cocultured OCL. OCL of 7-14d cocultures on glass were voltage-clamped at 21-24 C in the whole-cell (WC) patch-clamp mode, using an extracellular solution ECS (mainly NaCl) in the bath and an intracellular solution ICS (mainly KCl) in the pipet. OCL were identified from their multinuclear (2-10) morphology and by neutral red staining. 13 WCs obtained from 4 cocultures showed an inward rectifier K^+ (IRK) conductance (G_{IRK}, 10->100nS), rapidly activated (within 10ms) by hyperpolarization below its reversal potential $E_r = E_K$, also with ICS in the bath. In 7 of these WCs we also found an outwardly rectifying conductance (G_{or}, 20->100nS), which rapidly (within 10ms) activated at $V_m > 0$mV, had an $E_r \sim 0$mV and was not affected by extracellular K^+. No other conductances were found. G_{IRK} and G_{or} strikingly resemble a G_{IRK} and chloride conductance G_{or} in OCL from rat or mouse bone marrow, found by others. These results open the way to study the role of G_{IRK} and G_{or} in OCL differentiation and activity.

W-Pos133

CELL TO CELL COUPLING AND ELECTRICAL ACTIVITY ON MOUSE PANCREATIC ISLETS OF LANGERHANS.

((E. Andreu, B. Soria and J.V. Sanchez-Andres)) Departamento de Fisiologia & Instituto de Neurociencias. Universidad de Alicante, 03080 SPAIN.

Pancreatic beta cells are grouped in islets and coupled through gap-junctions. When coupled in the islet these cells show a typical bursting pattern. Coupling in this system is very important as far as isolated cells are unable to oscillate. The membrane conductances of the cell and the intracellular agents are not enough to explain the oscillatory behaviour. Characterization of coupling conductances at different glucose levels and during different electrical patterns has been performed in order to elucidate the role of coupling in this system. Double intracellular recording of beta cells in intact mouse islets of Langerhas was done. Coupling conductances were studied at substimulatory and physiological ranges of glucose in neighbour cells at close distances. Coupling conductance increases as a function of glucose arriving to a maximum level defined on the active phase of the oscillatory pattern. Increasing the glucose concentration up to 20 mM makes the islet to remain more time on active phase, but the relative coupling conductance does not increase. Different coupling domains were defined along the whole islet in relation with the decay of the propagated voltage deflections. Synchronicity between cells belonging to different coupling domains was studied. In the most part of the cells (90%) of the islet a perfect synchrony was observed even if there is no recordable coupling. We hypothesize a role for cell to cell coupling on the arising of oscillatory patterns and syncrony of the system. Small changes on relative coupling conductance can perturb the whole system dynamics thus modifying the global electrical response of every cell on the islet.

W-Pos134

CHANNEL BLOCK BY THIOL REAGENTS IN A CYSTEINE REPLACEMENT MUTANT OF CONNEXIN46.

((A. Pfahnl, B. L. Moss and G. Dahl)) Department of Physiology and Biophysics, University of Miami, School of Medicine, Miami, FL 33101.

Cysteine replacement mutagenesis has identified two positions in the first transmembrane domain of connexins as contributors to the pore lining of gap junction hemichannels (Pfahnl et al. Biophys. J. 70, A31). Oocytes expressing mutant cx46 with cysteines in positions (34 or 35) exhibit a membrane conductance which is sensitive to extracellularly applied thiol reagents. Dye flux studies have suggested that the thiol reaction in these cysteine mutants restricts the pore size of the gap junction hemichannels. Single channel analysis corroborates this conclusion. Patch clamping of inside-out patches from oocytes expressing wild type cx46 yields channels with a complex voltage dependence as described by Trexler et al. (PNAS 93, 5836, 1996). At -30 mV the single channel conductance is 330 pS in symmetric potassium gluconate solutions. These channels are pH-dependent and close with acidification. Channel activity is unaffected by thiol reagents. The cystelne replacement mutant cx46 L35C yields channels that are both voltage- and pH-dependent like the wild type channels. They differ, however, in their single channel conductance, which is 270 pS. Upon application of maleimido-butyryl-biocytin (MBB, MW 537) to the patches the channel conductance is reduced to about 20% of the full open state. This modification is irreversible as expected from the nature of the maleimide reaction. The modified channels can still be gated by voltage and pH. These findings are consistent with a steric block of the channel pore by the thiol reagent.

W-Pos135

CONNEXIN CHANNEL ACTIVITY MODULATION BY CYCLIC NUCLEOTIDE ANALOGS, pH BUFFERS & MAGNESIUM IONS

((C.G. Bevans and A.L. Harris)) Biophysics Department, Johns Hopkins University, Baltimore, MD 21218.

The effects of nucleotide analogs, pH buffers, and divalent cations on liposome-reconstituted connexin (Cx) channel activity were determined. Cx channel activity was assessed as large changes in liposome sucrose permeability (*J. Membr. Biol.* 109:243). Cx channel activity was inhibited by exposure prior to reconstitution of detergent-solubilized Cx to nM etheno-cAMP or N-methylanthraniloyl-cGMP, both fluorescent analogs of the cellular second messengers. At this level cAMP and cGMP, but not other nucleotides, completely inhibit Cx channel activity (1996 ASCB abstract, *in press*). The data show that the high-affinity regulatory interaction by purine cyclic nucleotides with Cx is mimicked by these fluorescent analogs. These will be used to determine the binding constants by changes in fluorescence anisotropy upon binding (*Anal. Biochem.*, 224:330, 1995).

It was found that heteromeric Cx26/Cx32 (from mouse liver) channel activity significantly increased with increased pH over the range 5-9 when 10mM amino-sulfonate buffers (HEPES, MES, or TAPS) were present. Surprisingly, half-maximal increase in channel activity was at a pH value equal to the pKa of each buffer. pH5-9 solutions containing maleate or Tris, both organic weak-acid buffers with pKa's in the same range, did not modulate activity. An earlier study (*Mol. Biol. Cell*, 6:1106a, 1995) showed that homomeric Cx32 (from rat liver) was pH-insensitive in HEPES buffer. This suggests that Cx26, but not Cx32, confers to the channels a modulation mediated by the amino-sulfonate buffers; Cx26-containing channels are sensitive to the protonation state of these buffers, not to [H+] directly. The chemical basis for this modulation is being explored.

300μM ionic calcium or magnesium in the presence of 100μM each of EDTA and EGTA did not affect Cx channel activity in the channel activity assay. When [Mg++] was raised to 1 mM there was a complete inhibition of channel activity; raising [Ca++] to 1mM did not affect Cx channel activity. This suggests a low affinity interaction between Cx channels and magnesium cations. Support:NIH GM36044

W-Pos136

EFFECT OF SARCOPLASMIC RETICULUM (SR) CALCIUM CONTENT ON SR CA RELEASE ELICITED BY SMALL DEPOLARIZATIONS IN FROG CUT SKELETAL MUSCLE FIBERS. ((P.C. Pape)) *Département de physiologie et biophysique, Faculté de médicine Université de Sherbrooke, Sherbrooke (Québec) Canada J1H5N4.*

To assess whether Ca coming from an open SR Ca release channel can influence its own open state or that of nearby channels, the EGTA-phenol red method (Pape et al., 1995, *J. Gen. Physiol.*, **106**, 259-336) was used to measure SR Ca release and the Ca content of the SR ([CaSR]) over a range of resting [CaSR] ($[CaSR]_R$). The pulse protocol consisted of small voltage-clamp depolarizations ranging from -80 to -57 mV and lasting 400 to 150 ms, which were interspersed by 400 ms intervals at the holding potential of -90 mV; the protocol ended with a strong depolarization in order to estimate $[CaSR]_R$. Pulses to -80, -75, -72, -69, and -66 mV were repeated, respectively, 16, 16, 8, 4, and 2 times during the protocol and averaged in order to improve the signal to noise ratio. Because the rate of SR Ca release was small, [CaSR] remained closed to $[CaSR]_R$ during the small depolarizations. The depletion-corrected rate of SR Ca release vs [CaSR] data were reasonably well-fitted by straight lines with intercepts close to zero. In the two experiements done, there was a 78% and a 79% reduction (average over range of voltages) in the depletion-corrected rate of SR Ca release associated with a change in [CaSR] from 900 to 200 μM. This reduction was approximately the same for pulses between -75 and -57 mV (resolution was inadequate for -80 mV). Intramembranous charge movement was not significantly changed over this range of [CaSR] values. Two possible explanations for these results are that SR Ca release channels are regulated by [CaSR] or that there are binding sites accessible to Ca exiting the SR which increase the open time or conductance of voltage-activated channels and/or which activate channels in the vicinity of a voltage-activated channel. In favor of the latter explanation, earlier studies showed that SR Ca release is significantly reduced by high concentrations of fura-2 in the myoplasm (see p. 379-380, Jong et al. 1995, *J. Gen. Physiol.*, **106**, 259-336). *Supported by FRSQ and by MRC grant MT-12552.*

W-Pos137

LOCAL CONTROL MODEL OF CALCIUM RELEASE IN SKELETAL MUSCLE ((M.Stern, G. Pizarro and E.Ríos)) NIA, Univ. of Montevideo and Rush University. Chicago, IL 60612.

In skeletal muscle SR Ca^{2+} release remains under stable control by membrane potential at all times. One of its putative mechanisms, Ca^{2+}-induced Ca^{2+} release (CICR), is instead intrinsically unstable. We sought to solve the paradox using "local control" models (Stern, Biophys. J. 1992) in which the coupling of release channels by means of short-lived nanoscale $[Ca^{2+}]$ gradients, combined with the stochastic gating of the channels, stabilize CICR. We have examined the channel dynamics of a simple model of Ríos and Pizarro (NIPS, 1988) with alternating voltage-controlled (V) and calcium-controlled (C) release channels arranged in a staggered double row at the triad junctions. V channels are assumed to be allosterically coupled to sarcolemmal voltage sensors (Ríos *et al*, J.Gen.Physiol. 1993), while C channels have a gate opened by binding of two Ca^{2+}, and an inactivation gate closed by binding of one Ca^{2+}. The channels are coupled instantaneously by steady-state local Ca^{2+} diffusion gradients, and effects of background $[Ca^{2+}]$ are ignored. The model (30 C +30 V channels) is equivalent to a single Markov process with 10^{48} states. Monte Carlo simulation of this process showed that the model can qualitatively reproduce the kinetics of release, including its peak and its plateau, stable control of release by voltage, and the bell-shaped dependence of the peak/plateau ratio on voltage. The model did not reproduce incremental inactivation of release as a function of voltage ("Simon's paradox", Pizarro and Ríos, Biophys. J. 1994).

W-Pos138

BDM INHIBITS Ca RELEASE INDEPENDENTLY OF CHARGE MOVEMENT IN FROG MUSCLE ((R. De Armas, S.Gonzalez, G. Brum and **G. Pizarro**)) Fac. de Medicina, Montevideo, Uruguay.

Butanedione monoxime (BDM) suppresses the Iγ component of intramembraneous charge movement (ICM) and Ca release in frog muscle (Hui and Maylie, 1991, De Armas et al. 1993). We studied the effect of 10 mM BDM on ICM and Ca transients in voltage clamped frog cut fibers. In stretched fibers (that lacked Iγ) the maximun peak SR Ca permeability (P_{SR}) attained at maximally activating clamp depolarizations was reduced to 50% and the steady P_{SR} to 58%. The charge moved by the highest pulse that did not elicit Ca current was not significantly reduced. In slack fibers with a 5mM EGTA internal solution showing a distinct hump in the ICM current, peak P_{SR} was reduced to 54 % and steady P_{SR} to 65%. In slack fibers with 10 mM BAPTA in the myoplasm to abolish Iγ, maximun charge was not reduced. We conclude that Ca release suppression by BDM is mostly beyond the voltage sensing step, and that both kinetic components of P_{SR} are equally suppressed.
Funded by CONICyT.

W-Pos139

A Ca FRACTION IN SKELETAL MUSCLE CONSERVED IN 0 Ca RINGER. ((**Brian A. Curtis**)) University of Illinois College of Medicine at Peoria, Peoria, IL 61656

^{45}Ca efflux into 0Ca, 10Mg, 3EGTA Ringer solution can be described by the same two compartment model as ^{45}Ca efflux into Ringer. The rate of loss of the fast compartment was unchanged. 0.38 ± .15 pmol Ca/fiber (n=8) more Ca^{+} was associated with the slow compartment throughout the efflux into 0 Ca. Since the slowly exchanging compartment (SR) took up the same amount of ^{45}Ca during the preceding influx period, this conserved Ca must be a portion of the fast compartment which did not exchange with Mg. 0.38 pmol Ca/fiber represents 10 Ca/DHPR or 4Ca/+ charge moved, and might be Ca bound within the ionic channel of the L type Ca channel of the transverse tubular wall. After 1hr in 0Ca, a K^{+} contracture doubled the time constant of efflux in the 6 bundles tested. These bundles were then paralyzed until the bundle was returned to Ca containing Ringer. The conserved component is very similar in magnitude to the 0.36±.15 pmol Ca/fiber (n=54) which transfers from the fast to the slow compartment coincident with contracture. Instantaneous release of all this 0.38 pmol Ca into the spaces between the rows of feet, could rise the Ca concentration to 8 mM. Any kinetic scheme would lower [Ca] into a range which would activate CICR from RyR channels. I suggest that E-C coupling via CICR in 0 Ca solutions can be explained by this conserved Ca fraction.
Supported by American Heart Association, Illinois Affiliate

W-Pos140

MYOPLASMIC pH AND Ca^{2+} CONCENTRATION DURING A MALIGNANT HYPERTHERMIA EPISODE ((J.R. Lopez[12] N. Linares[1], H. Padrino[1], G. Cordovez[1], **P.D. Allen**[2].)) [1]Centro de Biofisica y Bioquemica, Instituto Venezolano de Investigationes Ceintificas, Caracas, Venezuela. and [2]Department of Anesthesia, Brigham and Women's Hospital, Boston, MA, 02115

Malignant Hyperthermia (MH) is a pharmacogenetic syndrome associated with a dysfunction in intracellular calcium regulation. We measured, *in vivo*, the intracellular calcium concentration ($[Ca^{2+}]_i$) and intracellular pH (pH_i) in muscle fibers from control (Yorkshire) and MH susceptible (Poland China) swine using double barreled Ca^{2+} and pH selective microelectrodes. In control fibers the resting $[Ca^{2+}]_i$ was 110±10 nM and pH_i was 7.08±0.01 (n=14), while in MH susceptible muscle $[Ca^{2+}]_i$ was 380±20 nM* and pHi was 7.00±0.01* (n=15) (*p<0.01). Exposure to 2% halothane in control swine did not induce a significant change in either $[Ca^{2+}]_i$ or pH_i. However, exposure of MH susceptible swine to 2% halothane induced a significant increase in $[Ca^{2+}]_i$ to 6800±260 nM* that was followed by a decrease in pH_i to 6.82±0.07* (n=11). Administration of Dantrolene reverted the elevation of $[Ca^{2+}]_i$ and the decrease in pH_i to levels similar to those seen in control swine. These results indicate that MH skeletal muscle has a higher $[Ca^{2+}]_i$ and lower pHi than control skeletal muscle. The acidification of the intracellular medium may be related to the higher $[Ca^{2+}]_i$. During the MH episode there was a further acidification of the myoplasm, which appears to be related to the substantial elevation of $[Ca^{2+}]_i$. Dantrolene was able to revert the increase in $[Ca^{2+}]_i$ and decrease pH_i observed during the MH episode. These findings provide support for the idea that acidification of the intracellular medium in MH skeletal muscle is related to the high resting $[Ca^{2+}]_i$.

Supported in part by Angelini Pharmaceuticals.

W-Pos141

ALTERED VOLTAGE DEPENDENCE OF CALCIUM RELEASE IN MYOTUBES OF MALIGNANT HYPERTHERMIA SUSCEPTIBLE PIGS

((**B. Dietze**, H.M. Eichinger*, W. Erhardt+, W. Melzer)) Dept. Applied Physiology, University of Ulm, D-89069 Ulm, *Experimental Center Thalhausen, TU Munich, D-85402 Kranzberg, +Institute of Experimental Surgery, TU Munich, D-81675 Munich, Germany (Spon. by A. Herrmann-Frank)

Malignant hyperthermia (MH), a hypermetabolic state triggered by volatile anesthetics, can originate from a variety of point mutations in the skeletal muscle ryanodine receptor. Pigs carrying a mutation (Arg615Cys) homologous to one of those found in humans show similarly altered responsiveness of skeletal muscle. Gallant et al. (Muscle Nerve 19: 68-73, 1996) demonstrated a lowered voltage threshold for contracture activation in cultured myotubes of MH-susceptible (MHS) pigs. To further pursue the effect of Arg615Cys on excitation-contraction coupling we applied the whole cell patch clamp technique in combination with fura-2 fluorescence measurements to primary myotubes of pigs homozygous for this mutation. Cells were loaded by diffusion from the patch pipette with an intracellular solution containing 200 μM fura-2 and 5 mM EGTA to prevent saturation of the indicator dye. Depolarization-induced Ca^{2+} transients were obtained by recording the fluorescence intensity at 510 nm during excitation at 380 nm, which was divided by the resting fluorescence at 358 nm excitation (isosbestic point of fura-2) measured 1 s before each depolarizing pulse. Simultaneously the L-type Ca^{2+} inward current was recorded. The measurements showed no change in the voltage dependence of Ca^{2+} conductance in MHS myotubes in accordance with Gallant et al. (Muscle Nerve 19: 450-455, 1996) while a significant negative shift (ca. 15 mV) in the voltage dependence of Ca^{2+} release activation could be noted. We conclude that the mutant ryanodine receptor in porcine MHS muscle is not only more sensitive to certain Ca^{2+}-releasing ligands but also to the physiological control input from the transverse tubular voltage sensor (dihydropyridine-receptor).

W-Pos142

REGULATION OF MALIGNANT HYPERTHERMIA-SUSCEPTIBLE (MHS) Ca^{2+} RELEASE CHANNELS BY Mg^{2+} ((**E.M. Balog**, N.H. Shomer, B. Fruen, J.R. Mickelson, and C.F. Louis)). University of MN, St. Paul, MN 55108.

The sarcoplasmic reticulum (SR) Ca^{2+} release channel (CRC) is regulated in a biphasic manner by cytoplasmic Ca^{2+}, suggesting a high affinity activating and a low affinity inhibitory Ca^{2+} binding site. The concentration of Ca^{2+} required to inhibit single MHS channel percent open time 50% is significantly greater than that required to inhibit normal channels, indicating that the MH mutation affects the inhibitory Ca^{2+} binding site in intact muscle. It has been proposed that Mg^{2+} binds to the low affinity CRC inhibitory site and MHS results from a decreased Mg^{2+} inhibition of SR Ca^{2+} release. To examine this possibility, Mg^{2+} was added to the cis side of single MHS (n=10) and normal (n=9) CRCs in lipid bilayers. Single channel open time decreased, although the half-inhibitory Mg^{2+} concentration was not significantly different for the two channel types (normal: 680±100 μM, MHS: 525±95 μM). Similarly, inhibition of ^{3}[H]ryanodine binding by Mg^{2+} was not significantly different between MHS and normal SR. Rather than acting in an additive manner at the low affinity site, millimolar Ca^{2+} reversed the Mg^{2+}-dependent inhibition of single CRC activity. Thus, Mg^{2+} appears to inhibit CRC activity by competing with Ca^{2+} at the high affinity activating site. Further, the sensitivity of the CRC to Mg^{2+} inhibition is not significantly affected by the MHS mutation, indicating MH does not result from a decreased Mg^{2+} inhibition of the CRC. Supported by NIH grant GM-31382.

W-Pos143

THE DURATION OF THE INTRACELLULAR Ca^{2+} TRANSIENT INCREASES WITH INCREASED SARCOMERE LENGTH IN INTACT FROG SKELETAL MUSCLE FIBERS. ((**D.R. Claflin**, R. Vandenboom, D.L. Morgan* & F.J. Julian)) Department of Anesthesia, Brigham & Women's Hospital, Boston, MA 02115; *Monash Univ., Australia.

Most studies of the time-course of the intracellular free Ca^{2+} transient (ICT) in skeletal muscle are performed on preparations that are stretched to long sarcomere lengths to minimize motion artifact. Others have reported reductions in the amplitude of the ICT with increasing sarcomere length. The effect of stretch on ICT duration has not, however, been reported. The purpose of this study was to determine the effect of sarcomere length on the duration of the twitch ICT. Experiments were performed on intact fibers isolated from the tibialis anterior muscle of the frog (*R. temporaria*) and maintained at 3.0°C. The ICT was monitored using the Ca^{2+}-sensitive fluorescent dye mag-fura-2 excited at wavelengths of 344nm and 375nm and reported here as the ratio of the two responses (344/375). Twitch contractions were elicited by electrical stimulation once every 120s. Recordings were made at sarcomere lengths of 2.8μm, 2.2μm, 3.4μm, and again at 2.8μm, in that order. Relative to the pre-stimulus (resting) level, the peak reached by the ICT was 69.9±1.7, 65.7±1.8 and 46.2±3.8 $\times 10^{-3}$ ratio units (mean±SEM, n=4) at sarcomere lengths 2.2μm, 2.8μm and 3.4μm, respectively. The ICT duration, defined as the interval between the 50% rise time and the 50% fall time, was 17.3±0.5ms, 23.3±0.9ms and 25.1±1.2ms at sarcomere lengths 2.2μm, 2.8μm and 3.4μm, respectively. The original and repeated measurements at 2.8μm were not different from each other. We thus confirm that the ICT associated with twitch contractions are reduced in amplitude and conclude that they are increased in duration as sarcomere length is increased. One possible mechanism for the increase in ICT duration with reduced filament overlap is a decrease in the Ca^{2+} buffering capacity of the thin filament due to Ca^{2+}-myosin cooperativity. Supported by NIH HL35032 (FJJ).

W-Pos144

SUPERCHARGING COMMAND PULSES PERMIT RAPID VOLTAGE STEPPING OF T-TUBULE MEMBRANES IN SKELETAL MUSCLE FIBERS
((J.L. Vergara and A.M. Kim)) Department of Physiology, UCLA, Los Angeles, CA 90095
(Spon. by J.R. Monck)

In voltage-clamp, there exists a lag between application of a command voltage and the resulting membrane potential change due to limitations in the charging time of the membrane capacitance imposed by resistances in series. Since this lag is considerable in the case of the T-tubule (TT) membranes of skeletal muscle fibers, we investigated the effects of supercharging pulses in attaining fast changes in the TT membrane potential. Single frog skeletal muscle fibers were mounted and voltage-clamped in a triple vaseline gap chamber. External and internal solutions were chosen to block Cl^-, K^+, Na^+, and Ca^{2+} conductances. The standard triple-gap voltage-clamp circuitry was modified to permit the addition of a supercharging component to the command voltage. This consisted of a compound exponential function with three decay time constants adjustable in duration and amplitude. *Weighted-average* TT potential changes were recorded by staining with 0.5-5 μM concentrations of the potentiometric fluorescent indicator di-8-ANEPPS. Epifluorescence signals were recorded with a PIN photodiode, using a 610 nm barrier filter, a 440-490 nm band-pass excitation filter, and a 510 nm dichroic mirror. Stimulation with conventional voltage-clamp steps yielded fluorescence transients of ~6% ΔF/F per 100 mV, with kinetics representative of the slow charging process of TT membrane capacitance. Typical fluorescence records indicated that the average potential of the TT system reached 70% of the command voltage within ~5 ms, and settled to 95% of the command voltage within ~12 ms. Supercharging allowed to boost the speed of the *weighted-average* TT potential changes such that they achieved 95% of the command voltage in < 2 ms. These rapid response times may be useful in unmasking kinetic and voltage-dependent properties of excitation-contraction coupling previously confounded by temporally compromised input functions to the system. Supported by NIH AR25201.

W-Pos145

COMPONENTS OF CHARGE MOVEMENT IN SKELETAL MUSCLE FIBERS OF THE FROG. ((F. Francini, C. Bencini and R. Squecco)) Dpt. di Scienze Fisiologiche, Università di Firenze, 50134 Firenze, Italy.

Intramembrane charge movement (ICM) shows two components (Q_β and Q_γ) in normally polarized skeletal muscle fibers of the frog. The aim of this work was to further investigate the components of ICM to better evaluate their kinetics and the relation between them. Single cut fibers were dissected from semitendinosus muscle and voltage clamped in a double Vaseline-gap chamber. All ionic currents were minimized except for calcium current (I_{Ca}). ICM and I_{Ca} were detected at 16 °C using voltage pulses of different duration (0.5-5000 ms) from a holding potential (HP) of -100 mV. Linear capacitive and leak currents were subtracted using scaled current in response to depolarizing control pulses of 20 mV from a HP of 0 mV. I_{Ca} activation was evaluated by a multiexponential fit to I_{Ca} time course recorded by 5-s pulses. For each voltage pulse, the time course of ICM was determined by subtracting the calculated time course of I_{Ca} activation from the original current traces. Two transient ICM components were observed in the voltage range of Q_β: one fast, never described before, resolved in about 2-3 ms and the other in 30 ms. Q_γ threshold was at about -55 mV. The time integral of ICM vs. voltage plot showed another component too elicited above 20-30 mV. These results agree with the presence of four components of ICM. This involves at least seven microscopic transitions. But, the time integral of ICM, time to peak and time constants vs. voltage curves can be better reproduced by using a model that involves twelve microscopic transitions.
Supported by grant n° 625 from Telethon-Italy.

W-Pos146

FAST CALCIUM CLEARANCE BY PARVALBUMIN DURING SINGLE TWITCHES IN SKELETAL MUSCLE FIBERS. ((**C.Caputo**, P.Bolaños and A.L.Escobar)). Centro de Biofisica y Bioquimica. Instituto Venezolano de Investigaciones Cientificas, IVIC, Caracas.Venezuela

We have studied the effects of low frequency stimulation, 0.1 to 5 Hz, on the fluorescence signals time course in normal and thapsigargine, TG, poisoned muscle fibers. Intact muscle fibers, dissected from *Leptodactylus insularis* were loaded with Fluo-3 AM. In single twitches the decay phase of fluorescence signals shows an early fast exponential component (τ = 15 to 20 ms), followed by a slower one that keeps basal fluorescence, F, (and basal $[Ca^{2+}]_i$), at higher than normal levels for tens of ms (Cannell, 1986; Caputo et al. 1994). During repetitive stimulation, restoration of resting $[Ca^{2+}]_i$ is attained with increasingly longer delays depending on stimulation frequency and duration. However in normal fibers, after few twitches (< 10), the increase in resting $[Ca^{2+}]_i$ is relatively small (0.1 to 0.4 μM) and is not accompanied by appreciable changes in the kinetics of the early exponential decay phase. After TG treatment, repetitive stimulation causes a much larger change, (0.5 to 1.5 μM), in the basal $[Ca^{2+}]_i$ which is accompanied by a sizeable decrease in the rate of decay of the early component (τ = 20 to 40 ms). Interestingly the increase in the basal fluorescence and the increase in the time constant of the early component are reversed with rest, and could be abolished by loading the fiber with EGTA-AM. This indicates that the slowing of the early exponential component is caused by the increase in the resting Ca^{2+} levels, and is not a direct consequence of the SR Ca-ATPase poisoning, suggesting a primary role for intracellular buffers, other than the SR, in the fast clearance of Ca^{2+} loads necessary for relaxation of single twitches. These results could be reproduced by a distributed mathematical model in which saturation of parvalbumin during repetitive stimulation causes the slowing of the decay phase. Supported by CONICIT grant S1-95000 493.

W-Pos147

EFFECTS OF 4-CHLORO-M-CRESOL ON EC COUPLING IN VOLTAGE-CLAMPED MUSCLE FIBRE SEGMENTS
((W. Melzer, A. Struk)) Dept. Applied Physiology, University of Ulm, D-89069 Ulm, Germany

4-Chloro-m-cresol (4-CmC) has been shown to be a very potent activator of the calcium release channel (ryanodine receptor) in skeletal muscle (e.g. Herrmann-Frank et al., BBA 1289, 31-40, 1996). We investigated the effects of low concentrations of 4-CmC on excitation-contraction coupling in voltage-clamped cut muscle fibres of the frog. Intracellular Ca^{2+} concentrations were measured with fura-2 in the presence of 15 mM internal EGTA to prevent saturation of the fluorescent indicator. After correction for kinetic filtering due to non-instantaneous binding of fura-2 the time course of the rate of release was estimated using the method of Baylor et al. (J.Physiol. 344, 625-666, 1983). Ca^{2+} transients were elicited with 50ms depolarizing pulses; intramembrane voltage sensor charge movements were determined simultaneously by using a -P/4 paradigm; their voltage dependence was fitted with a standard Boltzmann relation. 5 μM 4-CmC, applied externally, caused an instantaneous increase of the resting Ca^{2+} concentration by 67% at a holding potential of -80mV, indicating a steady activation of the release channels even in the absence of electrical stimulation. 4-CmC also potentiated the voltage-dependent Ca^{2+} release and altered its time course. The release rate at the end of a pulse to 0 mV increased by about 50%, while the potentiation of the peak release rate was significantly smaller (26 %). Maximum charge movement increased by 19 % while the parameters describing its voltage dependence remained unchanged. A similar potentiation of early release was caused by 0.5 mM caffeine. This concentration has been reported to induce a loss of the voltage control after short (10-20 ms) depolarizing pulses, resulting in an continuing increase of Ca^{2+} transients after repolarisation (Simon et al., Biophys. J. 55, 793-797, 1989). Under our conditions, no signs of delayed deactivation could be detected neither with 4-CmC nor with caffeine.

W-Pos148

NUMERICAL SIMULATION AND IMAGE ANALYSIS OF THE Ca^{2+}-TURNOVER IN SKINNED SKELETAL MUSCLE FIBRES. **((D. Uttenweiler, C. Weber and R.H.A. Fink)) II. Institute of Physiology, University of Heidelberg, INF326, 69120 Heidelberg, Germany.**

A numerical model was developed for the simulation of the spatial and temporal time course of Ca^{2+}-ion movement in caffeine induced calcium transients of skinned fibre preparations. These preparations allow direct diffusional access to the myoplasm via the perforated sarkolemma, while the sarcoplasmic reticulum (SR) remains fully intact, and calcium transients can be induced by application of a high dose of caffeine. The results were compared with experimental data which we obtained from fluorescence photometry and imaging measurements using the ratiometric dye fura-2. Small bundles (one or two fibres) of Balb-C EDL mouse muscle, chemically skinned with Saponin (50 μg/ml) were mounted in a perfusion chamber and the fluorescence signal was recorded simultaneously with isometric force.

The model with cylindrical symmetry calculates the radial profile of Ca^{2+}-ion concentration by solving the diffusion equation for Ca^{2+}-ions and various mobile buffers with a finite difference algorithm. The various rate equation terms for Ca^{2+}-buffering (mobile and immobile buffers), SR Ca^{2+}-release and uptake were also incorporated into the model, thus allowing the direct investigation of the different contributions of these processes to the overall Ca^{2+}-transient.

As the fast initial rise in Ca^{2+}-concentration at the beginning of the transient is mainly governed by the initial amount of Ca^{2+} stored in the SR and the buffering of Ca^{2+}-ions by calsequestrin, the relaxation is mainly due to the diffusional loss of Ca^{2+}-ions through the permeabilized sarcolemma. The rate of relaxation is strongly influenced by EGTA, as the main Ca^{2+}-buffer in the release solution as well as by the amount of Ca^{2+}-uptake by the SR Ca^{2+}-pump. **(Supported by the EC and the Minister for Science and Research Baden-Württemberg.)**

W-Pos149

DYNAMIC REGULATION OF CALCIUM HANDLING BY PHOSPHORYLATION AND DEPHOSPHORYLATION IN MOUSE SINGLE FAST-TWITCH AND SLOW-TWITCH SKELETAL MUSCLE FIBERS ((Y.Liu, E.G.Kranias,M.F.Schneider)) Dept.Biochem.and Molec.Bio.,Univ.of MD School of Med.,Baltimore,MD 21201 and Dept.Pharmacol and Cell Biophysics, Univ.of Cincinnati,OH 45267

The effects of phosphorylation and dephosphorylation on calcium release and calcium removal were examined in normal mouse fast-twitch flexor digitorum brevis (FDB) and slow-twitch soleus fibers and in phospholamban knockout mouse soleus fibers. Calcium transients were measured using the fluorescent calcium indicator fura-2 in intact dissociated single fibers. [Ca^{2+}] transients (Δ[Ca^{2+}]) triggered by 1 ms duration electrical stimulation were calculated from fluorescence ratio records corrected for non-instantaneous reaction of fura-2 with Ca^{2+} (k_{on}=3.66 x 10^8 $M^{-1}s^{-1}$; k_{off}=26 s^{-1}). In normal FDB and soleus fibers dibutyryl-cAMP had little effect on the peak of calcium transients or on the speed of decay of calcium transients. In FDB fibers cAMP-dependent protein kinase inhibitor H-89 significantly decreased the amplitude of calcium transients but had no effect on the decay of calcium transients. In soleus fibers H-89 decreased the amplitude of calcium transients and slowed the decay of calcium transients. In both FDB and soleus fibers dibutyryl-cAMP reversed these effects of H-89. In soleus fibers from phospholamban knockout mice H-89 decreased the amplitude of calcium transients and dibutyryl-cAMP antagonized this decrease, whereas H-89 or dibutyryl-cAMP had no effect on the decay phase of the calcium transients. Our results, combined with results by others, suggest that there is a high basal state of phosphorylation of both the calcium release channel and phospholamban and that phosphorylation/dephosphorylation modulate calcium release in both fast- and slow-twitch skeletal muscle fibers but modulate calcium uptake only in phospholamban-expressing slow-twitch fibers. (Supported by AHA Maryland Affiliate, MDA and NIH:NS33578)

W-Pos150

THE DIFFERENTIAL EFFECT OF ANTRACENE ON THE ELECTRICAL PROPERTIES OF NORMAL AND ENLARGED MAMMALIAN MUSCLE.
((**F.W. Banks** and Y. Chen.)) Department of Biological Sciences, Chicago State University. Chicago, Il. 60628

We have previously reported that the input impedance, as measured by two electrode intercellular recording, increases by seventy three percent while the space constant increased thirty three per cent in enlarged rat muscle. This anomaly we explained, resulted from a possible change in the population of chloride channels in the membrane. Normally, there is a mixed population of both high conducting and low conducting chloride channels. When the muscle fiber enlarges, there is a shift to low conducting channels. This shift would account for our anomalous result and maintain the safety factor for transmitter release. By increasing the input impedance, the normal amount of neurotransmitter could bring about muscle fiber excitation in an enlarged muscle fiber. To test this hypothesis we applied 1μM 9-antracenecarboxylic acid to control and enlarged muscle fiber. If antracene blocks differentially the two channel types, the differential block should evidence itself in an increased or decreased percent block in the test muscle over control. We will show that this is indeed the case. With the introduction of antracene to the bath of five week post operative control muscles, the input impedance increased 300%. When introduced into the bath of five week enlarged muscle fibers, the input impedance increased 340%. Suggesting that the low conducting channels have a greater affinity for antracene. These results suggest that the muscle fiber size regulation controls are closely linked with the membrane channel control mechanisms. (Supported by NIH grant GM 08043)

MUSCLE ULTRASTRUCTURE AND PHYSIOLOGY

W-Pos151

EFFECTS OF SMOOTH MUSCLE MYOSIN LIGHT CHAIN 2 EXCHANGED INTO SKINNED SKELETAL FIBERS. ((R. Levine, Z. Yang, H.L. Sweeney)) Allegheny Univ. & Univ. of PA, Philadelphia, PA

Phosphorylation of myosin light chain 2 (LC2) regulates smooth muscle contraction and modulates that of skeletal muscle. In the latter, the ordered, helical array of myosin heads on relaxed thick filaments may be due to charge interactions between the positively-charged N-terminus of LC2 and other myosin sites, which is attenuated by phosphorylation of serine 15. This would increase S1 mobility, and chances of actin-myosin interaction, accounting for the observed leftward shift of the pCa-tension relation. Disordering of the myosin head array on filaments seen after LC2 phosphorylation supports this suggestion. If it is correct, we would expect that LC2 with fewer positively charged N-terminal residues would produce permanent (a) potentiation of tension at low Ca^{2+} and (b) disorder of thick filament structure; the converse would be seen with more positively-charged residues in the same region. Here we demonstrate the effect of exchanging smooth muscle LC2 (SmLC2) (expressed in E. coli), which has a more positively-charged N-terminus than skeletal muscle LC2 (SKLC2), for the latter in permeabilized rabbit psoas fibers. A modified procedure enabled 100% LC2 exchange. Thick filaments separated from exchanged fibers appeared more highly ordered than native ones under relaxing conditions. Potentiation of tension at low calcium was greater in hybrid fibers than native ones (with SkLC2) after LC2 phosphorylation. We are examining the effects of LC2 phosphorylation on filament structure in these fibers.

W-Pos152

LATTICE COMPRESSION OF FROG SKINNED MUSCLE FIBERS ON PHOTOLYSIS OF THE CAGED CALCIUM COMPOUND NP-EGTA
((**B.K.Hoskins**, P.J.Griffiths, C.C.Ashley and G.Rapp*)) Univ. Lab. of Physiol., Oxford, OX1 3PT, UK. *EMBL Outstation, DESY, 22603 Hamburg, Germany.

Single cut end sartorius muscle fibers from the frog (Rana temporaria) were skinned for 2 minutes in 1% Triton X-100, then bathed in a loading solution containing the caged compound o-nitrophenyl EGTA (Molecular Probes Inc.) at 3°C for 5 minutes until tension reached a plateau at ca. 20% of maximum isometric tension (144 $kN.m^{-2}$). The loading solution contained 3.12mM NP-EGTA, 1.18mM calcium, plus an ATP regenerating system (10mM creatine phosphate, 20 $units.ml^{-1}$ creatine phosphokinase). Fibers were flashed in air using a single U.V. pulse (pulse power, 240kW) from a xenon flash lamp (XF-10, G.Rapp Optoelektronik, Heidelberg). This resulted in a step liberation of 116μM bound calcium within 20μs of the flash (computer simulation using data from Ellis-Davies et al. (1996, Biophys. J., 70: 1006-1016)). A rapid tension increase was observed ($t_{½}$ ≈45ms). A second flash after 3s did not elicit any further tension increase, indicating that maximum isometric tension had been achieved. Equatorial X-ray diffraction patterns were simultaneously recorded (8keV, wire per wire detector, beamline X-13, DESY, Hamburg) with 10ms resolution. The intensities of the 10 and 11 reflections changed with a similar time course to that of isometric tension development, while lattice spacing decreased. This decrease, which was initially rapid ($t_{½}$ ≈29ms, 2.3nm) and preceded tension development, was followed by a slower lattice compression.

W-Pos153

EFFECT OF PHOSPHORYLATION OF C PROTEIN ON CROSS BRIDGES IN CARDIAC MUSCLE.((A. Weisberg, S. Winegrad)) Dept of Physiology, School of Medicine, University of Pennsylvania, Philadelphia, Pa. 19104

In cardiac muscle, C protein, a component of the thick filament, has 4 phosphorylation sites that are phosphorylated by PKA, PKC, and a Ca-calmodulin regulated kinase. Studies of the functional consequence of phosphorylation of C protein in whole cardiac myocytes have failed to produce unambiguous results. We have isolated natural thick filaments from young, euthyroid adult rat cardiac cells and examined the effect on structure of the filament produced by PKA mediated phosphorylation of C protein in the presence of inhibitors of phosphorylation of the light chain of myosin. The dimensions of negatively stained isolated filaments were measured in electron micrographs in control filaments and those with phosphorylated C protein. Optical diffraction patterns of the two populations of filaments were also examined. From the thickness of the filaments where there are cross bridges and the reflections on the 43 nm layer line in the diffraction pattern, the position of the cross bridges with respect to the axis of the filament can be estimated. Both the ends and the center of mass of the cross bridges were extended approximately 3 nm as a result of the phosphorylation. PKA treatment of cardiac cells from young euthyroid rats, in which the myosin contains entirely or almost entirely alpha myosin heavy chain (MHC) increases actomyosin ATPase activity. In old or hypothyroid animals, the isoform of MHC is predominately beta, and PKA phosphorylation does not increase ATPase activity. To determine if the extension of the cross bridge from C protein phosphorylation is required for the increase in ATPase activity, the effects on the structure of thick filaments from old and from hypothyroid hearts from C protein phosphorylation were examined. Phosphorylation of C protein did not extend the cross bridges in these filaments. These results indicate that the change in cross bridge structure produced by C protein phosphorylation may be required for the increase in ATPase activity from PKA activity. Additional data suggest that a decrease in the distance between the cross bridge and the thin filament is necessary but not sufficient to produce the increase in ATPase activity; presumably some additional structural change in the thick filament and/or thin filament is required. (Supported by NIH grant HL16010 to S.W.)

W-Pos154

CONFORMATIONAL DEPENDENCE OF CROSSLINKS INTRODUCED INTO SKELETAL MUSCLE TITIN. ((Vincent A. Barnett, Tamba Johnson*, Jennifer Hayes, and Cheryl A. Miller))Department of Physiology, University of Minnesota.

We have previously shown that incubation of full-overlap skinned rabbit psoas fibers with para-phenylenediamaleimide (pPDM) introduces crosslinks into the titin filaments that increase titin's resistance to stretch (Barnett & Schoenberg, 1992; Miller & Barnett, 1996). In our earlier reports we focused on the response of the muscle fibers to a large stretch (10% fiber length) after incubation with pPDM. In the current study we examine the titration of the crosslinking sites on titin with pPDM, the effect of the modification on the length-extension curve for single psoas fibers and the effect of treatment at sarcomeres where there is no overlap of the thick and thin filaments. The results at full-overlap show that the sites on titin are finite and saturable. The length-extension relationship when all sites are occupied is shifted to the left as should be anticipated. The length-extension curve shows hysteresis between the stretch and the release pathways for both control and pPDM-treated fibers. This is suggestive of a need for a recovery period as the titin attempts to refold after being strained. Stretch of the cells to non-overlap of the thick and thin filaments leads to modification of the sites responsible for the increase in stretch resistance without a change in the muscle's elastic behavior. Length-extension curves for cells treated while stretched are nearly identical to those of control fibers. Therefore, while the sites of modification are accessible at non-overlap sarcomere lengths, the conformation of titin under strain does not allow the cross-linkages to be formed. These studies are a part of our ongoing efforts to determine the molecular mechanism of the extensibility of this giant muscle protein. This work was supported in part by a grant from the Minnesota Medical Foundation (G0-8-95).*T. Johnson was supported by the Health Sciences Summer Research Opportunities Program.

W-Pos155

ACTIN-TITIN INTERACTION IN THE I-BAND OF RAT CARDIAC MYOCYTES. ((TROMBITÁS, K., GRANZIER, H.)) DEPARTMENT VCAPP, WASHINGTON STATE UNIVERSITY, PULLMAN, WA 99164-6520.

Recent ultrastructural studies have indicated that thin and titin filaments are laterally associated (Funatsu et al., 1993). This raises the possibility that dynamic interaction may take place between actin and titin along the entire length of the thin filament. Such interaction is expected to be weak in the region of the I-band where titin behaves elastically. On the other hand, titin may bind strongly to actin in the ~100 nm wide region adjoining the Z-line where titin is inextensible. To study the interaction between titin and actin, techniques for selective removal of actin from different regions of the I-band are needed. Here we report biochemical and ultrastructural studies in which a gelsolin fragment (FX-45) was used to extract actin from rat cardiac myocytes. By using immuno-electron microscopy we studied the epitope location of the anti-titin antibodies T12 and 9D10, as a function of sarcomere length, in both control and actin-extracted myocytes.

Depending on the extraction time, we could remove either the full actin filament, or just the segment of the actin filament that extends beyond the inelastic region of titin that adjoins the Z-line. The actin filament-free I-band contained titin filaments, as indicated by their immuno-reactivity with T12 and 9D10, with one filament extending from each thick filament.

T12 and 9D10 epitope behavior was unaffected by the limited extraction. After full actin extraction, the T12 epitope moved further away from the Z-line in sarcomeres longer than ~2.2 μm. Findings with 9D10 were consistent with the observed T12 behavior. The results suggest that titin's Ig-domains between the N-terminus and T12 epitope unfold at sarcomere lengths longer than ~2.2 μm, and that in control cells unfolding is prevented by the presence of actin.

Actin removal significantly affected slack sarcomere length. Slack sarcomere length of control cells was 1.85 ± 0.04 μm and decreased to 1.71 ± 0.05 after full actin extraction. This length reduction may be caused by contraction of the titin segment that becomes exposed following actin removal near the Z-line. In agreement with this is the obsevered movement of the T12 epitope towards the Z-line in sarcomeres shorter than ~1.9 μm. Thus, close to the Z-line, ttitin is not only attached to the actin filament, but it is also under tension.

W-Pos156

DIFFUSION OF ANTIBODIES INTO MECHANICALLY SKINNED MUSCLE FIBERS. ((**K. Trombitás***, M.L. Greaser#, and G.H. Pollack^)) *Central EM Laboratory, Medical University, Pécs, Hungary; #Muscle Biology Laboratory, University of Wisconsin, Madison WI 53706; ^Center for Bioengineering, University of Washington, Seattle WA 98195

Since diffusion is an essential feature of cell function, the movement of macromolecules in the cytoplasm has been a topic of some interest. In this study, we examined the penetrability of antibodies against myofibrillar proteins in skinned muscle cells. Single muscle fibers of frog tibialis anterior were mechanically skinned and labeled with IgG or IgM type antibodies in native or slightly fixed state. Both the axial and the radial movement of the antibodies was followed. Antibody localization was detected by electron microscopy, using indirect immunolabeling with or without silver intensification. Antibody diffusion was highly restricted. Only the outermost myofibril layers of the fiber were labeled. The sharp labeling gradient was different for the IgG and IgM type antibodies. IgG molecules could diffuse approximately twice as deep as the IgM molecules. Penetration showed neither an obvious time or concentration dependence: after prolonged labeling and elevated antibody concentration (up to 72 hrs; 1 mg/ml), the penetration depth did not increase significantly. Axial and radial diffusion distances were essentially the same. In both cases penetration did not exceed several micrometers. The cytoplasm appears to behave as a molecular filter. Large molecules are apparently retarded because of the limited pore size and possibly of further reduction because of pore size due to antibody binding. The absence of anticipated time or concentration dependence implies that antibody penetration into skinned muscle cells may involve something other than simple diffusion.

W-Pos157

ORIENTATIONS OF THE REGULATORY DOMAIN OF MYOSIN STUDIED BY SITE-DIRECTED SPIN LABELING OF REGULATORY LIGHT CHAINS IN SCALLOP MUSCLE FIBERS. ((Ingrid Brust-Mascher, Josh E. Baker, Leslie LaConte, Sampath Ramachandran, Renne Lu*, David D. Thomas)) *BBRI, Boston, MA 02114 and University of Minnesota, Minneapolis, MN 55455.

We have used electron paramagnetic resonance (EPR) to study orientations of the myosin light-chain binding domain. The native regulatory light chain (RLC) was extracted from scallop adductor muscle fibers, and the fibers were reconstituted with site-directed spin-labeled gizzard RLC. Several spin labels were used to modify a single cysteine on both native (Cys 108) and mutant (S28C and G165C) gizzard RLCs in order to define global orientations of the regulatory domain. The EPR spectra of some RLC/spin label pairs indicated stereospecific probe binding, with two resolved probe orientations. In muscle fibers exchanged with FDNASL-native gizzard RLC, the two components were equally populated in relaxation. Trapping weak-binding intermediate states with nucleotide analogs did not perturb this orientational distribution. During contraction there was a redistribution between the two components. We conclude that (1) the regulatory domain shows two distinct orientations, neither of which can be assigned to a single biochemical state, and (2) force generation involves the reorientation of the light-chain binding domain.

W-Pos158

MECHANISM FOR COUPLING FREE ENERGY IN ATPASE TO THE ACTIVE SITE OF MYOSIN. ((**S. Park**, K. Ajtai, C. R. Zayas and T. P. Burghardt)) Department of Biochemistry and Molecular Biology, Mayo Foundation, Rochester, MN 55905.

Acrylamide quenching of tryptophan 510 (Trp510) fluorescence in rabbit skeletal myosin subfragment 1 (S1) indicates the conformation of the probe binding cleft, containing the highly reactive thiol (SH1) and Trp510, in the presence of nucleotide or nucleotide analogs trapped in the active site of S1[1]. The Trp510 quenching efficiency of S1 shows that the probe binding cleft closes slightly in the presence of MgADPBeF_x and most tightly in the presence of MgADPVi in the following order MgADPBeF_x > MgADPAlF_4 > MgADPScF_x > MgADPVi. Correlation of the separation distance (*d*) of the *γ*-phosphate analog metal from the oxygen connecting it to the *β*-phosphate in ADP, to the extent of cleft closure, suggests that this distance in the nucleotide transition state determines the conformation of the probe binding cleft. The Trp510 quenching efficiency of S1 was also measured as a function of the base moiety of the vanadate trapped Mg-nucleotide diphosphate (MgNDPVi-S1), and as a function of the size of the divalent cation trapped in the active site of S1 with ADPVi. These experiments suggest that the changing conformation of ATP during hydrolysis, parameterized by the increasing separation distance (*d*), is communicated to the active site of S1 through protein-nucleotide contacts at the *γ*-phosphate and at the nucleotide base. Supported by NIH (AR 39288), AHA (GIA 930 06610), and Mayo Foundation.

1. Park et al., 1996, *Biochem. Biophys. Acta 1296*, 1-4.

W-Pos159

THE EFFECTS OF HYPERBARIC OXYGEN ON INJURED SKELETAL MUSCLE
((**Paul Gregorevic**, Alan Hayes, Ian Millar* and David A. Williams)) Muscle & Cell Physiol. Lab., Dept. of Physiology, The Univ. of Melbourne, * Hyperbaric Unit, Alfred Hospital, Prahran, Vic., Australia.

Despite lack of medical and scientific support, use of Hyperbaric oxygen (HBO) therapy (periodic inhalation of 100% oxygen at pressures > 1 atmosphere absolute), in the treatment of skeletal muscle injuries continues to grow, particularly among professional athletes. The aim of this study was to examine the effects of HBO upon functional aspects of skeletal muscle injury and recovery.
EDL muscles of young Sprague Dawley rats were injected under light anaesthesia with Bupivacaine, a myotoxic agent, to induce rapid, significant muscle damage. Half of these animals underwent three sessions a day of HBO therapy during the first two days, commencing approximately 2 hours post-injury, and two sessions a day every day thereafter until killed for testing. Each HBO session consisted of 90 minutes exposure to 100% O_2 at 2 ATA in a hyperbaric chamber, with daily sessions separated by 2 hours. STD (exposed only to room air) and HBO-treated animals were killed by diethyl ether overdose at 2, 4 and 7 days post-injury ($z = 9$ all groups). Bupivacaine injection caused a significant reduction (20-40%, $P < 0.0001$) in maximum isometric tension (P_o) of all muscles 2 days post-injury. By 4 days post-injury P_o had returned to control (pre-injury) levels, but only in HBO treated animals. After 7 days in room air tetanic force levels were still significantly depressed indicating a marked functional improvement following HBO treatment. We are presently determining whether HBO: i) minimised the extent of the initial damage events and/or ii) increased the rate of structural and functional recovery from damage, by investigating histochemical evidence of fibre damage, lipid peroxidation levels and PCNA staining of muscle precursor (satellite cell) proliferation. Supported by NH&MRC of Australia.

W-Pos160

THE EFFECT OF STRETCH ON THE ADAPTATION OF ADULT GUINEA-PIG VENTRICULAR MYOCYTES IN CULTURE: CYTOARCHITECTURE VISUALIZED BY CONFOCAL MICROSCOPY.
((**M. Horackova**)) Faculty of Medicine, Dalhousie University, Halifax NS B3H 4H7, Canada

Using laser confocal scanning microscopy and immunofluorescent markers we have investigated the effect of stretch (applied by varying the plating density) on the adaptation of adult guinea-pig cardiomyocytes in culture. Providing that the preparation of freshly isolated cardiomyocytes consists mostly (>80%) of rod-shaped, Ca-tolerant, and quiescent cells and these are plated under optimal conditions and density (10^5/cm^2), these myocytes have the following characteristics: 1) they remain elongated with regular ultrastructural characteristics and quiescent for several days; 2) within 10–14 days, they reestablish their cell-cell contacts and resume contractile activity, which becomes synchronous all through the confluent layers; 3) they retain their regular myofibrilar striation all through the adaptation to culture conditions without any sign of dedifferentiation or redifferentiation; 4) these characteristics are lost when the cells are plated at too low (<10^4/cm^2) or too high (2 x 10^5/cm^2) a density and they exhibit signs of dedifferentiation and the myofibrilar apparatus is restructured; 5) the cells resume normal activity and express atrial natriuretic peptide (ANP); 6) this activity seems to be directly related to the surface area of the myocytes in contact with the substrate (i.e. to the stretch of the myocytes). Long-term cultures of adult guinea-pig ventricular myocytes (alone or in their cocultures with cardiac neurons) provide a valuable model for studying the cellular and molecular regulation of myocardial function under acute or chronic effects of various intrinsic and extrinsic factors.

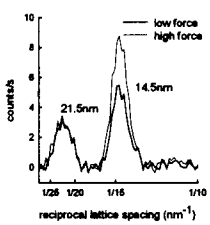
W-Pos161

ALTERED NUCLEAR CONTROL DOMAINS IN AGING SKELETAL MUSCLE.
((**C. E. Kasper** and L. V. Thompson)) School of Nursing, UCLA, Los Angeles, CA 90095; and, Dept. of Physiology, Univ. of Minnesota, Minneapolis, MN 55455

The nuclear domain (NCD)(i.e., the amount of cytoplasm associated with a single nucleus) has been characterized in adult fibers where it has been shown to be larger in fibers which express the fast myosin heavy chain (MHC) and low oxidative capacity, than in fibers which express the slow MHC and high oxidative capacity. It is unknown however, whether this ratio is modulated during with aging. The NCDs in Soleus (SOL) fibers from 12 and 30 month old rats were examined and compared to mature adult controls(CON; aged 6 mo). Enzyme histochemistry (pH 10.4, 4.6, 4.4, 4.2) revealed in the 30 mo. SOL a distribution of 92% type I (N = 586 fibers) and 8% type II (N = 52 fibers) as compared to CON of 83% type I and 17% type II. SDS-Page gels of MHC confirmed these data with 91% of the fibers (N = 423) expressing MHC type I and 9% co-expressing MHC I and MHC IIa. Overtime, there were no differences in fiber cross-sectional area or volume. However, the number of myonuclei / mm significantly increased from 154 ± 11 at 6 months to 280 ± 6 at 30 mo. and the NCD ($\mu m^3 \times 10^3$) decreased from a mean of 33 ± 2 at 6 mo. to 22 ± 7 at 12 mo. and 17±5 at 30 mo. of age. It has been shown that during altered loading, aged muscle appears to be less able to maintain contractile characteristics than CON adult rats. These data may indicate a decreased ability of each myonuclei to express sufficient mRNA to maintain the contractile protein systems resulting in the necessity to compensate by increasing myonuclear number and decreasing the size of the NCD. (Supported by NIH NINR/NIAMS NR 02922 to CEK; Am. Heart Asso.-MN, MN Medical Found., & Grant-in-Aid Univ. of MN to LVT)

W-Pos162

TENSION DEPENDENT STRUCTURAL CHANGES OF ACTIN-MYOSIN CROSS-BRIDGES IN RIGOR
((I. Dobbie[1], M. Irving[1], M. Reconditi[2], M. Linari[2], N. Koubassova[2], **G. Piazzesi**[2] & V. Lombardi[2])) [1]The Randall Institute, King's College London, London WC2B 5RL, U.K., [2]Dip. Scienze Fisiologiche, Università di Firenze, I-50134 Firenze, Italy (Spon. by F. Francini)



The effect of stretching a muscle fibre in rigor, where myosin heads are thought to be in a conformation corresponding to the end of the working stroke, was investigated by x-ray diffraction at SRS (Daresbury, UK). Single muscle fibres from tibialis anterior of Rana temporaria (2.1 μm sarcomere length, 4 °C) were stimulated to measure the isometric tetanic tension (T_0). Rigor was induced by MgATP depletion after permeabilization of the membrane (Linari et al., this meeting) and tension was repetitively increased from 0.1 T_0 up to a maximum of T_0 by slow ramp stretches (complete in 1 s). After 8 s the fibre was released to recover the low tension level. The intensity of the 14.5 nm meridional reflection, arising from the axial repeat of myosin heads, increased by ca. 50% for a tension change of 0.7 T_0 (Fig. 1, data from three fibres, total exposure 230 s). There was no significant change in the intensities of the other equatorial and meridional reflections or actin-based layer lines out to 5.1 nm. Similar tension changes produced by high frequency (3 kHz) sinusoidal oscillations in length changed the 14.5 nm reflection intensity of only 10-15% These results suggest that the structural change in the intensity of the 14.5 nm reflection produced by slow stretch cannot be solely attributed to elastic distortion. Reversal of the structural change occurring in the transition to the rigor state may also contribute.
Supported by EC, CNR (Italy) and MRC (UK).

MUSCLE MECHANICS I

W-Pos163

THE INFLUENCE OF INTERCELLULAR CONNECTIVE TISSUE ON SMOOTH MUSCLE SHORTENING.
((**R. A. Meiss**)) Indiana University School of Medicine, Indianapolis, IN 46202

In smooth muscle tissues, where the contractile cells are very small compared to the whole tissue, connective tissue forms an important part of the system for force transference. Some of the connective tissue is ordered longitudinally and transmits contractile force, and some is ordered radially and maintains tissue integrity. Previous work has hypothesized that this radial connective tissue also functions to limit the shortening of smooth muscle by resisting the increase in diameter at very short lengths. Here I report two further tests of this *radial constraint hypothesis*. To accentuate the effects of radial connective tissue, small bands cut from silastic tubing were placed around thin strips of canine tracheal muscle at a number of positions along its length. They were sized to fit loosely at L_o. The muscles were stimulated electrically to shorten under very light afterloads while longitudinal stiffness was measured and displayed as a function of instantaneous length. Compared with the strip in its unbanded condition, a constrained strip shortened significantly less, while developing considerably more length-dependent stiffness at longer lengths; deviations from control behavior were correlated with the internal diameter of the bands. Isometric length-tension curves showed a similar correlation with band diameter, with isometric force being significantly reduced below the critical length. To *reduce* the effects of radial connective tissue, muscle strips were subjected to mild collagenase digestion after their shortening properties were determined. The digested muscle shortened significantly more and did so with a smaller increase in stiffness at short lengths. Supported by the Dept. of OB/GYN, Indiana University School of Medicine.

W-Pos164

THE STIFFNESS VS. FREQUENCY CHARACTERISTICS OF SMOOTH MUSCLE.
((**G. Shue** and F.V. Brozovich)) Department of Physiology and Biophysics, Case Western Reserve University, Cleveland, OH 44106

To investigate the mechanical properties of smooth muscle, stiffness was measured during Ca^{2+} stimulated contractions of single β-escin permeabilzed smooth muscle cells isolated from the rabbit portal vein. Both at rest (pCa 9, 5 mM MgATP) and after Ca^{2+} activation (pCa 4), the force and length of single vascular smooth muscle cells were measured with a five second length perturbation sequence which combined several single cycle sine waves with fixed amplitude (< 0.4 % of cell length) and frequencies ranging from 1 to 32 Hz. The frequency response of muscle stiffness was calculated over 1 to 32 Hz with a 0.33 Hz resolution. To reduce the influence of passive properties, the stiffness was referenced to the stiffness in the relax state and the frequency responses calculated from five consecutive five second measurements were averaged. After Ca^{2+} activation, stiffness increased to 0.08 N/m as frequency increased from 1 to 5 Hz, then remained constant until 12 Hz, after which stiffness decreased and crossed zero between 15 and 20 Hz. The temporal resolution of the combined sine wave sequence demonstrates the this technique may be used to calculate the frequency response of smooth muscle stiffness at consecutive time points during force activation and maintenance.

W-Pos165

EFFECTS OF MgADP ON ACTIVATION AND SHORTENING VELOCITY OF CHEMICALLY SKINNED CARDIAC TRABECULAE FROM THE GUINEA PIG.

((**R. M. Fulbright** and R. J. Barsotti)) The Graduate Hospital, Bockus Research Inst., Philadelphia, PA 19146.

Triton-skinned trabeculae with diameters from 150-350 μm can produce tension and undergo shortening in the absence of Ca^{2+} (pCa > 8) in a solution with a low [MgATP] (1 mM) and a high [MgADP] (4 mM) (REL1T4D). Trabeculae in 1 mM MgATP without added MgADP (pCa > 8) do not produce tension, indicating that this effect requires the presence of a high [MgADP]. Typically a trabecula placed in the REL1T4D solution develops tension slowly, taking 100-150 s to reach its maximum value which is ~40 % of the steady active tension developed in a normal activating solution (5 mM MgATP, pCa 4.5). Isotonic releases of trabeculae in various solutions, including REL1T4D, were performed to determine MgADP effects on shortening velocities at 0 % of active isometric tension. Stable tension levels were achieved in < 10 ms after release and data were recorded out to ~80 ms. Because the change in length with time was not linear, shortening velocities were determined at both 10 ms and 60 ms after release. By 60 ms after release, shortening velocities were nearly constant. To see how shortening velocity resulting from activation of the thin filament by (presumably) MgADP-bound myosin heads compares with shortening velocity resulting from thin filament activation by Ca^{2+}, shortening velocities in REL1T4D were compared with those in 1 mM MgATP with 4.5 < pCa < 8 such that the levels of isometric tension of a given trabecula in the two solutions were almost the same. At 10 ms after release for trabeculae in REL1T4D, the shortening velocity was $(0.61 \pm 0.06) L_o\ s^{-1}$ compared with $(0.60 \pm 0.08) L_o\ s^{-1}$ for trabeculae in 1 mM MgATP with 4.5 < pCa < 8 (mean ± s. e. m., N = 6), where L_o is the prerelease length. The values at 60 ms were $(0.11 \pm 0.01) L_o\ s^{-1}$ for REL1T4D and $(0.12 \pm 0.02) L_o\ s^{-1}$ for the 1 mM MgATP with 4.5 < pCa < 8 solution. These results suggest that MgADP-bound crossbridges can activate the thin filament without presenting a significant internal load to shortening cardiac muscle. (This work was supported by the American Heart Association, Southeastern Pennsylvania Affiliate (fellowship to R. M. F.) and National Institutes of Health grant HL40953 (to R. J. B.).)

W-Pos166

DETHIOPHALLOIDIN, A WEAK F-ACTIN STABILIZER, IS A STRONGER ACTIVATOR OF SKINNED CARDIAC MUSCLE THAN PHALLOIDIN

((**A.E. Bukatina**[1,2], R.D. Kirkpatrick[1] and K.B. Campbell[1])) [1]Dept. of VCAPP, WSU, Pullman, WA 99164 and [2]ITEB, Russian Acad. Sci., Pushchino, Russia 142292. (Spon. by M.J. Smerdon)

Phallotoxins (PHTs) are known muscle-specific modifiers of contraction (review: Bukatina, 1996). Among a number of PHTs, the ability to modify skeletal muscle tension is qualitatively correlated with the ability to stabilize F-actin structure. An exception is dethiophalloidin (DTPH), which is a poor F-actin stabilizer but still has considerable phalloidin(PH)-like effects on contraction (Son'kin et al, 1983). Here, we studied the effects of DTPH on skinned bovine cardiac muscle. Like PH, DTPH (30-150 μM) caused an increase in active tension at all pCa and an increase in Ca^{2+} sensitivity (~0.3 pCa units). Over these [DTPH], the increase in tension did not depend on concentration but the rate of tension increase was slower at the lower concentrations. The addition of PH to the muscle activated by DTPH caused about a 50% decrease in the DTPH-induced tension increase. Pre-incubation with PH suppressed the DTPH effect. Unlike PH, the effects of DTPH were reversible. These results suggest: 1) DTPH is a stronger activator of contraction than PH in cardiac muscle even though it binds to actin more weakly; Actin sites for PH and DTPH overlap, and both compounds likely work by a similar mechanism. PHTs do not cause irreversible changes in cardiac muscle structure; 2) Unexpectedly strong DTPH effects on contraction in both cardiac and skeletal muscle indicate similarity in mechanism of PHTs action in spite of qualitative difference in the PHTs induced responses in these two muscles; PHTs induced strengthening of contacts between actin monomers is not likely to be a major factor in the mechanism by which contraction is modified. Supported by NIH grant HL-21462.

W-Pos167

THE EFFECT OF STRONG BINDING MYOSIN CROSS-BRIDGES ON THE LENGTH-DEPENDENCE OF CALCIUM SENSITIVITY OF TENSION IN SINGLE SKINNED CARDIAC MYOCYTES

((**D.P. Fitzsimons and R.L. Moss**))
Dept. of Physiology, University of Wisconsin Medical School, Madison WI 53706

Reductions in sarcomere length (SL) and resulting increases in interfilament lattice spacing (ILS) have been shown to decrease the Ca^{2+} sensitivity of tension in myocardium. ILS could exert its influence on the SL-dependence of tension by mediating the probability of strong interaction of myosin cross-bridges with actin. We hypothesized that decreased submaximal Ca^{2+}-activated tensions at short SL are due to decreased cooperative activation of the thin filament as a consequence of increased ILS and reduced numbers of strongly-bound cross-bridges. To test this hypothesis, N-ethylmaleimide-modified myosin subfragment 1 (NEM-S1), a non-tension generating analog of strongly-bound cross-bridges, was applied to skinned single myocytes to promote the formation of strongly-bound endogenous cross-bridges. Single myocytes from enzymatically digested rat ventricles were attached to a force transducer and piezoelectric translator, and tension-pCa relationships were measured at short and long SL, respectively. The Ca^{2+}-sensitivity of tension (pCa_{50}) increased in control myocytes when SL was increased from 1.90 μm to 2.25 μm. At SL 2.25μm, application of 6 μM NEM-S1 increased the pCa_{50} and nearly eliminated the SL-dependence of Ca^{2+}-sensitivity of tension. These data support the hypothesis that the reductions in Ca^{2+}-sensitivity of tension at short SL are due in large part to reduced cooperative activation of the thin filament as a consequence of increased ILS. **Supported by NIH HL54581**

W-Pos168

IS CARDIAC TnC A LENGTH-SENSOR IN THE FRANK-STARLING MECHANISM ?

((**J. Gulati**))
The Molecular Physiology Laboratory, Einstein College of Medicine, Bronx, NY

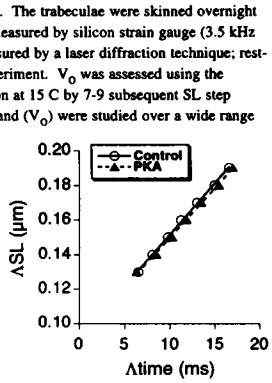
The Ca-sensitivity of tension in cardiotrabeculae is greater at 2.4μm sarcomere length than that at 1.9μm, and this length-dependence provides the molecular basis for Starling's law of the heart. Thus, there is interest in locating the length sensor in cardiac muscle. Following the observation that cTnC/sTnC exchange in cardiotrabeculae diminished the length-dependence of Ca-sensitivity of tension, the TnC moiety was suggested as central to the length-sensing process (Babu et al, Science, 1988). But, this TnC-hypothesis was challenged primarily because Moss and coworkers had failed to observe the TnC-effect (1) in fast-twitch skeletal fibers and (2) in isolated cardiac cells from Parmacek-Leiden transgenic mice with cardiac expression of sTnC (see Solaro and Van Eyke, JMCC, 1996). There was additional concern that slow-twitch fibers, which naturally contain cardiac TnC and exhibit the expected enhanced length-dependence similar to cardiac muscle, show deviations in their Ca-binding properties. We will summarize data showing that fast-twitch fibers with cTnC exhibit cardiac-type response under optimal conditions for cTnC regulation. The possible shortcomings of the available transgenic results will also be discussed, as will be the consequences of differences with slow fibers. Additionally, new results of TnC exchange in cardiotrabeculae with chimeras of cardiac-skeletal TnCs will be presented. We find that the cardiac type length responsiveness is located within 1- 41, and possibly 1-96, cardiac N-terminal residues. These findings provide further support for the TnC hypothesis, revealing moreover that the length-sensing response of cTnC may require fundamental interactions between sites I and II of the trigger domain.

W-Pos169

LACK OF EFFECT OF PROTEIN KINASE A ON UNLOADED SHORTENING VELOCITY IN SKINNED RAT CARDIAC TRABECULAE

((**P.M.L. Janssen** and P.P. de Tombe)) Dept. of Physiology and Biophysics, University of Illinois at Chicago, Chicago IL 60612

Whether ß-adrenergic stimulation affects the cross-bridge cycling rate independent from its effect on calcium handling by the cardiac myocyte is still unknown. Accordingly, we studied the impact of PKA stimulation on isometric tension and unloaded velocity of sarcomere shortening (V_0) in five cardiac trabeculae isolated from rat hearts. The trabeculae were skinned overnight (1% Triton-X100 in a relaxing solution). Force was measured by silicon strain gauge (3.5 kHz resonant frequency). Sarcomere length (SL) was measured by a laser diffraction technique; resting SL was maintained at 2.25 μm throughout the experiment. V_0 was assessed using the "Edman slack test" during a single 45 second activation at 15 C by 7-9 subsequent SL step releases (from 0.12 to 0.20 μm; see Figure). Tension and (V_0) were studied over a wide range of free calcium concentrations both before and after exposure to the catalytic subunit of PKA (3 μg/ml;45 min;20C). Treatment with PKA caused a reduction in calcium responsiveness (pCa_{50} 5.72±0.02 vs. 5.60±0.05; p<0.05), while maximum tension was not affected (54.8±3.6 vs 55.2±4.6 mN/mm^2; p=0.9). The figure shows the determination of V_0 in a typical experiment during maximal activation by calcium. PKA treatment did not affect V_0 in any of the trabeculae studied (6.5±0.5 vs 6.6± 0.8 μm/s; p=0.9). We conclude that ß-adrenergic stimulation does not affect cross-bridge cycling rate in myocardium.

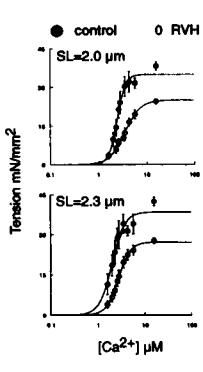


W-Pos170

DECREASED MYOCYTE TENSION DEVELOPMENT AND CALCIUM RESPONSIVENESS IN RAT RIGHT VENTRICULAR PRESSURE OVERLOAD

((**D.S. Fan**, T. Wannenburg, P.P. de Tombe)) Dept. Physiology & Biophysics,Univ. of Illinois at Chicago, Chicago, IL 60612 and Section on Cardiology, Bowman Gray School of Medicine, Winston-Salem, NC 27157.

We studied the force pCa relationship in single skinned myocytes isolated from rats with chronic right ventricular hypertrophy (RVH) induced by pulmonary artery constriction (36 wks) and from control rats. RVH was associated with significant myocyte hypertrophy (p<0.05). Myocytes were attached to micropipettes that extended from a force transducer and motor, respectively. Isometric force was measured over a wide range of calcium concentrations at a high sarcomere length (SL=2.3 μm), and a low sarcomere length (SL=2.0 μm). RVH was associated with a significant increase in maximal force development (1.2 ± 0.10 vs. 1.6 ± 0.13 mg at SL =2.0 μm; 1.3 ± 0.10 vs. 1.8 ± 0.15 mg at SL =2.3 μm). Myocyte cross-sectional area increased in RVH (3.2 ± 0.32 vs 6.7 ± 0.56 mm^2; p<0.05). Tension development (force divided by cross-sectional area) decreased significantly in RVH (p<0.05; cf. Figure). RVH was also associated with a significant decrease in calcium responsiveness as indexed by the EC_{50} of the force-[Ca^{2+}] relation (by 31% at SL=2.0 μm, and 28% at SL=2.3 μm; Figure). Thus, the impact of changes in SL on the force-[Ca^{2+}] relation was not affected by RVH. These results suggest that diminished contractile function in myocardial hypertrophy is due, in part, to an alteration in contractile protein function.

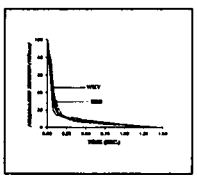


W-Pos171

SLOWING OF RELAXATION RATE, FOLLOWING PHOTOLYSIS OF DIAZO-2, IN SKINNED, GLYCERINATED TRABECULAE FROM HYPERTROPHIED SHR LEFT VENTRICLE.

((EC **Johns**, EAM Hodson, H Fish[1], KO Ryder[2], JS Lymn[3], G Hart[2], H Thurston[1], IP Mulligan[2] and CC Ashley)). Univ. Lab. of Physiology, Oxford, OX1 3PT, UK. [1]Leicester Royal Infirmary, UK. [2]John Radcliffe Hospital, Oxford, OX3 9DU. [3]St. Mary's Hospital Medical School, London, UK.

In the spontaneously hypertensive rat (SHR) myocardial tissue hypertrophies as a reaction to increased afterload imposed by the hypertensive state, and diastolic dysfunction generally follows. Rat models of cardiac hypertrophy are characterized by a shift in myosin isoform from V1 (adult) to V3 (foetal), the latter being associated with a slowing of the acto-myosin ATPase rate. The aim of this study was to examine effects of hypertrophy on the relaxation of a skinned cardiac preparation from the SHR, where the sarcoplasmic reticulum (SR) is rendered nonfunctional and the myofibrils can be studied in isolation. We have used the technique of laser flash photolysis of the Ca^{2+}-chelator diazo-2 to investigate the rate of relaxation in glycerinated, skinned (1% v/v Triton X-100; 20 minutes) trabeculae from the hypertrophied left ventricle (LV) of 12 week old SHR. Experiments were performed at 12°C, with fibres from the LV of age-matched, normotensive WKY rats used as controls (average heart weight/tibial length ratio for WKY:0.0225; and for SHR: 0.0248 ($p<0.01$)). On comparing the two mean relaxation transients in the figure (WKY n=7; SHR n=6), it can be seen that the SHR LV fibres relax at a slower rate than their age-matched WKY counterparts. We suggest that, since regulatory protein isoforms are probably not affected by cardiac hypertrophy in the rat, this result can be attributed to the shift in LV myosin isoform - to the foetal form.The reduced relaxation rate, demonstrated here, could be the result of a slowing of the dissociation of actin and myosin. Similar experiments using skinned LV trabeculae from aortic-banded guinea-pigs have revealed no change in the relaxation rate, when compared to sham-operated controls. This finding supports our theory as, despite regulatory protein alterations, there is no shift in myosin isoform with cardiac hypertrophy in the adult guinea-pig which normally expresses the V3 isoform.



W-Pos172

CHANGES IN PASSIVE TENSION AFFECT Ca^{2+} SENSITIVITY IN SKINNED RAT CARDIAC CELLS.

((Cazorla O., Vassort G.*, Garnier D. & Le Guennec J-Y.)) Labo. de Physio. Animale, CNRS EP 21, Fac. des Sciences, 37200 TOURS (France) *Labo de Physiopatho. Cardiovasc. INSERM U-390, CHU A. De Villeneuve, 34295 MONTPELLIER (France).

There is increasing evidence that force applied to the contractile machinery during a stretch may play a role in the length-tension relationship of skeletal and cardiac muscles. We have shown that in intact single cardiac cells, resting tension modulates active tension (Cazorla *et al, Biophys. J.*, 1995, 70 :A347). To locate the origin of this observation, we used skinned rat cardiac cells and, by mild trypsin digestion, specifically degraded titin, the major protein involved in the high resting tension of cardiac cells (Granzier & Irving, *Biophys. J.*, 1995, 68 : 1027-1044). Cells were enzymatically isolated and skinned using Triton-X100. To change their length and measure the force developed during different pCa solution application or during a stretch, cells were attached with an optical glue to glass micropipettes fixed to a force transducer (Puceat *et al, Circ. Res.*, 1990, 67 :517-524). Tension-pCa relationships were built in different experimental conditions : sarcomere length (S.L.) of 1.9 and 2.3 µm before or after different times of digestion by trypsin (0.25 µg/ml). We observed that in control conditions, stretching the cells from 1.9 to 2.3 µm of S.L. induced a mean shift of the pCa_{50} of 0.39 ± 0.09 pCa unit (n = 20 cells). As already described by Granzier & Irving (1995), we found that a mild digestion of skinned cells by trypsin reduced the passive resting tension of the cells ($t_{1/2}$ of about 12 min). For the first 30 % of lost resting tension, no significant reduction of Ca^{2+}-sensitivity was observed. However, above this value, a decrease in the shift of pCa_{50} induced by stretch was linearly related to the reduction of passive tension. This phenomenon was not accompanied by a significant decrease of the maximal tension provoked by a pCa 4.5 solution indicating that the contractile machinery did not seem to be particularly degraded by trypsin. In conclusions, we think that our results confirm an involvement of the resting tension in Ca^{2+} sensitivity of the cardiac contractile machinery.

W-Pos173

ACTIN-TITIN INTERACTION AFFECTS PASSIVE FORCE DEVELOPMENT IN RAT CARDIAC MYOCYTES ((HELMES, M., GRANZIER, H.)) DEPARTMENT VCAPP, WASHINGTON STATE UNIVERSITY, PULLMAN, WA 99164-6520.

The finding that thin and titin filaments are laterally associated (Funatsu et al., 1993) suggests that titin binds to actin *in situ*. To understand the functional significance of this interaction, we studied the effect of actin extraction on passive force developed by cardiac myocytes. Myocytes were studied before and after actin extraction with the gelsolin fragment FX-45 (0.25 mg/ml). By controlling the extraction time, we achieved either limited or full actin extraction.

Limited actin extraction. When the extraction duration was 10 min, only the segment of the actin filament that extends beyond the ~100 nm inelastic region of titin that adjoins the Z-line was extracted (see also poster by Trombitás and Granzier). Passive force was unaffected between the slack length (~1.85 µm) and a sarcomere length of ~ 2.0 µm. At longer lengths passive force was reduced by ~30-40%.

Full actin extraction. When the extraction time was extended to 100 min, the full actin filament was extracted. The slack sarcomere length was now only ~1.70 µm. Passive force at sarcomere lengths between slack and ~ 2.1 µm was higher than in the control cells. At longer lengths passive force was ~20% less than that measured after limited actin extraction.

We recently proposed a two-stage mechanism of passive force development by titin where force is developed between the slack length and a sarcomere length of ~2.0 µm as a result of straightening of the elastic segment and at longer sarcomere lengths as a result of unfolding of molecular subdomains (*Biophys. J.*,70, 430-442). Our results suggest that after limited actin extraction only the 'unfolding-based passive force' is reduced. After full actin extraction, the increase in passive force at short sarcomere lengths may be caused by contraction of the titin segment that becomes exposed following actin removal near the Z-line. The reduction in passive force at sarcomere lengths longer than ~2.1 µm seen when cells go from limited to full extraction, may result from the increased contour length of the elastic titin segment. This decreases titin's strain for a given degree of sarcomere stretch, and hence decreases passive force.

We conclude that in control cells, interaction between actin and the elastic region of titin is functionally important at sarcomere lengths where titin unfolds. Actin may stabilize titin and increase its threshold for unfolding, resulting in enhancement of passive force.

W-Pos174

Viscoelastic properties of passive cardiac myocytes ((HELMES, M., GRANZIER, H.)), VCAPP, WASHINGTON STATE U., PULLMAN, WA 99164-6520.

It is now well established that titin generates passive force in stretched myocytes. This force is viscoelastic in nature and, therefore, its magnitude will vary with the velocity of stretch imposed on myocytes. In the heart, the stretch velocity of myocytes changes with the stroke volume and the beat frequency. Thus, in order to understand the contribution of titin to the diastolic wall tension, the visco-elastic properties of titin have to be studied first.

Stress relaxation was investigated in rat cardiac myocytes. Myocytes were stretched from slack to a pre-determined sarcomere length (sl) in 0.1 s and held at the stretched length for a prolonged period of time (300 seconds) while measuring force. We also studied the speed-dependence of passive force, and imposed stretch-release protocols with varying amplitude and velocity.

Both stress-relaxation and the dependence of passive force on stretch velocity were most pronounced at sarcomere lengths >2.0 µm. Furthermore, both stress-relaxation and the velocity dependence of passive force could be described with a double exponential fit. Stress-relaxation had a fast component that was dominant in the first half second after stretch and a slow component that determined the stress relaxation over the remainder of the 300 seconds. Time constants of the exponential fits varied linearly with force. For velocity dependence of passive force, the fast component was dominant at stretch velocities faster than 0.15 µm/sl/s (sl: 2.3 µm).

The velocity range where passive force is strongly dependent on velocity includes the physiological range of stretch velocities that has been estimated to occur in the rat heart. This suggests that any change in stretch velocity, due, for example, to a change in beat frequency, may lead to a considerable change in titin's contribution to diastolic wall tension.

W-Pos175

TITIN ELASTICITY: INTERACTION AND EXTENSIBILITY OF PEVK SEGMENT OF HUMAN FETAL SKELETAL TITIN. ((**G. Gutierrez**, A. van Heerden, J. Wright and K. Wang)). Dept. of Chem. and Biochem. Univ. of Texas, Austin. Tx 78712

The extension of elastic titin filaments, especially in the segment that spans the I-band in the sarcomere, is thought to be responsible for generation of resting tension of skeletal and cardiac muscle. The I-band titin consists predominately of two types of potentially elastic structural motifs: immunoglobulin repeats and a newly discovered PEVK segment (Labeit and Kolmerer, Science *270*, 293). We have previously obtained a 2.5 kb cDNA clone by screening a human fetal muscle library with a monoclonal antibody (RT11) that recognizes an I band epitope. The sequence of 823 amino acids shows high degree of similarity with published PEVK sequences of rabbit muscle. We have expressed and purified a 53 kD fragment of this proline rich fragment in *E coli* as a soluble protein. Studies of protein interaction with solid phase assays indicate that these domains interact with actin, nebulin and titin (T2). These data suggest that PEVK region of titin may be involved in interfilament association with actin, nebulin and titin, which in turn may modulates titin extensibility. ImmunoEM localized the PEVK specific RT11 epitope to an extensible region of the I band titin in rabbit psoas and semitendinosus muscle. Further evaluation of the conformational transition and extensibility of PEVK constructs is likely to facilitate understanding of the segmental extension of titin filaments (supported by NIHAR43514 and FFR).

W-Pos176

THE PROJECTIN MUTANT *BENT^D* /+ HAS REDUCED FLIGHT ABILITY AND ALTERED INDIRECT FLIGHT MUSCLE KINETICS ((J. **Moore**[1], J.Vigoreaux[2], D. Maughan[1])) [1]Dept. Molecular Physiology & Biophysics and [2]Dept. Biology, U. Vermont, Burlington VT 05405

Drosophila projectin is homologous to twitchin and titin. Projectin is postulated to play a role in stretch activation and oscillatory power output of IFM. Through genetic studies, twitchin is believed to play a role in regulating muscle function, whereas titin is believed to have primarily a structural role. The mutant *bent ^{D}* (*bt ^{D}*) allele has been shown to have a breakpoint that removes the carboxy terminal kinase domain. Homozygotes for *bt ^{D}* are lethal, and heterozygotes, which we studied, are flightless (flight index of 1.0±0.4 vs. 5.6±0.7in wild type). The wingbeat frequency winds down rapidly (1-2 s) from wild type values (~200 Hz at 22°C). Electron microscopy of *bt ^{D}* /+ IFM fibers show normal ultrastructure, suggesting that the *bt ^{D}* mutation does not interfere with the structural role of projectin, if any, in the IFM. Isolated skinned IFM at 12°C show normal power output. However, the frequency at which power is maximum (f_{max}) is significantly higher ($P<0.05$) in *bt ^{D}* /+ (112±17 Hz) than in wild type (79±35 Hz). If f_{max} no longer matches the natural resonant frequency of the flight system, less power will be transmitted from the IFM to the wings, which could explain the flight impairment. These results suggest projectin has a regulatory role in IFM function. Supported by NIH R01 AR 40234.

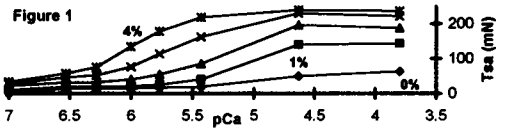
W-Pos177

STRAIN ACTIVATION IN INSECT FIBRILLAR FLIGHT MUSCLE

((J.W. Regini & D.C.S. White)) Department of Biology, University of York, YO1 5DD, UK

Insect fibrillar flight muscle (IFM) shows the property of strain activation: small applied strains increase active tension and ATPase markedly. A possible mechanism for the length sensing element is that the extended arm of troponin-H may be bound to the thick filament and act, say, to move tropomyosin or troponin mechanically. To test these ideas, fibres from *Lethocerus indicus* at rest length were immersed in a series of solutions in which the free Ca^{2+} was raised from pCa = 7 to pCa = 4. A six second 1% step length change was applied at each $[Ca^{2+}]$ and the peak tension of strain activation (Tsa) measured. The fibres were then extended to a new starting length of 1% and the process repeated. Further 1% increments were given to a final starting length of 4%. Figure 1 shows Tsa plotted against pCa; the curves clearly shift to left at higher lengths, indicating a change in Ca^{2+} sensitivity. Moreover, at low Ca^{2+} concentrations the fibres have to be extended further before activation occurs. One interpretation of these results is that at high Ca^{2+} concentrations the troponin-tropomyosin switch is permanently on, and thus a number of myosin binding sites on the actin filament are exposed. When the fibre is stretched, further sites are exposed, possibly by means of a troponin-H lever. At low Ca^{2+} levels the troponin-tropomyosin switch is off, thus the fibre needs to be extended further in order to expose enough binding sites for tension generation.

Figure 1



W-Pos178

UNLOADED VELOCITY OF SHORTENING IS HIGHER DURING THE ONSET OF ACTIVATION THAN DURING THE TETANUS PLATEAU IN FROG MUSCLE

((K.A.P. Edman and R.K. Josephson)) Department of Pharmacology, University of Lund, S-223 62 Lund, Sweden.

Maximum speed of shortening measured at steady state in frog striated muscle has previously been shown to be very little influenced by changes in sarcomere length and degree of activation of the muscle fiber (Edman, J. Physiol.1979, 291: 143-159). We here report that the velocity of unloaded shortening (V_0) is not constant during tetanic stimulation but is substantially higher during the onset of mechanical activity than during the tetanus plateau. Single fibers from the anterior tibialis muscle of *Rana temporaria* were studied (1-3 °C, resting sarcomere length 2.2 μm). The fibers were released to shorten against different loads (load-clamp) at selected times during the rising phase and during the plateau of isometric tetani. The force-velocity relation determined soon (< 30 ms) after the onset of tension rise was steeper and exhibited higher velocities in the low-force range than did that derived during the plateau of the tetanus. V_0, measured by the slack test method (Edman 1979, see above), was up to 25 % higher within the interval 10-30 ms after the latency period than on the tetanus plateau. This difference in V_0 between onset and plateau of the tetanus was also found when the fiber was restimulated immediately after a preceding tetanus suggesting that accumulation of metabolic products does not contribute to the decrease of V_0 from its initially high value during a tetanus. The results support the idea that myosin cross-bridges attach to the thin filament in a position where they are ready to develop force, i.e. in a pulling position. During the onset of mechanical activity, when new cross-bridge connections are rapidly being formed, the number of pulling cross-bridges will temporarily exceed the number of bridges that are in a braking position. The myofilaments will therefore initially accelerate to a higher velocity than that assumed later when the pulling and braking bridges are in equilibrium. This explanation predicts that V_0 should be lower than the plateau value during relaxation as activation declines, and indeed V_0 values were found to be depressed during the linear phase of relaxation after tetanic stimulation.

W-Pos179

ZERO LENGTH REDUCTION IN S1 AND FILAMENT FORCE IN A MECHANICAL UNIT CELL MODEL.

((FRANS VAN KAAM)) MIJLBERG 38,5508EN VELDHOVEN, NETHERLANDS.

A conformational change in unloaded S1 after energy transfer (WAKABAYASHI ET AL, 1992, SCIENCE 258: 443; RAYMENT ET AL ,1995, SCIENCE 261: 50) is mechanically interpreted as a reversible deformation of an active contractile elastic element CE in S1. During a loaded contraction part of the energy is stored in CE itself to resist the force it builds up by stretching the elastic passive structures PE in series with it. PE comprises non contractile structures of S1; S2 (HUXLEY & SIMMONS,1971,NATURE 233:533) and filaments (HE HUXLEY ET AL,1994,BIOPH J 67: 2411; WAKABAYASHI ET AL; 1994, BIOPH J: 67:2422). Energy storage in CE is possible when it behaves like an Hookean spring of which the zero length L_0 can reduce upon activation (VAN KAAM, J MUSLE RES CELL MOT, ABSTRACT IN PRESS). L_0 is a vector as defined for proteins by FLORY (1956, SCIENCE, 124: 53).

The zero length reduction can have its atomic origin in reduction of the characteristic Van der Waals radius (r_0) of contractile atom pairs in S1. Different contractile atom pairs could result into different stages of S1 (EISENBERG & HILL ,1978, PROG BIOPHYS MOL BIOL,33:55) but loaded deformation of S1 is assumed to take place smoothly. Reduction of r_0 can explain rotational as well as translational deformation in S1.

Zero length reduction is applied to a mechanical unit cell model defined with one actin filament pulled by up to 57 crossbridges protruding from myosin filaments in trigonal position (VAN KAAM,1996, SUPPL PROG BIOPHYS MOL BIOL,P172). During the energy transfer, CE develops force in equilibrium with PE force. The final steady state force of CE+PE arrives at a minimum potential energy level of the crossbridge. Steady state force is lower when PE is more compliant. Larger filament compliance and higher stiffness of S2 result into a larger strain relieve in S1 due to force contributions of crossbridges attaching afterwards. Strain relieve in S1 is most effective when the second head can attach and pulls on one and the same passive structure in S2.

Zero length reduction in S1 gives activated muscle a stretched spring character.

W-Pos180

CONTRACTION-INDUCED INJURY TO EXTENSOR DIGITORUM LONGUS MUSCLES OF *mdx* MICE.

((Susan V. Brooks)) Inst. of Gerontology, Depts. of Physiology and Biomedical Engineering, Univ. of Michigan, Ann Arbor, MI, 48109-2007.

In control muscles, contraction-induced injury is initiated by the mechanical disruption of specific sarcomeres and is a function of high force and stretch. A protocol of ten maximal contractions with 20% stretches resulted in greater force deficits, [(1 - post-stretch maximum isometric force/pre-stretch max. isometric force) x 100], for *in situ* muscles of *mdx* mice than for those of control mice, both immediately and 1 hr after the protocol. The greater force deficits observed for muscles in *mdx* mice could be due to more severe myofibrillar disruptions or to types of damage not observed in control muscles. In control muscles, severe myofibrillar disruptions initiate events resulting in a secondary injury that peaks 1 to 3 days later, depending on the severity of the initial injury. In the present study, the time course of the force deficit for muscles in *mdx* mice was followed for 3 days following the initial damage to investigate the mechanism for the more severe initial injury to muscles in *mdx* compared with control mice. I hypothesized that the greater force deficits induced in muscles of *mdx* compared with control mice were the result of greater myofibrillar disruptions, and, consequently, muscles in *mdx* mice will continue to show greater force deficits at 3 days. Extensor digitorum longus muscles in 8-10-week-old *mdx* and control mice were exposed to stretches from the plateau of 10 maximum isometric contractions. Stretches were initiated from optimum length for force (L_o) and were of 20% strain relative to optimum fiber length (L_f). Force deficits were measured at 10 min, 12, 24, 48, and 72 hr. In contrast to the significantly greater force deficits observed for muscles in *mdx* compared with control mice immediately and 10 min after the contraction protocol, the force deficits were not different for the two groups after 12 hr. While muscles in control mice still showed ~20% force deficits at 3 days, forces developed by muscles in *mdx* mice had recovered to pre-injury values. The dramatically differing time courses for the recovery of force by muscles in *mdx* and control mice suggest that the more severe initial injury in dystrophic muscle is due to the manifestation of an injury unique to these muscles rather than an amplification of the injury which occurs in control muscle. The most attractive hypothesis is that, in addition to sarcomeric disruption, fibers in the muscles of *mdx* mice experience disruption of the sarcolemma.

W-Pos181

THE EFFECT OF REPEATED LENGTHENING CONTRACTIONS ON OPTIMUM LENGTH AND Ca^{2+} SIGNALS IN FROG SINGLE FIBRES.

((D.L. Morgan*, D.R. Claflin & F.J. Julian.)) Department of Anesthesia, Brigham & Women's Hospital, 75 Francis St., Boston, MA, 02115; *Monash University, Clayton, Vic., 3168, Australia.

Interest has recently been renewed in an observation by Katz (J. Physiol. 96:45-64, 1939) that a series of muscle contractions with applied lengthening (eccentric contractions) causes an immediate shift in optimum length for tension generation of whole muscles. Proposed explanations involve either disrupted sarcomeres or reduced calcium ion release. Using intact single fibres from the tibialis anterior muscles of *Rana temporaria* at 3.0°C, a similar shift was apparent. The magnitude of the shift was not well correlated with the accompanying fall in optimum tension, to the extent that at long fibre lengths, tension was sometimes greater after the eccentric contractions than before. Measurement of the intracellular free Ca^{2+} transient (ICT) with the rapidly responding dye mag-indo-1 did not show a significant decrease in the amplitude of the ICT after eccentric contractions, but did show a significant prolongation. The twitch potentiator nitrate increased both the amplitude and the width of the ICT, but did not reverse the shift in optimum length. Measurement of the resting $[Ca^{2+}]$ with the more sensitive dye fura-2 showed that a rise in resting $[Ca^{2+}]$ was associated with damage to the cell, but not with shift in optimum length. Stiffness measurements using a small 2kHz sinusoidal length change showed that eccentric contractions lead to a decrease in isometric stiffness much greater than the decrease in isometric tension. All these results are consistent with proposals that the shift is produced by disruption of filament overlap in some half-sarcomeres, not by impairment of activation. Supported by NIH HL35032 (FJJ) and Monash University outside studies program grant (DLM).

W-Pos182

KINETICS OF FORCE RECOVERY AFTER LENGTH CHANGES IN ACTIVE SKINNED SINGLE FIBERS FROM RABBIT PSOAS MUSCLE.

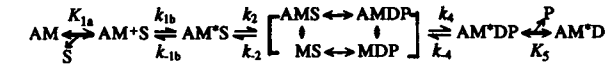
((K. Burton)). STC, Carnegie Mellon University, Pittsburgh, PA, 15213.

The rate of force recovery following several length-change protocols has been studied in skinned single fibers from rabbit psoas muscle during maximum activation at 5°C. The protocols included (1) isometric step-release or stretch, eliciting "phase 4" recovery, (2) slack test or ramp shortening to a stop, (3) ramp shortening of 70-150 nm/hs at high velocity terminated by a rapid stretch (0.2-0.5 ms) of 2-200 nm/hs, and (4) ramp shortening with restretch to the initial length, followed within 0.1-20 ms by step-release to reduce force at the beginning of recovery. Recovery from near zero was described by two exponentials for all protocols, with rate constants of 1.7-2.4 s^{-1} for the slow rise and 7.7-9.7 s^{-1} for the fast rise. The amplitudes of the two components were similar. A slow falling component of ~0.6-0.8 s^{-1} was present following either ramp/restretch or small isometric step-stretch (3 nm/hs). The similarity of phase 4 to force recovery after ramp shortening suggests that a transient reduction in load shifts crossbridges to a distribution similar to that at the end of steady shortening. The rate of recovery following a step-release applied immediately after a large restretch (70-150 nm/hs) was similar to recovery without a restretch, showing that forcible disruption of cross-bridges *per se* has little effect on the kinetics of recovery. A model is proposed in which the fast component is controlled by a transition from non-force generating to force generating states, similar to the proposal of Brenner and Eisenberg (1986) based on single exponential fits. The slow rise and fall are suggested to result from detachment of crossbridges under strains *less* than and *greater* than average for isometric contraction, respectively. The ATPase rate for isometric fibers at 5°C is between these two rate constants, and they span changes in muscle ATPase activity observed during steady shortening and lengthening.

W-Pos183

EFFECT OF IN-SERIES THIN FILAMENT COMPLIANCE ON THE ELEMENTARY STEPS OF THE CROSS-BRIDGE CYCLE IN SKINNED RABBIT PSOAS MUSCLE FIBERS. ((**Gang Wang** and Masataka Kawai)) Dept of Anatomy, Univ of Iowa, Iowa City, IA 52242.

To investigate how in-series thin filament compliance affects the elementary steps of the cross-bridge cycle, sinusoidal analysis was carried out in skinned rabbit psoas muscle fibers at sarcomere lengths (SL) 2.1 μm and 2.4 μm. These SL were chosen because in-series compliance is varied by 1.6 fold (X) while maintaining the same overlap between thick and thin filaments (Higuchi, Yanagida, and Goldman, *Biophy. J.* 1995, 69: 1000-1010). Single fiber preparations were activated at pCa 4.66, 200 mM ionic strength, pH 7.00, and at 20°C. The effect of MgATP on exponential process C and that of phosphate (Pi) on exponential process B were studied and the kinetic constants at these two different SL were compared. The results are explained in the following cross-bridge scheme:



As SL was increased from 2.1 μm to 2.4 μm, the kinetic constants of the elementary steps changed in the following manner: K_{1a} 0.6X, k_{1b} 2X, k_{-1b} 1.1X, K_{1b} 1.8X, k_2 0.8X, k_{-2} 1.1X, K_2 0.8X, k_4 1.1X, k_{-4} 1.3X, K_4 0.9X, and K_5 1.1X. Thus, the effect of the SL change is largest on k_{1b}, followed by K_{1b} and K_{1a}. The effect is smaller or absent in other kinetic constants. From these we conclude that the in-series thin filament compliance affects MgATP binding and cross-bridge detachment steps slightly, but it does not affect the kinetic constants of the force generation and Pi release steps.

W-Pos184

CORRELATING TITIN ISOFORM EXPRESSION WITH MECHANICAL PROPERTIES OF CARDIAC AND SKELETAL MYOFIBRILS.

((M. Ivemeyer[1], N.Olivieri[2], A. Freiburg[2], B. Kolmerer[2], S. Labeit[2] and W.A. Linke[1]))
[1]Physiology II, University of Heidelberg, Im Neuenheimer Feld 326, D-69120 Heidelberg and [2]EMBL, Meyerhofstr. 1, D-69012 Heidelberg, Germany

In relaxed myofibrils from vertebrate striated muscle, titin is the principal determinant of elasticity. The elastic I-band portion of titin primarily consists of two structural motifs, tandem-immunoglobulin-domains (Ig) and PEVK-rich sequences. To confirm previous data on splicing pathways of titin in human muscle (Labeit & Kolmerer, *Science*, 270:293,1995), we sequenced I-band titin from rabbit muscle and used RT-PCR on a panel of rabbit skeletal mRNAs. Six splice variants of I-band titin Ig domains have been identified so far, three for cardiac muscle and three others, specific for *soleus*, *psoas*, and *longissimus dorsi*. Muscle-type specific length expression was also found for the PEVK region. Differential splicing of I-band titin specifies characteristic mechanical features of a relaxed muscle tissue, such as the sarcomere length (SL), at which stiffness upon myofibril stretch begins to increase, and the stiffness magnitude at a given SL.
To address the molecular basis of I-band titin elasticity, we studied the effect of variations in pH and ionic strength (IS) on myofibril stiffness. Small myofibril bundles were mounted between a piezoelectric micromotor and a sensitive force transducer, and stiffness was estimated from the force response to sinusoidal oscillations (20 Hz) recorded at either 2.4 (cardiac) or 2.8-3.2 (skeletal) μm SL. To exclude a possible contribution of actomyosin interactions to myofibril stiffness, we selectively removed actin by treatment with gelsolin, which generally decreased the stiffness of both cardiac and skeletal specimens. Preliminary results show a lack of pH dependence of actin-depleted myofibril stiffness, whereas low IS buffer markedly increases this stiffness. In *longissimus dorsi*, for example, stiffness at low IS (20 mM) was ~3 times higher than that at physiological IS (170 mM). We conclude that the observed stiffness changes reflect an IS dependence of titin elasticity. Although these results will ultimately help understanding the molecular basis of titin elasticity, further studies at different SLs and in a variety of muscle tissues are necessary to establish the contribution of Ig domains and of PEVK segments to the IS-dependence of myofibril stiffness.

MUSCLE REGULATORY PROTEINS III

W-Pos185

A METHOD TO STUDY PUTATIVE EF-HAND Ca^{2+} BINDING SITES USING A MODEL Ca^{2+} BINDING PROTEIN ((Qi Li, Georgianna Guzman, Paul D. Allen*, and James D. Potter)) Dept. of Mol. & Cell. Pharmacology, Univ. of Miami School of Medicine, Miami, FL 33136, *Dept. of Anesthesia, Brigham & Women's Hospital, Boston, MA 02115

There are many reported putative EF-hand sequences in the literature whose Ca^{2+} and Mg^{2+} binding properties are unknown. They are often found in large membrane associated proteins that are insoluble, present in low abundance, and therefore, difficult to study. To measure the metal binding properties of these sites, we have developed (Li, *et al.*, Biophys. J. 68:A403, 1995) a mutant of carp parvalbumin (PVCD) which contains only one active metal-binding site (CD site). To test putative Ca^{2+}-binding site sequences, we have replaced the helix-loop-helix (***hlh***) sequence (33 res.) of the CD site with the ***hlh*** sequences of Ca^{2+}-specific site II of chicken skeletal TnC (PVCD-TnCII), and putative ***hlh*** sequences from the ryanodine receptor (PVCD-RyR1-1) and the polycystic kidney disease protein (PVCD-*PKD2*) (Mochizuki, T. *et al.*, Science 272:1339, 1996). The Ca^{2+} binding properties of the site in PVCD-TnCII were essentially the same as in the native protein suggesting that the new environment for this site in PV did not alter its binding properties. Since the metal binding properties of this site were unchanged, it is likely that the metal binding properties of transplanted putative ***hlh*** sequences will also be unchanged. The metal binding properties of PVCD-RyR1-1 and PVCD-*PKD2* will be presented.

W-Pos186

INCREASING THE NUMBER OF EF-HAND ACID PAIRS INCREASES THE RATES OF CA^{2+} EXCHANGE. ((Songtao Wang*, Samuel E. George@ & J. David Johnson*))
*Dept. of Med. Biochem., The Ohio State Univ. Med. Center, Columbus, OH 43210 and @Dept. of Cardiology, Duke Univ. Med. Center, Durham, NC 27710.

The C-terminal of cardiac troponin C (TnC) has two Ca^{2+}-Mg^{2+} sites which exhibit ~ 20-fold higher Ca^{2+} affinity than the two Ca^{2+} specific sites in the C-terminal of calmodulin (CaM). Substitution of the third EF-hand of TnC for the third EF-hand of CaM in CaM[3TnC], produced an ~6-fold increase in C-terminal Ca^{2+} affinity (George et al. J. Biol. Chem. 268, 25213-20, 1993). To delineate the mechanism for this increased Ca^{2+} affinity, chimeric CaM's were produced with contained subcomponents of the third EF-hand of TnC: CaM[loop3TnC], CaM[helix5TnC] and CaM[helix6TnC]. We determined the Ca^{2+} affinity and the rates of Ca^{2+} exchange with these chimeric proteins. CaM[loop3TnC] exhibited a 35-fold faster Ca^{2+} on-rate and a 22-fold faster Ca^{2+} off-rate than CaM, but only a 1.6-fold higher Ca^{2+} affinity. Helix 6 and helix 5 of TnC's third EF-hand supported the rapid Ca^{2+} on-rate of loop 3 and produced a 17-fold reduction in loop 3 Ca^{2+} off-rate, explaining the high Ca^{2+} affinity of TnC's third EF-hand. The Ca^{2+} binding loop of the third EF-hand of TnC has 2 (X&Z) acid pairs while the corresponding Ca^{2+} binding loop of CaM has no acid pairs. Our results indicate that the introduction of these acid pairs into the third EF-hand of CaM produce dramatic increases in Ca^{2+} on and off-rates with little change in Ca^{2+} affinity. The greater helical hydrophobicity of the third EF-hand of TnC slowed Ca^{2+} off-rate and increased Ca^{2+} affinity. It is possible that electrostatic repulsion of acid pairs in the Ca^{2+} binding loop produce a more open conformation which allows faster rates of Ca^{2+} exchange. (Supported by HL48662 and DK33727)

W-Pos187

SOME COMMENTS ON THE ROLE OF THE SMOOTH MUSCLE CALDESMON. ((**Katsuhide Mabuchi**)) Muscle Research Group, Boston Biomedical Research Institute, Boston Ma 02114

Immuno-electron microscopy on isolated chicken gizzard native thin filaments revealed that caldesmon (CaD) does not distribute uniformly along the filaments, rather it forms clusters, leaving spaces (>200 nm) which appear wide enough for myosin to interact with the bear portions of actin filaments (AFs). Such interaction could enable muscles to contract without the inhibitory action by the C-end CaD being removed. Thus, the clustered distribution of CaD seems to explain the lack of the evidence for the CaD's inhibition on the actomyosin ATPase *in vivo*. The role of the C-end CaD, therefore, must be modulatory rather than an on/off switch. CaD molecules within a cluster arrange themselves in such way that N-ends of a CaD molecule on one actin strand and of the second CaD molecule on the opposite actin strand appear on the same side of an AF (made of two strands) with a half molecular length displacement of ~40 nm. With this arrangement, the ends of all CaD molecules in a cluster could face the same myosin filament (MF). The long, central helical region of CaD, specific to the muscle isoform makes this arrangement possible. The N-ends CaD could guide MFs from one cluster to another, forcing MFs to move somewhat obliquely to AFs. This possibility is based on the ability of the N-end CaD to enhance the non-force bearing actin-myosin interaction in the presence of ATP. Why is such guiding needed in smooth muscles? In skeletal muscles MFs move toward Z-discs, an arrangement that limits the degree of shortening to ~60% of the resting length. The smooth muscles can shorten down to 25% of the resting length, suggesting a need of MFs to slide longer distance without colliding to the dense bodies, structures presumably equivalent to the Z-discs (Supported by a grant form NIH, P01-AR41637).

W-Pos188

CHARACTERIZATION OF AN N-TERMINAL CALDESMON FRAGMENT EXPRESSED BY THE BACULOVIRUS EXPRESSION SYSTEM. ((S. Zhuang and C.-L. A. Wang)) Muscle Research Group, Boston Biomedical Research Institute, 20 Staniford St., Boston, MA 02114.

A recombinant fragment, N240, corresponding to the N-terminal region (from residue 1 to 240) of chicken gizzard caldesmon has been over-expressed in insect cells (High-Five™) using the baculovirus expression system. When purified without heat treatment by DE-52 column chromatography, N240 migrated on the SDS gel with an apparent molecular weight of 40,000. N240 exhibited weak interactions with F-actin, as evidenced by co-sedimentation experiments and electron microscopic visualization; such interactions were abolished upon chymotryptic digestion. Binding of N240 to myosin was also examined. Undigested N240 co-sedimented with both phosphorylated and unphosphorylated smooth muscle myosin. Based on the results of fluorescence titrations using N240 labeled with acrylodan at Cys-153 and smooth muscle HMM, the binding affinity was estimated to be around 1.8×10^8 M^{-1}. Binding of N240 to HMM was weakened by Ca^{2+}/calmodulin, although such an effect was not detected by co-sedimentation, and although N240 did not bind to calmodulin-Sepharose column. These observations are consistent with the previous findings that caldesmon contains both a myosin-binding site and an actin-binding site in its N-terminal region, and further suggest that such binding plays a physiological role in tethering myosin to the thin filaments. The exact location of the interacting sites on both N240 and myosin, as well as the mechanism of calmodulin's action, are now under investigation. Supported by a grant from NIH (AR41637).

W-Pos189

TRYPTOPHAN RESIDUES IN CALMODULIN-CALDESMON INTERACTION.((S.P.Graether, T.Y.K. Heinonen, W.H. Raharjo, J.-P. Jin and A.S. Mak)) Queen's University, Kingston, Ontario and University of Calgary, Calgary, Alberta, Canada.

Calmodulin has been shown to interact with the COOH-terminal domain of gizzard h-caldesmon at three sites, A (residues 658-666), B (residues 687-695) and B' (residues 717-725), each of which contains a Trp residue. To determine the contribution of each of the three Trp residues in the calmodulin-caldesmon interaction, we have mutated the Trp residues to Ala in the COOH-terminal domain of fibroblast caldesmon (CaD39) and studied the effects on calmodulin-binding by fluorescence measurements and using immobilized calmodulin. Wild type CaD39 binds with a Kd of 0.13 x 10^{-6} M and a stoichiometry of 1 mol calmodulin per mol caldesmon. Replacing Trp 659 at site A or Trp 692 at site B to Ala reduces binding by 22 and 31 fold, respectively and destabilizes the CaD39-calmodulin complex by 1.75 kcal mol^{-1} and 1.94 kcal mol^{-1}, respectively. Mutation of both Trp 659 and Trp 692 to Ala further reduces binding with a Kd of 6.1 x 10^{-4} M and destabilizes the complex by 2.17 kcal mol^{-1}. On the other hand, mutation of Trp 722 at site B' to Ala causes a much smaller decrease in affinity (< 5 fold) and results in a destabilization energy of 0.87 kcal mol^{-1}. To investigate the relative importance of the amino acid residues near each Trp residue in the caldesmon-calmodulin interaction, deletion mutants were constructed lacking site A, site B and site A+B. Although deletion of site A decreases binding of CaD39 to calmodulin by 13 fold, it results in tighter binding than mutation of Trp 659 to Ala at this site, suggesting that the residues neighbouring Trp 659 may contribute negatively to the interaction. Deletion of site B causes a similar reduction in binding as observed for replacing Trp 692 to Ala at this site, indicating that Trp 692 is the major binding determinant at site B. Deletion of both site A and site B drastically reduces binding by 62 fold. Taken together, these results suggest that Trp 659 and Trp 692 are the major determinants in the caldesmon-calmodulin interaction and that Trp 722 in site B' plays a minor role.

W-Pos190

THE FUNCTIONAL CONSEQUENCE OF TRYPTOPHAN MUTATIONS IN THE 10kDa C-TERMINAL FRAGMENT OF CHICKEN GIZZARD CALDESMON
((Pia A.J. **Huber**, *Barry A. Levine, Iain D.C. Fraser, O'neal Copeland, Mohammed EL-Mezgueldi and Steven B. Marston)) Cardiac Medicine, Imperial College School of Medicine at the National Heart and Lung Institute, Dovehouse St. London SW3 6LY, UK; *University of Birmingham, Birmingham B15 2TT, UK

The interaction of caldesmon with Ca^{2+}-calmodulin involves three sites, A, B and B', including the tryptophans 659, 692 and 722 respectively, in the C-terminal domain 4 of chicken caldesmon. We have now used the well described, fully functional, recombinant, 10 kDa C-terminal fragment, 658C, (residues 658-756) for the construction of a mutant with only one tryptophan left (722, in the B' site). This recombinant peptide, termed Cg1, has mutations in site B, EW^{692}LTKT to PGHYNN, in addition to the mutation in site A, $KSMW^{659}$ to GSMG, which is already present in 658C. We have measured the binding of Cg1 (and 658C) to Ca^{2+}-calmodulin by measuring changes in tryptophan fluorescence and by cosedimentation of Cg1 or 658C with calmodulin-Sepharose. Cg1 bound Ca^{2+}-dependent to calmodulin with an affinity of 1/7 compared to 658C. Cg1 and 658C bound to actin and to actin-tropomyosin with a similar affinity. These interactions were also probed in NMR experiments. The observed signal changes indicate that Ca^{2+}-calmodulin makes equal and intimate contact to both tryptophans 692 and 722 of 658C, however it restricts its interaction to tryptophan 722 in Cg1 and hardly affects the aromatic residues His or Tyr in the mutated B site. Some signal changes of His or Tyr in titrations with actin indicated however that the mutation affected the actin binding to a much lesser extent. In functional assays we found that Cg1 was able to inhibit the actin-tropomyosin activated myosin ATPase. 50% inhibition was achieved with 2µM 658C whereas the same level of inhibition required 7µM Cg1. Ca^{2+}-calmodulin was not able to reverse this Cg1 induced inhibition. We conclude that site B is not essential for actin or calmodulin binding but it is essential for the coupling of Ca^{2+}-calmodulin binding to the release of caldesmon induced inhibition.

W-Pos191

NMR SOLUTION STRUCTURE OF CALCIUM-SATURATED CARDIAC MUSCLE TROPONIN C. ((**Samuel K. Sia**[1], **Monica X. Li**[1]*, **Leo Spyracopoulos**[1], **Stéphane M. Gagné**[1], **John A. Putkey**[2], **and Brian D. Sykes**[1])) [1]MRC Group in Protein Structure and Function, Department of Biochemistry, University of Alberta, Edmonton, Canada T6G 2H7 and [2]Department of Biochemistry and Molecular Biology, University of Texas at Houston.

Contraction of the cardiac muscle is marked first by a transient 100-fold increase in the Ca^{2+} concentration in the cytosol of the muscle cell, followed by a cascade of events which leads to the activation of the Mg^{2+}-ATPase and the formation of myosin-actin cross-bridges. It is the function of cardiac troponin C (cTnC) to bind to the Ca^{2+} ions released from the sarcoplasmic reticulum, and to relay this signal to the rest of the troponin complex (*i.e.* TnI and TnT) via a conformational change. We have solved the three-dimensional structure of cardiac TnC, with the mutations C35S and C84S, in the Ca^{2+}-saturated state by using heteronuclear, multidimensional NMR spectroscopy. The structural C-domain of cTnC resembles the C-domain of skeletal TnC and calmodulin, however, contrary to all the predicted models for cTnC, the structure of its regulatory N-domain is significantly more compact relative to skeletal TnC. This phenomenon can be untimately attributed to the inactive Ca^{2+}-binding site I of cTnC which prevents Glu-40 from acting as a pivot to open and close the B-helix. In addition, only one strong hydrogen bond can be identified in the β-sheet of the regulatory domain, as opposed to two hydrogen bonds in the β-sheet of all other known pairs of Ca^{2+}-binding EF-hands. This single hydrogen bond may provide a pivot point which allows the β-strands cross at an acute angle. These findings will be important in explaining the different properties of cardiac versus skeletal muscle contraction, and will allow for more accurate understanding and design of cardiac sensitizer drugs.

W-Pos192

NEUTRON SCATTERING REVEALS A LARGE-SCALE CHANGE IN THE *IN SITU* SHAPE OF TROPONIN I, BUT NOT TROPONIN C, UPON CALCIUM ADDITION TO WHOLE TROPONIN. ((**R. A. Mendelson**[1], D. B. Stone[1], I. Krylova[1], F. Reinach[2], D. Schneider[3] and P. A. Timmins[4])) [1]C.V.R.I, and Dept. Biochem. & Biophys., Univ. Calif. San Francisco, CA 94143; [2]Univ. São Paulo, São Paulo, Brazil; [3]Brookhaven National Laboratory, Upton, NY; [4]Institut Laue-Langevin, 38042 Grenoble Cedex, France.

Small-angle neutron scattering was used to investigate the radius-of-gyration (R_g) of TnI and TnC within the whole troponin complex with and without regulating concentrations of Ca^{2+} present. Deuterated (chicken) fast skeletal muscle TnC and (SH-less) TnI, expressed in *E. coli* grown on deuterated algal hydrolysate in D_2O, were purified and combined with recombinant (protonated) chicken fast skeletal muscle subunits into a reconstituted whole Tn complex which displayed normal regulation of the actin-activated MgATPase of myosin subfragment 1. Scattering experiments were performed at BNL on beamline H9b (TnC) and at ILL on D11 (TnI). Samples for neutron scattering were in a 41% D_2O solvent in which protonated troponin subunits (C and T or I and T) were rendered "invisible". Results show that TnI in whole Tn is a highly elongate structure. When Ca^{2+} binds to the TnC regulatory sites, TnI undergoes a large-scale structural change ($\Delta R_g/R_g$= -12%) resulting in a less elongate structure. On the other hand, TnC in whole Tn exhibits no detectable large-scale change in structure when Ca^{2+} binds to its regulatory sites. Modeling of low- and wide-angle data is currently underway to elucidate the *in situ* shapes of TnI and TnC. (Supported by NSF MCB-9404705)

W-Pos193

EFFECTS OF TROPONIN C MUTANTS WITH ALTERED Ca^{2+} AFFINITY ON FORCE REGULATION IN SKELETAL MUSCLE. ((Erik R. Rennie, J. David Johnson, Lawrence B. Smillie* and Jack A. Rall.)) Depts. of Physiol. and Med. Biochem., Ohio State University, Columbus, OH, 43210 and *Dept.of Biochem., University of Alberta, Edmonton, Alberta, Canada T6G 2H7.

To investigate the role of Ca^{2+} affinity for troponin C (TnC) in contraction, avian TnC mutants with increased or decreased Ca^{2+} affinity were examined. Using F29W as a spectral probe, Ca^{2+} bound half-maximally at pCa 6.3, 5.8 and 5.4 to F29W/M82Q, F29W and F29W/N-helix deleted (residues 1-11 deleted) mutants, respectively at 20 °C. Consistent with their differing Ca^{2+} affinities, Ca^{2+} dissociated from these mutants at 28 s^{-1}, 138 s^{-1} and 265 s^{-1} respectively at 10 °C. TnC mutants were incorporated into skinned rabbit psoas fibers from which TnC was extracted until force in pCa 4 was reduced by an average of 90%. Reconstitution with mutants led to force recovery ≥ 90% of the maximum value in unextracted fibers. Isometric force versus pCa curves exhibited the following order of decreasing pCa_{50} values at 15 °C: M82Q/F29W (6.13 ± 0.01), unextracted (6.04 ± 0.01), wild-type (6.01 ± 0.01), N-helix deleted (5.69 ± 0.02). Unextracted and wild-type were not significantly different form each other but were significantly different from the mutants. Thus decreasing the affinity of TnC for Ca^{2+} produces a corresponding rightward shift in the force versus pCa curve. These preparations will be utilized to examine the effects of altered TnC Ca^{2+} affinity on the rates of contraction and relaxation using caged calcium and caged calcium chelators. (Support from NIH AR20792.)

W-Pos194

CROSSBRIDGE INHIBITION WITH Pi ANALOGS CAUSES LITTLE CHANGE IN SKELETAL TROPONIN C CONFORMATION ((**D.A. Martyn**, P.B. Chase, A.M. Gordon and L.L. Huntsman)). Center for Bioengineering, Dept. of Physiology and Biophysics and Dept. of Radiology, University of Washington, Seattle, WA 98195

Cycling acto-myosin crossbridges (XBr's) are thought to contribute to activation of the thin filament. To test this hypothesis we selectively extracted endogenous troponin C (sTnC) from single skinned rabbit psoas fibers and reconstituted the fibers with sTnC labelled with the fluorophore 5-dimethylaminonaphthalene-1-sulphonylaziridine (DANZ) at Met 25, near regulatory Ca^{2+} binding site I. All changes in fluorescence (FL) are expressed as FL obtained under the test conditions relative to that obtained in pCa 9.2 relaxing solution ($dFL/dFL_{9.2}$). Fiber FL was corrected for background by subtracting the FL without the fiber in the viewing field. Upon maximal Ca^{2+} activation of the fiber (pCa 4.0), $dFL/dFL_{9.2}$ increased to 1.56 ± 0.21 (± SEM, n = 4). At pCa 4.0 steady isometric force was inhibited with either 0.5 mM aluminofluopride (AlF_4^-) or 1.0 mM vanadate (Vi). AlF_4^- inhibited force to 0.07 ± 0.06 (± SEM, n = 4) of that obtained in pCa 4.0 without AlF_4^-, while the corresponding level of force inhibition with Vi was 0.15 ± 0.08 (± SEM, n = 3 fibers). On the otherhand, inhibition of force with AlF_4^- resulted in no significant change in $dFL/dFL_{9.2}$ (1.46 ± 0.07; ± SEM, n = 4), while inhibition of force with Vi resulted in a small decrease in $dFL/dFL_{9.2}$ (1.25±0.06; ±SEM, n = 3). $dFL/dFL_{9.2}$ was also measured during the recovery from force inhibition with either AlF_4^- (5-10 minutes for full recovery) or Vi (2-3 minutres for full recovery). In each case, $dFL/dFL_{9.2}$ remained unchanged from the level at pCa 4.0, even though force increased continuously throughout recovery from inhibition. Thus, if XBr's are an important contributor to thin filament activation, it occurs with minimal alteration of TnC structure. Supported by NIH Grants HL-51277, HL-52558 and NS 08384.

W-Pos195

STEADY-STATE AND TIME-RESOLVED FLUORESCENCE STUDIES ON BARNACLE TROPONIN C_2 ($BTNC_2$) WITH SINGLE TRYPTOPHAN. ((**Y. Zhang**, Z. Luo, A.M. Gordon, and C.K. Wang)) Dept. of Physiology & Biophysics, Univ. of Washington, Seattle, WA 98195.

Two recombinant $BTnC_2$s containing a single tryptophan F66W and F145W, respectively, have been studied with steady-state and time-resolved fluorescence spectroscopy. With excitation at 290 or 295 nm, emission peak of apo-mutant F145W was at 345 nm. Addition of Mg^{2+} and/or Ca^{2+} to the mutant resulted in a blue shift by 15 nm with an increase in fluorescence intensity. Emission peak of apo-mutant F66W was at 340 nm and remain unchanged in the presence of cations with an insignificant change in emission intensity. The results suggested that (1) $BTnC_2$ is sensitive to not only Ca^{2+}-binding, but also Mg^{2+}-binding; (2) neighboring environment of Trp145 becomes more hydrophobic in the presence of Mg^{2+} and/or Ca^{2+}; and (3) Trp66 was in a polar environment and remained unchanged in the presence of Mg^{2+} and/or Ca^{2+}. In the studies of time-resolved fluorescence decay, the protein was excited and its emission was detected at 295 and 340 nm, respectively. The anisotropy decay for apo-$BTnC_2$(F145W) was best represented by a bi-exponential function with two rotational correlation times ϕ_1=0.79 ns and ϕ_2=6.10 ns. ϕ_1 is attributed to the Trp side chain motion and ϕ_2 reflects the overall motion of the protein. The rotational correlation time of 6.10 ns (ϕ_2) is comparable to the value of 6.46 ns calculated for a 17 kDa hydrated sphere with a partial specific volume of 0.73cm^3/g and a 20% hydration, suggesting that $BTnC_2$ protein is likely symmetric. Mg^{2+} binding resulted in an decrease in ϕ_2 with no change in ϕ_1, suggesting that Mg^{2+} induced a conformational change and enhanced the mobility of whole molecule. ϕ_2 became 6.47 ns and ϕ_1 remained unchanged when Ca^{2+} was added to Mg^{2+}-bound $BTnC_2$. Anisotropy decay measurements for $BTnC_2$(F66W) showed that (1) changes in ϕ_2 were similar to those of $BTnC_2$(F145W) in response to binding of cations; (2) the mobility of Trp66 side chain was much faster than that of Trp145; and (3) the motion of Trp66W side chain was slowed downed by Mg^{2+} and Ca^{2+}. I$^-$ quenching results showed that Trp66 was more accessible than Trp145, suggesting that neighboring environment of Trp66 was more positively charged.
(Supported by NIH HL52558 & NS 08384).

W-Pos196

COMMUNICATION BETWEEN TROPONIN-C and -I REVEALED BY SINGLE MOLECULE FLUORESCENCE SPECTROSCOPY AND FRET
((Y.Ishii, T.Funatsu, **T.Wazawa**, T.Yoshida, J.Watai M.Ishii and T.Yanagida)) Biomotron Project, ERATO, JST, Mino, Osaka 562, Japan

Signal transduction involves changes in protein-protein interactions. The changes in the interaction between troponin components, troponin-C and troponin-I in response to Ca^{2+} was monitored in a single molecular level, by directly visualizing fluorescence resonance energy transfer (FRET) from a single donor molecule to a single acceptor molecule in aqueous solution. Cys-98 of troponin-C was labeled with tetramethylrhodamine as a donor and Cys-133 of troponin-I with Cy5 as an acceptor. The fluorescence spectrum for single molecules was taken successfully with the total internal reflection fluorescence microscopy (Funatsu et al. *Nature* 374, 555 (1995)) and the FRET was monitored with this technique. The FRET efficiency was almost the same in average between in the presence and absence of Ca^{2+}. However, its diversity over individual molecules and its variation with time were greater in the absence of Ca^{2+} than in its presence, indicating that troponin-C and -I interact more flexibly in the absence of Ca^{2+}. Thus, we could monitor the FRET in a single molecular level and demonstrate how the diversity and dynamics of the protein-protein interaction is modified by Ca^{2+}.

W-Pos197

FUNCTIONAL MAPPING OF CHARGED RESIDUES IN THE N-DOMAIN OF TROPONIN C ((T. Kobayashi, Y. Maéda and J.H. Collins)) Int'l Inst. Advanced Res., Matsushita El. Ind. Co., Kyoto, 619-02 JAPAN, and Univ. Maryland Biotechnology Inst., Baltimore MD, 21201.

In order to investigate the role of charged residues of the N-terminal, regulatory domain of troponin C (TnC), we have expressed and purified five mutants of rabbit fast skeletal muscle TnC: TnC(R44A), TnC(E53A/E54A), TnC(E60A/E61A), TnC (R81A) and TnC(E85A/D86A). Each mutant TnC has one or two charged residues replaced by uncharged Ala residue. The mutated residues surround the hydrophobic patch in the N-terminal domain of Ca-saturated TnC. All mutants, like wild type TnC, bind to TnI in a Ca-dependent manner and release the TnI inhibition of acto-S1 ATPase activity. Ternary Tn complexes containing TnC(E53A/ E54A) or TnC(E85A/D86A) inhibited the acto-S1 ATPase activity even in the presence of Ca. The ability of these two mutant TnCs to bind to TnT was impaired, judging from native gel electrophoresis. In ternary Tn complexes containing these TnC mutants, TnI did not undergo expected conformational changes, as detected by limited chymotryptic digestion. On the other hand, TnC(E60A/E61A), which failed to activate acto-S1 ATPase activity in the presence of Ca, did bind to TnT. These results indicate that TnT interacts directly with the N-terminal domain of TnC in a Ca-dependent manner, as part of the extensive protein-protein interaction network in the ternary complex of TnC, TnI, and TnT.

W-Pos198

CALCIUM-INDUCED STRUCTURAL TRANSITION IN CARDIAC TROPONIN-C. ((Leo Spyracopoulos, Monica X. Li, Samuel K. Sia, Stephané M. Gagné, Murali Chandra*, R. John Solaro*, and Brian D. Sykes)) MRC Group in Protein Structure and Function, Department of Biochemistry, University of Alberta, Edmonton, Alberta, T6G 2H7 and *Department of Physiology and Biophysics, University of Illinois at Chicago, Chicago, Illinois, 60612.

Muscle contraction is mediated by calcium-induced structural changes within the troponin complex of the thin filament. In cardiac muscle, the binding of calcium to the N-domain of troponin C (cNTnC) is the principal regulatory event which leads to muscle contraction. Despite the high sequence similarity of the skeletal and cardiac proteins, the cardiac isoform is unable to coordinate calcium in one of the two binding sites in the N-domain and the implications for myocardial contraction are unclear. In order to investigate the molecular basis of the regulation of contraction in cardiac muscle, we have solved the three-dimensional solution structures of the apo- and Ca^{2+}-saturated states of cNTnC using multidimensional multinuclear magnetic resonance experiments. The solution structures of the two states reveal that the structural transition upon calcium binding exposes residues within the hydrophobic core of the regulatory domain. However, the structural details of this opening differ significantly from the analogous opening in the skeletal N-domain. The solution structures provide, for the first time, the detailed molecular mechanism by which myocardial contraction is triggered.

W-Pos199

CALCIUM REGULATION OF SKELETAL MUSCLE THIN FILAMENT MOTILITY *IN VITRO*. ((A.M. Gordon, M.A. LaMadrid, Y. Chen, Z. Luo and P.B. Chase)) Department of Physiology and Biophysics and Department of Radiology, University of Washington, Seattle, WA 98195.

Using an *in vitro* motility assay, we investigated Ca^{2+} regulation of sliding of thin filaments reconstituted from rabbit fast skeletal actin (labeled with rhodamine-phalloidin), troponin (Tn), and tropomyosin (Tm) on nitrocellulose surfaces coated with rabbit fast skeletal HMM. Filaments were visualized by epifluorescence and sliding measured for different [Ca^{2+}]'s at 30°C, 2mM MgATP, ionic strengths (Γ/2) of 0.085, 0.115 and 0.14M, and 0.4-0.7% methylcellulose. Intensified images were recorded on tape and analyzed using a Motion Analysis System. Reconstituted thin filaments exhibited well-regulated behavior when Tm and Tn were added to the motility solutions with no directed motion without Ca^{2+}. In contrast to F-actin (for which speed was unaffected by [Ca^{2+}]), the speed increased in a graded manner with [Ca^{2+}] while the number of regulated thin filaments moving was more steeply regulated. With increased Γ/2, Ca^{2+}-sensitivity of both the number of filaments moving and their speed shifted towards higher [Ca^{2+}] and was steepest at the highest Γ/2. The force-pCa relationship for rabbit psoas skinned fibers obtained under similar conditions (30°C, 0.14M Γ/2) paralleled the speed-pCa relationship for the *in vitro* regulated filaments. These results suggest that relatively few crossbridges are needed to make filaments move, but that many have to be cycling to make the regulated filament move at maximum speed. (Supported by NIH HL52558.)

W-Pos200

THE Ca^{2+} DEPENDENCE OF DICHROISM IN SKELETAL FIBERS CONTAINING RHODAMINE LABELLED TROPONIN C AND THE EFFECTS OF CROSSBRIDGE INHIBITION. ((D.A. Martyn, C. J. Freitag, and A.M. Gordon)). Center for Bioengineering and Dept. of Physiology and Biophysics, University of Washington, Seattle, WA 98195

The fluorescence polarization (dichroism) of iodoacetamide tetramethylrhodamine (Rh) labeled troponin C (TnC-Rh, labeled at Cys 98) was measured to monitor changes in probe orientation or order during Ca^{2+} and rigor activation of TnC reconstituted skinned rabbit psoas fibers. To test for the contribution of actomyosin crossbridges (XBr's) to thin filament activation, changes in dichroism were measured with steady force inhibition by aluminofluoride (0.05 mM AlF_4^-) and during recovery from AlF_4^- inhibition. Dichroism was 0.14 ± 0.01 (± SEM, n = 5) in relaxing solution (pCa 9.2) and decreased to 0.004 ± 0.004 (± SEM, n = 5) when force was maximally activated at pCa 4.0. Dichroism was more sensitive to Ca^{2+} (~0.2 pCa unit), than the corresponding changes in force. During rigor contraction at pCa 9.2 dichroism decreased to 0.086 ± 0.01 (± SEM, n = 5), while force was 0.40 ± 0.05 (± SEM, n = 5) of pCa 4.0 control. At pCa 4.0 in rigor, dichroism decreased further to 0.025 ± 0.002 (±SEM, n = 5), slightly above the pCa 4.0 control level; force was similar to pCa 9.2 rigor. Thus, rigor XBr's are able to effect a change in the orientation or order of the Rh probe on TnC. Inhibition of cycling XBr's at pCa 4.0 with 0.5 mM AlF_4^- decreased force to 0.02 ± 0.01 of pCa 4.0 control (± SEM, n = 3). On the other hand, AlF_4^- inhibition caused dichroism to increase only slightly to 0.02 ± 0.01 (± SEM, n = 3), relative to pCa 4.0 control (0.014 ± 0.01; ± SEM, n = 3). Likewise, during the slow recovery from AlF_4^- inhibition of force, dichroism changed little from that found during steady state inhibition. Thus, cycling XBr's contribute much less to activation dependent changes in TnC-Rh dichroism than does Ca^{2+} binding to TnC. Supported by NIH Grants HL-51277 , HL-52558 and NS 08384.

W-Pos201

A ROLE FOR ACTIN IN MEDIATING TROPOMYOSIN-TROPONIN REGULATION OF STRIATED MUSCLE THIN FILAMENTS: *IN VITRO* MOTILITY ANALYSIS SHOWS ACTIN MUTANT E93K SWITCHES TROPOMYOSIN TO THE OFF STATE.

((Wu Bing*, Azam Razzaq ,† John Sparrow† and **Steven Marston***))
Cardiac Medicine, Imperial College School of Medicine
at the National Heart and Lung Institute, Dovehouse Street, LONDON SW3 6LY, U.K. † Department of Biology, University of York, York YO1 5YW, UK

According to current models tropomyosin can move so that it either blocks access of myosin to actin (the OFF state) and hence crossbridge cycling or it is clear of the myosin binding site and cycling carries on unimpeded (the ON state). Troponin I is positively charged whilst tropomyosin is negatively charged, therefore we have investigated the possibility that charge on the surface of the thin filament plays a role in regulation. In the *Drosophila* flight muscle actin mutant E93K there is a charge reversal on the surface of actin close to the proposed position of tropomyosin when it is in the OFF state. Using a quantitative *in vitro* motility assay we have found that the wild type *Drosophila* actin behaved like rabbit skeletal muscle actin when tropomyosin and troponin were added at pCa5 and pCa9. In contrast the effect of tropomyosin upon E93K actin filament movement at pCa5 was completely different : velocity did not increase and the fraction of filaments motile was reduced to less than 10%. To confirm that the 'switch off' of E93K actin filament movement was due to tropomyosin binding to the actin in the OFF state rather than a different mechanism we added NEM-S-1 to E93K actin-tropomyosin. When NEM-S-1 was mixed with E93K actin-smooth muscle tropomyosin filaments we observed that it restored motility of the filaments to the level observed with E93K actin alone .This reversal of inhibition required less than 0.4 NEM S-1 per actin, thus confirming that it was acting cooperatively upon tropomyosin in the same way as it does in ATPase assays . We conclude that electrostatic charge on the surface of domain 2 of actin plays a critical role in determining the state of actin-tropomyosin that is a central feature of the steric blocking mechanism of actin filament regulation.

W-Pos202

CROSSBRIDGES-INDUCED MOVEMENT OF TROPONIN I RELATIVE TO ACTIN IN SKELETAL MUSCLE THIN FILAMENTS

((H.-C. Li, T. Palm, K. Sale and P. G. Fajer)) Dept. of Biol. Sci., Institute of Molecular Biophysics, Florida State University, National High Magnetic Field Laboratory, Tallahassee, FL 32306

The movement of TnI between TnC and actin is postulated to be the molecular switch for Ca^{2+} activation of muscle contraction (Tao *et al.* 1989 and 1990). Our present study tests whether a similar mechanism applies to the activation of contraction by binding of myosin heads to the actin filament. Following the protocol developed by Tao et al. (1990) we measured distances between defined sites on actin and TnI and between TnI and TnC in reconstituted thin filaments using fluorescence energy transfer. The distance between Cys133 of TnI and Cys374 of actin increased from 43Å to 57Å on binding of myosin heads to F-actin in the absence of Ca^{2+} and from 54Å to 62Å in the presence of Ca^{2+}. The S1 induced distance increase is similar to the Ca^{2+} induced movement previously determined by Tao et al. (1990). The magnitude of this S1 induced change might be bigger than the change induced by Ca^{2+}, implying the structural changes induced by binding of the myosin heads are different from those induced by calcium (Li and Fajer, 1994, Li et al. 1996). The S1 induced distance change between TnI(Cys133) and TnC(Cys98) is currently under investigation.

Tao, T., Gowell, E., Strasburg, G.M., Gergely, J., and Leavis, P.C. (1989) *Biochemistry* 28,5902-8.
Tao, T., Gong, B.-J., and Leavis, P.C. (1990) *Science* 247,1339-41.
Li, H. -C. and Fajer, P.G. (1994) *Biochemistry* 33,14324-32.
Li, H.-C., Hideg, K., and Fajer, P.G. (1996) *J. of Mol. Recog.* In press.

W-Pos203

STRUCTURAL CHARACTERIZATION OF SKELETAL TNT AND FRAGMENTS T_1 AND T_2.

((M. R. Snyder, J. Potter*, and F. G. Prendergast))
Mayol Clinic, Department of Biochemistry, Rochester, MN 55905 and *University of Miami, Department of Molecular and Cellular Pharmacology, University of Miami School of Medicine, Miami, FL 33101.

Contraction and relaxation of skeletal and cardiac muscle is regulated through the binding of calcium to troponin C (TnC), one subunit of the ternary troponin complex. A signal, induced by calcium binding to TnC, is transmitted through the remaining two subunits, troponin T (TnT) and troponin I (TnI). Eventually, this signal is received by actin and myosin, and contraction occurs. TnC, in addition to relieving the inhibition controlled by TnI, also activates the myosin-ATPase through a direct interaction with TnT (Potter, et al. (1995) **J. Biol. Chem. 270**, 2557-2562). In order to understand how the calcium-induced signal is transduced through the troponin complex, we have chosen to study the structure of TnT. Initial experiments, performed with TnT isolated from rabbit cardiac and skeletal muscle, indicate that cardiac TnT is approximately 50 angstroms longer and 20% more α-helical that skeletal TnT (Snyder, et al. (1996) **Biophys. J. 70**, A381). This work is now being focused on a recombinant form of rabbit skeletal TnT, as well as two mutants which correspond to the fragments T_1 (residues 1-149) and T_2 (residues 150-250). These proteins have been expressed in a bacterial system and purified,and are now being studied with respect to their secondary and tertiary structures and interactions. We believe these experiments will lead to a better understanding of the structure of TnT, as well as the overall arrangement of subunits within the troponin complex.

This work has been supported by NIH Grant AR37701.

W-Pos204

RECOMBINANT CARDIAC TROPONIN T IS BIOLOGICALLY ACTIVE IN SKINNED MYOCARDIUM.

((**P.A.W. Anderson***, N. N. Malouf†, R.Nassar*, A. J. Saunders†, A. Greig†, A. E. Oakeley*, J. Ackler†, B. K. Kay†, R. J. Solaro§ and G. J. Pielak†)). *Duke University Medical Center, Durham, NC 27710; †University of North Carolina at Chapel Hill, Chapel Hill, NC 27599; §University of Illinois at Chicago, Chicago, IL 60612.

Cardiac troponin T (cTnT) is expressed in multiple isoforms in the mammalian myocardium. The relative expression of the isoforms changes with development and with heart disease. As a first step towards studying the putative differences in isoform function, we expressed two recombinant rabbit isoforms, $rcTnT_1$ and $rcTnT_4$, in Sf9 cells using the baculovirus system. We characterized the proteins using spectropolarimetry and Western blots and tested their activity by extracting endogenous cTnT in detergent-skinned myocardial bundles using rcTnT ($rcTnT_1$ in 2 bundles, $rcTnT_4$ in 3 bundles, and $rcTnT_1$+$rcTnT_4$ in one bundle) and reconstituting the troponin molecule by adding cTnI and cTnC. We verified extraction of the endogenous troponin by the absence of the bundles' response to calcium and by SDS-PAGE and Western blot analysis. We obtained a force *vs.* pCa curve from each of six bundles before and after extraction-reconstitution at the same sarcomere length. The pCa_{50} and Hill coefficient were respectively 5.82± .20 and 1.9±.2 (mean±sd, n=6) before extraction, and 5.87±.31 and 1.7±.2 after reconstitution. Conclusion: recombinant cTnT expressed in Sf9 cells can replace the endogenous protein in skinned myocardium and is biologically active.

W-Pos205

Specific Interactions of the central region of Troponin I with the N-domain of Troponin C.

(Brian Tripet*, Jennifer Van Eyk and Robert S. Hodges) Dept of Biochemistry, University of Alberta, Edmonton T6H 2H7, Canada. (Spon. R.S.Hodges)

It is well established that the regulatory complex of troponin (consisting of the three proteins, troponin T, I, and C) changes conformation upon the binding of calcium to trigger muscle contraction. Although extensive studies into each of the individual proteins have been able to identify several sites of interactions and functions, the exact mechanism of the conformational change transmitted throughout the three proteins in the ternary complex is still not completely understood. Synthetic peptide approaches have been very significant in delineating sites of interactions between the three proteins and the thin filament that depend upon the presence or absence of calcium, and thus the changes in the ternary complex.

Using synthetic peptide analogs of a specific region of the Troponin I protein (residues 96-148) in the present studies, we have been able to identify several regions which specifically interact with the actin thin filament and Troponin C in a Ca^{2+} dependent manner. More specifically, we have been able to identify that the region adjacent to the inhibitory region (residues 96-115 of troponin I which cause the inhibition of muscle contraction) are essential for the rapid and full release of the inhibitory region from actin. This effect appears to be a direct result of the specific interactions of this region with the N-domain of Troponin C in the Ca^{2+} state.

W-Pos206

ACTIVATION OF P_2 PURINORECEPTORS INDUCES CYTOPLASMIC CALCIUM MOBILIZATION IN CEREBELLAR BERGMANN GLIAL CELLS

((**Voitenko N**, Kirischuk S. and Verkhratsky A.)) Bogomoletz Inst. of Physiology, Kiev, Ukraine

ATP receptor mediated Ca^{2+} signalling was recorded from Bergmann glial cells in mice cerebellar slices. To measure the cytoplasmic concentration of Ca^{2+} ($[Ca^{2+}]_i$), either individual cells were loaded with fura-2 using the whole-cell patch-clamp technique or slices were incubated with fura-2/AM and the imaging system was focused on individual cells. Extracellular application of 100 μM ATP caused a transient elevation of $[Ca^{2+}]_i$. The rank order of potency for the purinoreceptor agonist was:ADP≥ATP> UTP>>AMP=Adenosine=α, βmethylene-ATP. ATP-triggered Na^{2+} transients were reversibly inhibited by P_2 purinoreceptor agonist suramin (100μM). The involvement of P_2 metabotropic receptors is inferred by the observation that ATP mediated cytoplasmic Na^{2+} transients were not associated with measurable change in membrane conductance. The $[Ca^{2+}]_i$ increase was due to release from intracellular stores since responses were still observed in Na^{2+}-free extracellular solutions and irreversibly blocked by thapsigargin.

W-Pos207

ANALYSIS OF CHANGE IN CIRCULAR DICHROISM INDUCED BY BINDING OF α–BUNGAROTOXIN TO nAChR FRAGMENTS *((* **Qing-luo Shi,** M.A Grant and Edward Hawrot*))* Dept. of Molecular Pharmacology, Physiology and Biotechnology, Brown University, Providence, RI 02912

Synthetic peptides α185-196 (the 12mer) and α181-198 (the 18mer) of the nicotinic acetylcholine receptor (nAChR) from *Torpedo* can bind to α-bungarotoxin (BGTX) with considerable affinity. Our previous studies have shown that an equimolar mixture of BGTX with either of the two fragments can produce a circular dichroism (CD) spectrum distinctly larger than the summation of the CD spectra for the two components in their unbound states. In order to explore the nature of binding-induced CD, we extendsd our study to include two larger nAChR fragments, the 32mer (α173-204) and the 68mer (α137-204). CD studies on mixtures of BGTX/nAChR fragments in the presence of 0.02% sodium dodecyl sulfate (SDS), which has been reported (Donnelly-Roberts & Lentz, Molecular Brain Reserach, 19:55,1993) to greatly enhanced the affinity of nAChR fragments for BGTX were also undertaken.
It is observed that fragments with higher affinity for BGTX (longer fragments) tend to produce less binding- induced CD. In the case of the 68mer, which shows an affinity for BGTX identical to that for intact nAChR, the binding-induced CD is too small to be significant. While the 18mer shows the largest binding-induced CD of the four nAChR fragments, this CD is drastically reduced in the presence of 0.02% SDS. The CD spectra of the components in their free states show that 0.02% SDS strongly affects the secondary structure of only the 18mer. A higher concentration of SDS (0.06% SDS) abolishes the β–type CD curve of BGTX, in correlation with reports that 0.06% SDS abolishes BGTX/nAChR fragment binding.
Our observations argue for a dominant contribution from nAChR fragments to the binding-induced CD seen upon complex formation. Supported by NIH Grant# GM 32629

W-Pos208

IN SITU DOWN REGULATION OF ATRIAL M_2 RECEPTORS ASSESSED BY IN VITRO MEASUREMENT OF $I_{K(ACh)}$. ((**M. Bünemann**, B. Brandts and L. Pott)) Dept. of Physiology, Ruhr University Bochum, D-44780 Bochum, Germany

Long lasting down regulation of M_2 receptors can be induced in cultured atrial myoyctes from hearts of adult guinea-pigs by 24 h exposure to Cabachol [Bünemann et al. (1996) J. Physiol. 494: 351-362]. Down regulation is characterized by a slowing of onset of muscarinic K^+ current ($I_{K(ACh)}$) by ACh and a shift of EC_{50} to higher concentrations of ACh. Using measurement of $I_{K(ACh)}$ as sensitive assay of M_2 activation, we studied to what extent the physiological vagal tone causes down regulation of M_2R in the intact animal. In myocytes cultured in the absence of agonist the EC_{50} for activation of $I_{K(ACh)}$ by ACh was shifted from 110 nM (day 0) to 27 nM (day 6), without a change in I_{max}. Sensitization was accompanied by a significantly increased rate of activation of $I_{K(ACh)}$ by step changes to a saturating concentration of ACh (2 μM), $t_{1/2}$ being reduced from 550 ± 125 ms to 395 ± 120 ms. Concomitantly deactivation following washout of the agonist was slowed with time in culture. Using our previously published formalism, it can be shown that sensitization of the M_2-G_K-$I_{K(ACh)}$ pathway in culture reflects an about fourfold increase in density of M_2-receptors. Density of other receptors converging on this signaling pathway (A_1, sphingolipid [Bünemann et al. (1996) EMBO J., 20: 5527-5534]) did not measurably change in culture. We suggest that sensitization of atrial myocytes in culture to ACh reflects recovery from down regulation of M_2 receptors caused by tonic vagal activity in the intact animal. Supported by DFG (Po212/6-2)

W-Pos209

A COMPACT STRUCTURE FOR IgG BOUND TO FcRn INDICATED BY FLUORESCENCE RESONANCE ENERGY TRANSFER MEASUREMENTS.

((**P.S. Pyenta**, T. Cameron, P.J. Bjorkman*, D. Holowka, B. Baird))
Cornell University, Department of Chemistry, Ithaca, New York 14853
*California Institute of Technology, Division of Biology, Pasadena CA

The Fc receptor for IgG found in the intestinal lining of neonates (FcRn) transports maternal antibodies from ingested milk into the newborn's circulatory system. Fluorescence resonance energy transfer (FRET) measurements were performed to compare head-to-tail distances of an anti-dansyl IgG in the absence and in the presence of recombinant extracellular FcRn (Gastinel, et al. *PNAS* 89:638, 1992). The fluorescent donor is fluorescein-5-maleimide, conjugated to a sulfhydryl in cysteine that replaces serine at the C-terminus of the C_H3 domain (Shopes, *J. Immunol.* 148:2918, 1992), and the acceptor is dansyl-eosin located in the Fab binding sites. The measured head-to-tail FRET efficiency is 0.12±0.02 and 0.24±0.02 in the absence and presence of FcRn respectively. This suggests that, in contrast to the extended and flexible conformation expected for IgG in solution, IgG is more compact when complexed to FcRn. Another indication that the bound structure is different is our observation that the dissociation time for dansyl-eosin bound to IgG-FcRn (130 s) is approximately three times slower than that for dansyl-eosin bound to free IgG in solution.

W-Pos210

SYNERGISM FOR Mg2+ AND GLUCOSE TRANSPORTS IN HEART FOLLOWING INSULIN ADMINISTRATION. ((A. Romani, V. Matthews and A. Scarpa)) Dept. Physiology and Biophysics - Case Western Reserve University, Cleveland, OH, 44106.

Insulin antagonizes several of the cellular effects mediated by β-adrenergic receptor activation and cAMP formation. Several reports also indicate that β-adrenoceptor stimulation induces a marked extrusion of Mg2+ from several mammalian cell types. To investigate whether insulin administration could counteract the observed mobilization of Mg2+, rat hearts were perfused in a retrograde Langerdoff system with a Ringer medium devoid of Mg2+, and the changes in extracellular Mg2+ content were measured by atomic absorbance spectrophotometry (AAS). The pre-treatment of the hearts with 6 nM insulin completely abolished the Mg2+ extrusion induced by the β-adrenergic agonist isoproterenol or by the cell permeant 8-Cl-cAMP. When similar experiments were performed in the presence of a broad range of Mg2+ concentrations (10 uM to 500 uM) in the extracellular medium, insulin induced a marked accumulation of the cation into the cells. This accumulation was small but detectable in the presence of 25 uM [Mg2+]ex and became more evident at 50 or 100 uM [Mg2+]ex. Insulin-mediated Mg2+ accumulation was abolished when the hearts were perfused with a medium devoid of glucose, when cytochalasin-B or phloretin were used to inhibit glucose transport, and when glucose was replaced with an equiosmolar concentration of 2-deoxy-glucose or 3-methyl-O-glucose, suggesting a link between Mg2+ accumulation and glucose transport. Insulin-stimulated 14C-glucose accumulation into the cells was measured in the presence of the reported concentrations of [Mg2+]ex. No accumulation of 14C-glucose occurred when 10-15 uM [Mg2+]ex was present, a minimal concentration of 25-35 uM Mg2+ being required to observe a detectable transport. Glucose transport considerably increased when 50 uM Mg2+ was used, and it was almost maximal at 100 uM [Mg2+]ex. These results suggest that the insulin-stimulated accumulation of glucose via Glut4 transporters is associated with an uptake of Mg2+ into cardiac cells, and that extracellular Mg2+ may modulate Glut4 operation. The absence of Mg2+ uptake under conditions in which glucose was replaced with transportable but not metabolizable analogs would indicate that Mg2+ accumulation is connected with the metabolic utilization of glucose rather than with its transport via Glut4 operation. [Supported by NIH HL 18708 and Ohio Border of Regents-Research Initiation Grant].

W-Pos211

EFFECTS OF ETHANOL ON Mg2+ HOMEOSTASIS IN HEPATOCYTES. ((P Tessman; A. Romani)) Department of Physiology and Biophysics Case Western Reserve University, Cleveland OH, 44105-4920.

Several published studies indicate that an acute or chronic ethanol intake induces a marked decrease in serum and tissue Mg2+ content. To investigate the mechanisms involved in this process, rat livers were perfused with various concentrations of ethanol directly added to a Krebs-Henseleit medium devoid of Mg2+, and the Mg2+ content of the perfusate (contaminant and released) was measured using an Atomic Absorption Spectrophotometer (AAS). In the absence of ethanol infusion, no extrusion of Mg2+ from perfused livers occurred. By contrast, when various ethanol concentrations (0.1%, 1%, and 10%) were administered for a short period of time, a marked, dose-dependent Mg2+ extrusion from the livers was observed. This extrusion was noticeable immediately after the addition of ethanol, reached the maximum approximately 6-8 minutes after the administration of ethanol, and persisted for a few minutes after withdrawal of alcohol from the system. No detectable release of K+ or LDH accompanied the release of Mg2+ induced by ethanol. Furthermore, when 1% DMSO was used in place of ethanol, no Mg2+ was extruded from the organs, suggesting that the ethanol-induced Mg2+ extrusion was attributable to a selective impairment of Mg2+ transport and/or regulatory mechanisms, rather than a non-specific effect. Previous observations from this and other laboratories indicate that cellular Mg2+ could be extruded following adrenergic stimulation, likely through the activation of a putative Na+/Mg2+ antiporter. To investigate whether the ethanol-induced Mg2+ extrusion occurs through the operation of this pathway, livers were perfused with ethanol prior to isoproterenol or cAMP infusion. Ethanol pre-treatment markedly reduced the amount of Mg2+ mobilized by the subsequent administration of isoproterenol or cAMP, thus suggesting the activation of a common mechanism of extrusion. To determine whether Mg2+ was transported by the Na+/Mg2+ antiporter, livers were perfused with ethanol in the presence of 1 mM amiloride, as an inhibitor of the Na+ dependent Mg2+ transporter. Under these conditions, the extrusion of Mg2+ induced by 1% ethanol was decreased by ~50%. Similar experiments were also performed on collagenase-dispersed hepatocytes. The addition of varying doses of ethanol also induces a dose-dependent Mg2+ extrusion in this experimental model. Following the achievement of a maximal Mg2+ extrusion, digitonin was used to permeabilize the plasma membrane, and release the residual Mg2+ content present in the cytosol. The cells treated with ethanol released a larger concentration of Mg2+ from the cytosol into the incubation medium, suggesting that a major redistribution of Mg2+ among cytosol and intracellular organelles occurred during ethanol treatment. Taken together, these experiments demonstrate that: a) ethanol causes an efflux of Mg2+ from the cell, by affecting the Mg2+ transport mechanisms either directly or indirectly through an increase in cytosolic cAMP; b) Mg2+ is extruded from the cell via the operation of the amiloride-sensitive Na+/Mg2+ antiporter and, possibly, an additional non-inhibitable mechanism; and c) ethanol appears to determine a redistribution of Mg2+ within the cell, thus enlarging the pool of Mg2+ which can be extruded across the plasma membrane. [Supported by NIH-HL 18708 and Ohio Board of Regents-Research Initiation Grant].

W-Pos212

EFFECTS OF EXTRACELLULAR NUCLEOTIDES ON $[Ca^{2+}]_i$ IN RAT OSTEOCLASTS. ((**A.F. Weidema**, S.J. Dixon and S.M. Sims)) Dept. Physiology and Div. Oral Biology, The University of Western Ontario, London, Canada N6A 5C1.

It has previously been shown that extracellular ATP induces elevation of $[Ca^{2+}]_i$ in mammalian osteoclasts. In other systems, P2Y receptors are often coupled via G proteins to release of Ca^{2+} from intracellular stores, whereas P2X receptors are ligand-gated cation channels which can allow influx of Ca^{2+}. The aim of this study was to determine the pathways underlying the ATP-induced increase of $[Ca^{2+}]_i$ in osteoclasts. Single rat osteoclasts were monitored using fura-2 fluorescence and patch-clamp. ATP and other nucleotides (2-methylthio-ATP, ADP, ADPβS, ATPγS, UTP) caused elevation of $[Ca^{2+}]_i$ which, in many cases, consisted of an initial peak followed by a plateau. The nucleotide-induced elevation of $[Ca^{2+}]_i$ persisted upon removal of Ca^{2+}_o, indicating a dominant role for Ca^{2+} release from intracellular stores. Furthermore, inhibition of the endoplasmic reticulum Ca^{2+}-ATPase, with cyclopiazonic acid (1 μM) in zero Ca^{2+}_o, caused complete and reversible abolition of nucleotide-induced Ca^{2+} elevations. Following depletion of stores, readdition of Ca^{2+}_o caused transient elevation of $[Ca^{2+}]_i$, consistent with activation of capacitative Ca^{2+} influx. In patch-clamp studies, ATP activated a transient inward current (P2X) and then a Ca^{2+}-activated K^+current. Following decay of these currents, a third conductance became evident, which was inwardly rectifying and may be due to activation of CRAC channels. We conclude that extracellular ATP activates both P2X and P2Y purinoceptors on rat osteoclasts. Nucleotide-induced elevation of $[Ca^{2+}]_i$ appears to arise primarily through activation of P2Y purinoceptors leading to release of Ca^{2+} from intracellular stores. This study was supported by The Arthritis Society (Canada).

W-Pos213

VISUALIZING ODOR DETECTION IN SINGLE OLFACTORY CILIA BY MONITORING CALCIUM ENTRY THROUGH CYCLIC NUCLEOTIDE-GATED CHANNELS. ((**T. Leinders-Zufall**, M.N. Rand, G.M. Shepherd, C.A. Greer. and F. Zufall)) Sections of Neurobiology and Neurosurgery, Yale Univ. School of Medicine, New Haven, CT 06510.

Odor detection takes place in the cilia of olfactory receptor neurons (ORNs), resulting in a rapid rise in cAMP which activates cyclic nucleotide-gated (CNG) channels allowing Na^+ and Ca^{2+} to enter the cell. Transient elevations of intracellular Ca^{2+} levels play critical roles in the regulation of the odor sensitivity as part of the process that underlies olfactory adaptation, but such elevations have not been demonstrated in the olfactory cilia. We have analyzed the Ca^{2+} transients that occur in ORNs as a result of odor stimulation and CNG channel activity. To visualize ciliary Ca^{2+} signals, ORNs were loaded with fluo-3/AM and fluorescence was measured with a laser scanning confocal microscope. Brief odor stimuli increased fluorescence in the cilia and in other neuronal compartments but the ciliary signal occured first and was more transient. This fluorescence increase could be mimicked by the phosphodiesterase inhibitor IBMX, and was abolished by lowering external Ca^{2+} levels or by pharmacological blockade of CNG channels. This indicates that Ca^{2+} entry was the primary source of fluorescence increases. Membrane depolarization through elevated external K^+ was not sufficient to increase ciliary Ca^{2+} fluorescence. Interestingly, an odor pulse led to the generation of Ca^{2+} transients with very similar temporal characteristics simultaneously in all cilia. This suggests that the cilia of a given cell possess similar properties with regard to their odor sensitivity, and that a given ORN integrates signals from different cilia. Our results demonstrate the existence and spatiotemporal properties of Ca^{2+} transients in single olfactory cilia and show conclusively that CNG channels serve as a major pathway for Ca^{2+} entry into ORNs. Supported in part by NIH grants DC02227, DC00086, DC00210 and NS10174.

W-Pos214

IMMUNOLOCALIZATION OF β_1-ADRENERGIC RECEPTORS ON RAT VENTRICULAR MYOCYTES ((**Michael A. Laflamme**, H. Bindu Vanapalli, and Peter L. Becker)) Dept. of Physiology, Emory University School of Medicine, Atlanta, GA.

Ventricular cardiomyocytes possess a wide variety of G_S-coupled receptors, among these are the β_1- and β_2-adrenergic, prostaglandin E_1, histamine, and 5-HT receptors. Despite a common signal transduction pathway involving activation of adenylyl cyclase, not all such receptor systems are equally capable of enhancing contractility, even with an equivalent rise in total cellular cyclic AMP. We were interested in whether the distribution of the β_1-adrenergic receptor (AR) relative to other cellular structures might underlie its unique predominant influence over inotropy. To this end, we employed immunofluorescent techniques and confocal microscopy to localize β_1-ARs and other proteins involved in excitation-contraction coupling on isolated rat cardiac myocytes. Indirect labeling using a rabbit polyclonal anti-mouse β_1-AR IgG reveals that receptor to be widely distributed throughout the sarcolemma, the intercalated discs, and the transverse-tubules. Thus, it appears that a substantial number of β_1-ARs are located close to those proteins known to mediate adrenergic modulation of contractility.

W-Pos215

IMMUNOLIPOSOME TARGETING TO ACTIVATED VASCULAR ENDOTHELIAL CELLS. ((**D. Alford**[1], D. Spragg[2], R. Greferath[1], C. Larsen[1], C. Nicolau[1] and M.A. Gimbrone, Jr.[2])) [1]CBR Labs, Harvard Medical School, Boston, MA 02135; [2]Brigham & Women's Hospital, Harvard Medical School, Boston, MA 02115.

Vascular endothelial cells are potentially important targets for site-specific delivery of drugs or genes for modifying cell function. Targetable markers on these cells include E-selectin and VCAM-1, two endothelial cell-surface adhesion molecules which are upregulated in response to a variety of pathophysiological, proinflammatory stimuli. E-selectin is characteristically expressed at sites of acute inflammation, while VCAM-1 is expressed in early atherosclerotic lesions. Both molecules can be upregulated in cultured human umbilical vein endothelial cells (HUVEC) by exposure to certain reagents (e.g., IL-1). We developed immunoliposomes targeted to E-selectin by chemical attachment of the mAb H18/7. Both neutral and cationic immunoliposomes targeted to E-selectin bound to activated (pre-treated with IL-1, 4 h) HUVEC by an average of 50-fold more than to unactivated HUVEC. Specific association occurred at both 4 °C and 37 °C in the presence of serum-containing medium. The addition of excess free H18/7 antibody specifically blocked immunoliposome association to activated HUVEC. Liposomes without antibody or conjugated to a non-binding control mAb did not associate significantly to either activated or unactivated HUVEC. Neutral liposomes targeted to E-selectin and loaded with hydroxypyraninetrisulfonate (HPTS) appeared in intracellular, low-pH compartments two hours after binding to activated HUVEC, suggesting that immunoliposome entry into HUVEC occurs, at least in part, by a receptor-mediated endocytic pathway. Gene transfection studies using cationic immunoliposomes targeted to E-selectin and VCAM-1 are underway.

W-Pos216

IMAGE CORRELATION SPECTROSCOPY AND FLUORESCENCE PHOTOBLEACHING RECOVERY ANALYSIS OF THE SYNTHETIC GANGLIOSIDE NBD-GD1A. ((**Jonathan V. Rocheleau** and Nils O. Petersen)) Department of Chemistry, University of Western Ontario, London, Ontario, Canada N6A 5B7

Many animal viruses contain a membrane envelope surrounding their nucleic acid. In order for a membrane enveloped virus to infect a host cell, its membrane envelope must fuse with a target cell membrane. The first step in the fusion process is a binding step, a process that brings the virus membrane and cell membrane into close proximity. Sendai virus, a typical membrane virus, is thought to carry out this process with the aid of the membrane envelope bound virus protein Hemmaglutinin-Neuraminidase (HN). HN binds to sialic acid bearing moieties on the cell surface. One likely ligand for HN is the ganglioside GD1a, since it contains two sialic acids. It is our hope to carry out studies with a fluorescent derivative of GD1a, termed NBD-GD1a, that might provide mechanistic information about the binding of SV to live cells. The work presented will cover the characterization of an exogenously added fluorescent derivative of GD1a in terms of both its mobility and state of aggregation using both photobleaching recovery and Image Correlation Spectroscopy techniques.

W-Pos217

DOPAMINE RECEPTOR-MEDIATED CALCIUM RELEASE IN RAT PINEAL CELLS ((E. Rey and **L. Tabares**)) Dept. Physiology and Biophysics. Univ. of Seville. E-41009, Sevilla. Spain. (Spon. by J. López Barneo)

We have found that in pineal cells DA-induces intracellular Ca^{2+} increases. Intracellular Ca^{2+} was measured in single loaded Fura-2 cells by microfluorometry. The signal consisting in a transient peak response that declines to a sustained phase above the basal level. In the presence of EGTA (1mM), and without Ca-added in the extracellular solution, the transient response was maintained, whereas the plateau was eliminated. These results indicate that the DA-induced transient response was mostly due to mobilization of intracellular Ca^{2+}, whereas the sustained phase was dependent on extracellular Ca^{2+}. DA concentrations above 5 μM were required to elicit a detectable intracellular Ca^{2+} signal. The pharmacological profile of the receptor(s) that mediate this response is complex. DA-induced Ca^{2+} signals were sensitive to SCH 23390, a selective D1 type antagonist (IC_{50}: 1.7 μM), to Eticlopride (1-2 μM), a specific antagonist for the D2-type receptors (35 ± 32 % inhibition, mean ± s.d. n=9), and to prazosin (1 μM), a selective α_1-adrenergic receptor antagonist. DA-evoked responses were completely blocked by haloperidol (1 μM), while, the stimulation of α_1-adrenergic receptors with phenylephrine (1 μM), a specific agonist, was not affected by haloperidol, indicating that the increase of Ca^{2+} induced with DA is not mediated by the activation of α_1-adrenergic receptors. Propranolol (5-10 μM), a β-adrenergic antagonist, did not preclude the Ca^{2+} response elicited with DA suggesting that the increase of intracellular Ca^{2+} is not due to cAMP elevation. These results suggest that the DA-evoked Ca^{2+} responses in pineal cells are mediated by atypical DA receptors.

W-Pos218

THE EXTRACELLULAR LOOPS OF THE TRH RECEPTOR ARE RESPONSIBLE FOR SELECTIVITY IN TRH BINDING. ((**Anny-Odile Colson**[1], Jeffrey H. Perlman[2], Marvin C. Gershengorn[2] and Roman Osman[1])) [1]Mount Sinai School of Medicine, New York, NY 10029, [2]Cornell Medical College, New York, NY 10021.

Studies of the thyrotropin-releasing hormone receptor (TRH-R) indicate that the binding pocket of TRH (pyroGlu-His-ProNH$_2$) is positioned in the transmembrane domain (Laakkonen et al. Biochemistry, 1996, 35, 7651). To study the process of TRH binding, we have constructed a receptor model with extracellular loops. The initial construct was energy minimized and used to perform fourteen 60ps simulated annealings from 1500K to 300K followed by 100ps constant temperature simulations at 300K. Seven of the resulting structures cluster into a family with structurally related folds. These structures indicate that access to the binding pocket is occluded by the loops, suggesting that a static representation is incomplete. A dynamic analysis of changes in curvature and electrostatic properties of the surface defined by the loops, points to an intermittent formation of a putative entry point between loop 2 and loop 3. This crevice is generated primarily by K182 which lines the bottom of the entry point, and by E298 and F296 which principally form the rim. The structural stability of this putative entry point is supported by H-bonding of Y181 to the backbone of residues in the amino terminus of loop 2. Mutation Y181F reduces the affinity of TRH by ca. 5000 fold. These results suggest Y181 as a static determinant of receptor selectivity. To analyze the internal motions of the extracellular loops, a 1 ns trajectory at 300K was used to extract the quasi harmonic vibrational modes. Low frequency motions show an anticorrelated opening of a putative entry point between loop 3 and the C-terminus of loop 2. These results point to F296 and R185 performing large amplitude oscillations that form a path large enough for TRH to access its binding pocket. Our results therefore suggest that both recognition of specific elements on the receptor surface and loop motion are necessary for the ligand to access its transmembrane binding pocket.

Supported by US PHS Grant DK43036

W-Pos219

CHARACTERIZATION OF THE MUSCARINIC RECEPTOR SUBTYPE MEDIATING CATIONIC CHANNEL OPENING IN GUINEA-PIG ILEUM. ((**A.V. Zholos** and T.B. Bolton)) Department of Pharmacology & Clinical Pharmacology, St. George's Hospital Medical School, London SW17 0RE, U.K.

Mixed populations of muscarinic receptors (M_2 and M_3 subtypes) are coexpressed in gastrointestinal smooth muscles which contract in response to acetylcholine. The contractile response occurs due to both intracellular Ca^{2+} release and membrane depolarization produced by cationic channel opening. The contribution of the major M_2 population to contraction as well as the identity of the receptor producing membrane depolarization have not yet been established.

Cationic current evoked by stable muscarinic agonist carbachol (CCh) was studied using patch-clamp recording techniques in single guinea-pig ileal smooth muscle cells in which $[Ca^{2+}]_i$ was clamped with 10 mM BAPTA to minimise the effects of the PLC/IP$_3$ system. Concentration-effect curves (cationic conductance plotted *vs.* log[CCh]) had a mean EC_{50} of 8 μM and a maximal conductance, G_{max}, at saturating agonist concentration of 27±1 nS (n=114). Muscarinic antagonists with higher affinity for M_2 receptors produced a parallel shift to the right without reduction in G_{max}. Values of pK_b derived from conventional Schild plots were: tripitramine 9.1, methoctramine 8.1, himbacine 8.0. Thus, competitive antagonism with apparent affinities typical for the M_2 receptor was evident. Atropine, a non-selective antagonist, both shifted the dose-response curve (pK_b of 9.0) and reduced G_{max}. Thus, cationic channel is primarily opened by the M_2 receptor activation whereas M_3 receptors may play an important but as yet unknown modulatory role.

Supported by The Wellcome Trust

W-Pos220

IMAGE CROSS-CORRELATION SPECTROSCOPY ANALYSIS OF DIFFUSION, FLOW AND INTERMOLECULAR INTERACTIONS. ((**Mamta Srivastava** and Nils O. Petersen)) Department of Chemistry, The University of Western Ontario, London, Ontario, Canada N6A 5B7.

There are number of techniques which can measure rates of diffusion and flow on the surface of small objects such as living cells. These often fail when the rate of movement becomes too slow. On surfaces of cells diffusive movements at rates less than about 10^{-16} m^2/s and flow processes less than about 10^{-6} m/s are difficult to observe with techniques such as fluorescence photobleaching. In this work, we demonstrate that cross-correlation calculations of images taken at successive times can detect both diffusion and flow process significantly slower than the limits indicated above. The theoretical background and examples from studies of transferrin receptors on surface of living cells will be presented in details. In addition, we will show that the principles that govern image cross-correlation spectroscopy can be extended to measure the colocalization between two different species (receptors) on the cell surface. This is done by labelling the cell surface receptors by two different chromophores. Intensity fluctuations arising from samples containing two distinct type of labelled molecules are cross-correlated using two detectors with different wavelength sensitivities. Selected examples (with systems PDGF-Transferrin & EGF-Transferrin) of this principle will also be presented.

W-Pos221

GPI-ANCHORED FcγRIIIb SIGNALING: A PROPOSED MECHANISM. ((F.Y.S.Chuang[¶], **M. Sassaroli**[¶], J.C. Unkeless*)) *Dept. of Biochemistry and [¶]Dept. of Physiology & Biophysics, Mt. Sinai School of Medicine, New York, NY 10029-6574.

We have shown previously that: (1) GPI-anchored FcγRIIIb (CD16) and FcγRIIa (CD32) induce equivalent $[Ca^{2+}]_i$ transients upon crosslinking; (2) wortmannin, a PI 3-kinase inhibitor, is equally effective (K_i = 2-5nM) at blocking both $[Ca^{2+}]_i$ transients. These findings support the hypothesis that CD32 functions as the transmembrane signaling element for CD16. However, whereas ligation of CD32 to IV.3 Fab covalently coupled to glass, causes spreading of neutrophils (*frustrated phagocytosis*), similar ligation of CD16 to 3G8 Fab is without effect. The surface distribution of fluorescein-IV.3 (Fl-IV.3) and rhodamine-3G8 (Rh-3G8) was determined by fluorescence microscopy. Crosslinking of CD16 results in a rapid co-localization of CD32 in the CD16 cap. However, the fluorescence decay times of the capped Fl-IV.3 conjugates are unchanged from those outside the aggregates, indicating that the Fl donors and Rh acceptors are separated by distances greater than R_0 (≈5nM) for resonance energy transfer (RET). The cationic membrane probe $DiIC_{16}$ also concentrates in the receptor cap, suggesting that the membrane in this locus has physical properties distinct from the bulk. We propose that aggregation of CD16 induces co-partitioning of CD32, resulting in tyrosine kinase activation and signaling. (*Supported by PHS AI-24322*)

W-Pos222

CHIMERIC SUBSTANCE P RECEPTOR-GREEN FLUORESCENT PROTEIN: VISUALIZATION OF THE DISTRIBUTION AND DYNAMICS OF G-PROTEIN COUPLED RECEPTORS IN *XENOPUS* OOCYTES. ((C. A. Haskell*, T. R. McKnight#, **R. M. Shigeta*** F. E. Curry#, and M. R. Hanley*)) Depts of Biological Chemistry (*), and Human Physiology (#), University of California at Davis School of Medicine, Davis, California 95616

The *Xenopus* oocyte expression system is conventionally used for ectopic expression and functional characterization of channels and receptors. However, its usefulness for visualizing G-protein coupled receptors has not been evaluated. To evaluate G-protein receptor organization and trafficking in oocytes, we have utilized the jellyfish green fluorescent protein (GFP) as a recombinant C-terminal extension of rat Substance P receptor (SPR). When expressed in *Xenopus* oocytes by nuclear injection of plasmid DNA and analyzed by current response under voltage clamp, SPR-GFP was shown to be indistinguishable functionally from wild-type SPR. Using confocal microscopy, SPR-GFP was highly polarized in oocytes, with expression restricted to the surface of the animal pole. In the unstimulated state, SPR-GFP diffused freely in the X-Y plane, and was constitutively internalized in vesicles. Specifically, although substance P stimulation (10nM) led to desensitization of expressed SPR-GFP, no increase in receptor internalization was noted. Overall, these results suggest that the basal rate of membrane trafficking may be problematic for examining receptor endocytosis. Presently, we are examining the comparative trafficking dynamics of regulatory proteins in oocytes using similarly GFP-tagged β-arrestins.

W-Pos223

LPS-INDUCED TNF SECRETION AND APOPTOSIS IN RAT CARDIOMYOCYTES ((Kevyn Comstock, **Donald Martin**, Melinda Pedraza, Veronica Gutierrez, Kevin Krown, P.J. Quintana and Roger Sabbadini)). Biology Dept., San Diego State Univ, San Diego, CA 92182 (Sponsored by R.Sabbadini)

Bacterial endotoxin (LPS, lipopolysaccharide) is responsible for the cardiovascular collapse and death seen in patients with sepsis. In this study, we provide evidence suggesting that septic shock may be explained by LPS-stimulated secretion of tumor necrosis factor alpha (TNF) from cardiomyocytes, and that the cardiotoxic effects of TNF result from an autocrine effect of TNF acting on myocyte TNF receptors. Data are presented which demonstrate the presence of LPS and TNF receptors on cardiomyocytes. TNF is an inflammatory cytokine which is known for its negative inotropic effects on cardiac performance, but has not until recently shown to be produced by cardiac cells. LPS was found to strongly stimulate in a dose-dependent manner the secretion of TNF from cultured cardiomyocytes. Further, LPS-induced TNF secretion was blocked by inhibitors of TNF processing. Importantly, both TNF and LPS (presumably acting via TNF) produce apoptosis in cultured cardiomyocytes, suggesting an autocrine function for TNF. Cardiac cell apoptosis was blocked by preincubation with the soluble receptor fragment of TNF (TNFRI:Fc), indicating that the TNF-induced apoptosis was specific and receptor mediated. The percentage of apoptotic cells in the population was quantified by single cell gel electrophoresis of nuclei exhibiting DNA fragmentation patterns characteristic of apoptosis (i.e. cardiac comets). We postulate that the cardiodepressant effects of LPS seen in septic shock are not only due to acute negative inotropic effects of TNF but also may be complicated by TNF-induced apoptotic cell death which effectively reduces the number of working myocardial cells. Supported by MDA, AHA and NIH.

W-Pos224

STUDIES ON THE ROLE OF CYTOPLASMIC FREE-CA^{2+} IN CHEMOTAXIS IN *ESCHERICHIA COLI* K-12.
((**Harry J. Guttman**[1], Louis S. Tisa[4], M. Thomas Record, Jr.[1,2] and Julius Adler[2,3])) Departments of Chemistry[1], Biochemistry[2] and Genetics[3], University of Wisconsin-Madison, Madison, WI 53706. Department of Microbiology[4], University of New Hampshire, Durham, NH 03824.

Escherichia coli normally runs (straight-line movement) and tumbles (random rotation in one place). In mutants that always tumble, use of fluorescent Ca^{2+} dye indicators (e.g. fura-2) showed that the steady-state cytoplasmic free-Ca^{2+} concentration is elevated. Also, in normal *E. coli* the cytoplasmic free-Ca^{2+} decreases and increases, respectively, from the addition of attractants (which stimulate running) and repellents (which stimulate tumbling). To further delineate the role of Ca^{2+} in chemotaxis, we have measured the cytoplasmic free Ca^{2+} (using fura-2) in other mutant strains. This work is supported by the NIH and NSF; HJG is supported by an NIH postdoctoral fellowship.

ION MOTIVE ATPases

W-Pos225

POWER FREQUENCY ELECTROMAGNETIC FIELDS STIMULATE THE STRESS RESPONSE IN CELLS. ((**M. Blank**[1] and R. Goodman[2])) Columbia University Health Sciences, Departments of Physiology and Cellular Biophysics[1], and of Pathology[2] New York, NY 10032.

Interest in biological effects of power frequency electromagnetic (EM) fields continues to be stimulated by epidemiological studies that correlate increased risk of certain cancers with environmental exposures. Our studies provide significant insights into both the cell biology and the cell biophysics of the problem:

- Changes in biosynthesis following exposure to EM fields show that **EM fields stimulate the stress response**, a cellular reaction to stimuli that cells interpret as harmful.
- Studies of Na,K-ATPase ("ion pump" enzyme of cell membranes) function in electric and magnetic fields show that **signal transduction depends directly upon enzyme activity.** The different effects of electric and magnetic fields can be explained by the different degrees of penetration of the two fields and their **interaction with mobile charges in the enzyme**. This mechanism suggests that EM field-stimulated initiation of the stress response may involve direct interaction with DNA.

Both biological systems studied (stress response, ion pump enzyme) are present in virtually all cells, and the effects occur in the power frequency range at field levels that are slightly above normal background.
(We thank the DOE and the Heineman Foundation for their support.)

W-Pos226

CO-RECONSTITUTION AND CO-CRYSTALLIZATION OF THE SKELETAL MUSCLE CA^{2+}-ATPASE AND PHOSPHOLAMBAN. ((**Howard S. Young**[1], Larry R. Jones[2], and David L. Stokes[1])) [1]Structural Biology Program, Skirball Institute and Dept. of Cell Biology, New York University Medical Center, NY 10016, and [2]Krannert Institute of Cardiology, Department of Medicine, Indiana Univeristy School of Medicine, IN 46202.

Muscle contraction is initiated by the release of Ca^{2+} from the sarcoplasmic reticulum (SR) through the Ca^{2+} release channel, and relaxation is affected by recovery of Ca^{2+} into the SR by the Ca^{2+}-ATPase. In cardiac muscle, this process is regulated by β-adrenergic stimulation which causes enhanced contractility due to higher rates of contraction and relaxation. This regulation is mediated by phospholamban (PLB), an integral membrane protein of cardiac SR, which supresses Ca^{2+} pumping at physiological Ca^{2+} concentrations; β-adrenergic stimulation causes phosphorylation of PLB which relieves the inhibition of the Ca^{2+}-ATPase. For our crystallization studies, we have reconstituted the purified skeletal muscle Ca^{2+}-ATPase with recombinant PLB (expressed using the baculovirus system and purified from Sf21 insect cells) at a 1:5 molar ratio and at very low lipid:protein ratios, and verified the functional association between PLB and the Ca^{2+}-ATPase. We found that tubular crystals could be formed by incubation with deca-vanadate, similar to the skeletal SR. The frequency of tubular crystals depended on the conformation of the Ca^{2+}-ATPase and the fusigenicity of the proteoliposomes. Thapsigargin or cyclopiazonic acid were effective in stabilizing the E_2 conformation; fusigenicity was promoted either by careful choice of phospholipids or by adding small amounts of detergent ($C_{12}E_8$) during crystallization. Interestingly, the co-reconstituted Ca^{2+}-ATPase and PLB crystalized more readily than the reconstituted Ca^{2+}-ATPase with less of a dependence on fusigenic lipids or detergent, suggesting that PLB has an effect on both the conformation of the Ca^{2+}-ATPase and the physical properties of the lipid bilayer. We are now focusing on the preparation of frozen-hydrated specimens for cryo-electron microscopy. Preliminary results indicate that the helical symmetry and molecular packing in these reconstituted preparations are similar to those for the Ca^{2+}-ATPase from native skeletal SR. (Supported by NIH Grants HL48807 and HL28556 and HL06308)

W-Pos227

PROTEIN KINASE C ACTIVATES THE PLASMA MEMBRANE CALCIUM PUMP ISOFORM 4B BY PHOSPHORYLATION OF AN INHIBITORY REGION DOWNSTREAM OF THE CALMODULIN BINDING DOMAIN ((**A. Enyedi***, A.G. Filoteo, A.K. Verma, and J.T. Penniston)) Mayo Clinic, Rochester, MN 55905, USA and *National Institute of Haematology and Immunology, Budapest, Hungary

The plasma membrane calcium pump (PMCA) is an important element in regulating intracellular calcium concentration by removing the excess calcium from cells during calcium signalling. It is known to be regulated by calmodulin, acidic phospholipids and by phosphorylation with protein kinases. The carboxyl terminal region of isoform 4b of this pump has been shown to have a high affinity calmodulin binding domain, a downstream autoinhibitory region and a site for phosphorylation with protein kinase C. Using constructs of hPMCA4b lacking various numbers of residues at the carboxyl terminus, we studied the degree of phosphorylation by protein kinase C and the resultant activation of calcium transport. A specific phosphorylation site of this isoform occurred in a region of about 20 residues downstream of the calmodulin binding domain. This region has also been identified as an autoinhibitory region which is removed from its inhibition by phosphorylation with protein kinase C. Phosphorylation at this region activated the pump only partially leaving the inhibition by the calmodulin binding domain unaffected. (Supported by NIH grant GM 28835 and by the Howard Hughes Medical Institute.)

W-Pos228

THAPSIGARGIN-SENSITIVE ^{45}Ca ACCUMULATION IN RAT BRAIN MICROSOMES IS AUGMENTED BY OXALATE. ((**K.M. Wells and R.F. Abercrombie**)) Department of Physiology, Emory University School of Medicine, Atlanta, GA, 30322.

In rat brain microsomes incubated for 5 minutes, 50 mM oxalate revealed a thapsigargin (Tg)-sensitive portion, and increased the total, ATP-dependent ^{45}Ca accumulation. In the absence of oxalate, 1 μM Tg had no significant effect; in the presence of oxalate, 1 μM Tg decreased ATP-dependent ^{45}Ca uptake by 64%. Regardless of the presence of oxalate, 10 μM of the Ca ionophore A23187 released 90% of the ATP-dependent accumulation. Addition of 100 nM FCCP, alone or with Tg, had no significant effect, indicating little or no mitochondrial contamination. Addition of 10 μM digitonin decreased ATP-dependent uptake by approximately 20%, suggesting that a small part of the ^{45}Ca accumulation may be due to plasma membrane vesicles. Subsequent time courses of ATP-dependent uptake were therefore generated in the presence of 10 μM digitonin. In the presence of 50 mM oxalate, 100 μM EGTA, 90 μM Ca, and ~300 nM Ca^{2+}, all ATP-dependent ^{45}Ca uptake was linear up to 10 minutes, and the Tg-sensitive slope (41±12 nmol $g^{-1}s^{-1}$) was about 5 times larger than the Tg-insensitive slope (8±2 nmol $g^{-1}s^{-1}$). In contrast, ^{45}Ca accumulation under similar conditions, but in the absence of oxalate, plateaued within 4-5 minutes, and appeared insensitive to Tg. We conclude that oxalate, in addition to trapping luminal Ca^{2+} and linearizing uptake, significantly increases the apparent fraction of ATP-dependent ^{45}Ca uptake that is Tg-sensitive. Supported by NIH NS-19194.

W-Pos229

TISSUE DISTRIBUTION AND MEMBRANE TOPOLOGY OF THE GAMMA CHAIN OF THE SODIUM PUMP ((A.G. Therien[1], R. Goldshleger[2], S.J.D. Karlish[2] and R. Blostein[1])) [1] Department of Biochemistry, McGill University, Montreal, Canada and [2]Weizmann Institute of Science, Rehovot, Israel.

The Na,K-ATPase comprises a catalytic α, and a glycosylated β subunit. Another membrane polypeptide, γ, first described by Forbush et al., (Biochemistry, 17: 3667-3676, 1978) associates with α and β in purified kidney enzyme preparations and is involved in forming the site for cardiac glycoside binding. Tryptic digestion of the γ chain in right-side-out microsomes isolated from pig renal outer medulla shows that the N-terminal is extracellular. The γ chain is detected in Western blots as two bands, of which primarily the upper band is digested by trypsin. Rb ions protected partially against digestion, implying mutual interactions between the γ chain and cation sites. In experiments aimed to elucidate the structural basis for tissue-specific differences in behavior of pumps of the same (α1β1) isoform composition, we tested various tissues and cell lines expressing these pumps for the presence of γ using Western blot analysis. The results indicate that γ is not an obligatory subunit. Thus, with similar amounts of anti-α1-reactive membranes from tubules of renal outer medulla (ROM) and renal cortex (RC), renal glomerular cells, cultured glomerular cells, axolemma, red blood cells and rat α1-transfected HeLa cells, γ was detected in only ROM and RC. Coimmunoprecipitation of γ with α1 from Triton X-100-solubilized ROM confirms its association with α1. Although the function of γ remains unknown, it is intriguing that in the rat, K^+/Na^+ antagonism at cytoplasmic Na^+ activation sites as described previously (Therien et al., J. Biol. Chem. 271: 7104-7112, 1996) is relatively high only in ROM and RC. (Supported by the MRC of Canada, the Weizmann Institute Renal Research Fund, and a pre-doctoral fellowship from the Fonds FCAR to A.G.T.).

W-Pos230

N908-A933 OF RAT COLON H/K-ATPASE IN A CHIMERIC NA/K-ATPASE α3 SUBUNIT CONFERS PREFERENCE FOR ASSEMBLY WITH NA/K-ATPASE β1 SUBUNIT OVER GASTRIC H,K-ATPASE β. ((**Shyang-Guang Wang** and Robert A. Farley)) Dept. of Physiology & Biophysics, U. Southern California School of Medicine, Los Angeles, CA 90033.

Suzuki, Y. *et al* (Jap. J. Physiol. (1993) 43, 291-298) proposed that the rat colon H,K-ATPase (HK) α subunit requires a β subunit similar or identical to the Na/K-ATPase (NK) for function. Amino acids N886-A911 of rat NKα3 subunit, which are involved in assembly with β subunits, were replaced with the corresponding region (N908-A933) of the rat colon HKα subunit in order to determine what β subunit(s) can assemble with this chimera (NCH26) in yeast. NKα3 subunits assemble equally well with NKβ1 and HKβ subunits. More [^{3}H]ouabain-binding complexes were formed by NCH26+NKβ1 than NCH26+HKβ, consistent with the suggestion of Suzuki. The K_d for ouabain binding to NCH26+NKβ1 (58 nM) was higher than K_d for NK α3+NKβ1 (7.4 nM). Mutations were made in NCH26 in the HKα sequence in order to identify residues that are important in β subunit selection. In one mutant, R898 in NCH26, which is Y, F, or W in all NKα subunits and gastric HKα, was replaced with tyrosine. The ouabain B_{max} for the mutant was higher when expressed with HKβ or with Rβ1 than B_{max} for either β subunit expressed with NCH26. The K_d for ouabain binding to the R898Y mutant with NKβ1 was 14 nM. The complementary mutation (Y898R) in NKα3 reduced the number of pumps assembled with both β subunits without affecting the ouabain K_d value. The data support the possiblity that the NKβ subunit assembles with the colon HKα subunit, and indicate the importance of Y898 of NKα3 in the functional assembly with β subunits. (Supported by NIH grant GM28673).

W-Pos231

NONENZYMATIC GLYCATION OF CA^{2+}-ATPASE FROM RABBIT SKELETAL MUSCLE SR. **((Bannikuppe D. Shivanna and Elizabeth S. Rowe)) VA Medical Center Kansas City, MO 64128 and Department of Biochemistry and Molecular Biology, University of Kansas Medical School.**

Nonenzymatic glycation of proteins by long-term incubation with glucose leads to the formation of toxic and immunogenic Advanced Glycation End-Products("AGEs"). AGEs are implicated in the pathophysiology of aging and the complications of diabetes. To investigate the potential mechanisms and the pathways by which integral membrane proteins are glycated during long term exposure to glucose in diabetes, we investigated the effect of nonenzymatic glycation on the function of Ca^{2+}-ATPase from skeletal SR using ribose *in vitro*. Ribose was used instead of glucose because the kinetics of glycation by ribose were considerably faster than by glucose (Khalifah et al., Biochemistry 35 4645-4654, 1996). Glycation of Ca^{2+}-ATPase in native SR with ribose showed inhibition of the ATPase hydrolytic function, decreased calcium transport ability, and concomitant formation of AGEs. The ribose reaction did not show any preferential effect toward the calcium and magnesium bound conformational states of the enzyme. The loss in ATPase function and the formation of AGEs followed different kinetics and showed dependence on the ribose concentration. Our results show that glycation of SR Ca^{2+}-ATPase significantly inhibits Ca^{2+}-ATPase function. In addition, it may represent a general effect of chronic hyperglycemia on membrane function. (supported by the Department of Veterans Affairs).

W-Pos232

TRANSIENT KINETICS OF ANTHROYLOUABAIN BINDING TO DIFFERENT PHOSPHOENZYME FORMS OF THE Na^+/K^+-ATPase. ((**B. Yacono**[1], E.H. Hellen[2], and P.R. Pratap[2])) [1]Dept. of Biology and [2]Dept. of Physics and Astronomy, Univeristy of North Carolina at Greensboro, Greensboro, NC 27412

The Na^+/K^+ ATPase can be phosphorylated by ATP in the presence of Na^+ and Mg^{2+} (front door conditions) or by P_i in the presence of Mg^{2+} (back door conditions). The cardiac glycoside ouabain binds to the phosphoenzyme obtained under either condition. We are using anthroylouabain, a fluorescent derivative of ouabain, to investigate the transient kinetics of glycoside binding to these two forms of the phosphoenzyme and to relate them to the kinetics of inhibition. Anthroylouabain undergoes a large increase (~10×) in its fluorescence quantum efficiency upon binding to the enzyme, thus making it a good probe for investigating inhibitor binding. We find that at 25°C, the on and off rates for anthroylouabain binding are faster for the front door form of the phosphoenzyme than for the back door form. In addition we find that anthroylouabain photobleaches more easily when bound to the front door form of the phosphoenzyme than to the back door form. We are also measuring the rates of inactivation of the enzyme upon addition of either ouabain or anthroylouabain. These rates will be compared with rates for anthroylouabain binding to different forms of the enzyme, measured above.
Supported by a grant from the NIH.

W-Pos233

Plasmalemmal Vacuolar Type H^+-ATPases (pmV-ATPases) Regulate Intracellular pH (pH^{in}) in Microvascular Coronary Endothelial Cells (MCEC). ((J. D. Rojas[1], G. M. Martinez[1], A. Bezner[1], C.J. Meininger[2], G. Wu [2], D. E. Wesson[1], **R. Martinez-Zaguilán**[1])).[1]Departments of Physiology , Texas Tech University., H.S.C., 79430 Lubbock, TX; and [2]Texas A&M University, College Station, Tx.

pH^{in} regulation in most eukaryotic cells is mediated by the Na^+/H^+ exchanger (NHE) and HCO_3^--dependent transporting mechanisms, but some specialized and highly invasive cells (e.g., macrophages, osteoclasts, and metastatic cells) also utilize pmV-ATPases (Martínez-Zaguilán, Am.J. Physiol., 265: C1015, 1993; Clin. Exp. Metastasis, 14: 176, 1996). The relative contribution of these pH^{in} regulating mechanisms in any given cell type is not clear. Because angiogenesis (i.e., new blood vessel formation) involves invasion of adjacent tissues by microvascular endothelial cells we investigated if pmV-ATPases contribute to pH^{in} regulation in MCEC obtained from adult Sprague-Dawley rats. Cells grown on coverslips were loaded with SNARF-1, a pH fluorescent indicator, and its signal monitored by fluorescence spectroscopy. The steady-state pH^{in} of MCEC was 7.18 ± .07 (n = 30), at a pH^{ex}=7.15 in a HCO_3^- free media. This pH^{in} value was similar in the presence and absence of HCO_3^-, suggesting that HCO_3^- based transporting mechanisms are not the major MCEC pH^{in} regulatory mechanism. This is further supported by the observation that HCO_3^- addition to naive cells elicited only transient pH^{in} decreases followed by rapid recovery towards resting pH^{in}. This pH^{in} recovery was suppressed by V-H^+-ATPase inhibitors (bafilomycin and NBD-Cl). To evaluate the contribution of Na^+/H^+ exchanger, we acutely removed extracellular Na^+. This maneuver also caused a rapid pH^{in} decrease of ca. 0.2 pH unit, followed by a rapid recovery towards resting values. The abscence of a sustained pH^{in} decrease is consistent with the presence of an alternative pH^{in} regulatory mechanism. This Na^+-independent pH^{in} recovery was fully suppressed by V-H^+-ATPase inhibitors. We then performed acid-loading experiments using NH_4Cl in the absence of Na^+ and HCO_3^-. The kinetics of the pH^{in} recovery were monitored at pH^{ex} values ranging from 6.5 to 8.0, since we have previously shown that pmV-ATPases have an optimum pH. In agreement with our previous study, no pH^{in} recoveries were observed at pH^{ex} = 6.5, however pH^{in} recoveries ocurred at pH^{ex} >7.1. The pH^{in} recovery rates were not different in the presence or abscence of Na^+ or Na^+ plus HCO_3^-. The Na^+- and HCO_3^--independent pH^{in} recovery in MCEC's was abolished by V-H^+-ATPase inhibitors. These data indicate that pmV-ATPases comprise the major pH^{in} regulatory mechanism of MCEC's.

W-Pos234

CHANGES TO Na,K-ATPase α-SUBUNIT E779 SEPARATE STRUCTURAL BASIS FOR V_M AND ION DEPENDENCE OF Na PUMP CURRENT. ((RD Peluffo, JB Lingrel*, JM Argüello*, JR Berlin)) Bockus Research Inst., Philadelphia, PA; *Dept. of Molec. Genetics, Univ. of Cincinnati, Cincinnati, OH

The effects of changing E779, located in the 5th transmembrane spanning region of the Na,K-ATPase α-subunit, on the properties of Na pump current were investigated. HeLa cells were transfected with cDNA coding for E779A, E779D or E779Q substitutions in an ouabain-resistant sheep α_1 subunit (RD). Na pump activity was measured in patch-clamped cells at membrane potentials (V_M) from -100 to +60 mV as a 10 mM ouabain-sensitive current upon increasing extracellular K^+ (K^+_o) in the superfusion solution from 0 to 0.2 - 50 mM (Na^+ plus K^+ = 145 mM). Na pump current in RD cells had a positive slope at negative V_M and a K^+_o-dependent negative slope at positive potentials, similar to wild-type Na,K-ATPase. E779Q showed no change in $K_{1/2}$ for K^+_o at 0 mV, but the V_M dependence of Na pump current was abolished (see Table). Conversely, Na pump current in E779D had a similar V_M dependence as in RD but a 5-fold change in $K_{1/2}$ for K^+_o. These data demonstrate that a carboxyl group on residue 779 is critical for V_M dependence of Na pump current but is unrelated to K^+_o activation of the Na pump. Thus, the requirements for electrogenic ion transport are structurally distinct from those for ion binding. The loss of V_M dependence of Na pump current with mutant enzymes may reflect changes in rates and/or electrogenicity of ion transport steps. Analysis of presteady state reaction kinetics will distinguish between these mechanisms.

SUBSTITUTION	RD	E779A	E779D	E779Q
V_M dependence	Yes	No	Yes	No
$K_{1/2}$ at 0 mV (mM)	2.1 ± 0.4	7.4 ± 1.8	10.4 ± 1.6	2.1 ± 0.3

W-Pos235

EFFECT OF pH ON CHARGE MOVEMENT BY THE Na^+/K^+ PUMP IN *XENOPUS* OOCYTES. ((**K.A. Khater**, A. Vasilyev and R.F. Rakowski)) Department of Physiology and Biophysics, Finch University of Health Sciences/ The Chicago Medical School, North Chicago, IL 60064, USA.

Ouabain-sensitive pre-steady state transient currents were first described by Nakao and Gadsby (*Nature*. **323**: 628-30, 1986) in cardiac myocytes under conditions favoring electroneutral Na/Na exchange. Here we describe the pH dependence of transient currents over the range 5.6 to 9.6 using the cut-open oocyte technique. Two components of transient current were observed. The slow component was unaffected by alkaline pH, but appeared to be speeded by acidic pH. As pH was decreased from 7.6 to 5.6, the relaxation rate coefficient increased for both positive and negative pulses from the holding potential (–40 mV) and the relaxation rate constants became more steeply voltage dependent. The midpoint voltage and steepness of the Q-V relationship for the slow component, however, did not vary with pH. At alkaline pH, an additional ("intermediate") component of transient current was seen. The Q-V relationship of the intermediate component gave an exponential steepness factor of 0.31. When external [Na] was reduced from 100 to 50 mM the mid-point voltage shifted from –75.5±7.8 mV to –150±15 mV (calculated well depth 0.23). One explanation of the low pH data is that access to the binding sites of the second and third Na^+ occurs across an energy barrier. Protonation increases the barrier height thereby causing a decrease in reaction rates. The slowing of the intermediate component makes it more difficult to separate the two components so that it appears that the slow component has been speeded. The apparent rate of charge relaxation predicted by such a model increases as pH is lowered from 7.6 to 5.6 as was seen with the data. The model also predicts an increase in the voltage dependence of the relaxation rates at positive voltages. The predicted pK_a of the titratable site is about 5. Supported by NIH Grant NS-22979.

W-Pos236

FLUORESCENCE PROBES BOUND TO ATP-PROTECTABLE LYSINE RESIDUES IN NA^+,K^+-ATPASE CAN MONITOR ATP BINDING TO HIGH- AND LOW-AFFINITY SITES.((T. Tsuda, S. Kaya, T. Yokoyama, and **K. Taniguchi**)) Biological Chemistry, Graduate School of Science, Hokkaido University, Sapporo 060, Japan.

Na^+,K^+-ATPase preparations from pig kidneys were treated with 50 μM pyridoxal 5'-diphospho-5'-adenosine (AP_2PL) in the presence of NaCl. Resulting preparations contained 0.5 mol of the probe at the Lys 480 /mol α-chain. Modifications reduced both Na^+,K^+-ATPase activity and the amount of Na^+-dependent phosphoenzyme from ATP to ~ 50%, but not that from acetyl-phosphate (AcP). The addition of 1 mM AcP to the modified enzyme induced phosphorylation (3.0/s) followed by an AP_2PL fluorescence increase (1.2/s). The addition of 10 μM ATP instead of AcP induced rapid phosphorylation (28/s) followed by a slow fluorescence increase (1.0/s). When modified enzyme preparations were treated with fluorescein 5'-isothiocyanate (FITC), the phosphorylation capacity from ATP was reduced to around 5% with little influence on either the AP_2PL fluorescence change by ATP or phosphorylation from AcP. The addition of increasing concentrations of ATP with 160 mM NaCl to the K^+-bound AP_2PL-FITC-labeled enzyme showed quite different rate of each fluorescence change and affinity for ATP of the changes. These data and others indicate that the AP_2PL probe at Lys-480 can monitor ATP binding to high- and low-affinity sites, and suggest the simultaneous presence of two different low-affinity sites for ATP.

W-Pos237

INFLUENCE OF *V*-TYPE PROTON PUMP AND OUTWARD RECTIFYING CHLORIDE CHANNEL ON VESICLE pH. ((**P.H. Schlesinger**, with technical assistance by Soraya Gado)). Dept. Cell Biology and Physiology, Washington University Medical School, St. Louis, MO 63110.

Transport of protons for the acidification of intracellular vesicles is mediated by a multisubunit heteropolymer protein complex, the *v*-type H^+-ATPase. This complex also mediates the acidification of the bone resorption compartment of vertebrate osteoclasts. For this organelle it has been possible to isolate both the proton pump and an outwardly rectifying ~20 pS chloride channel which participates in the acidification by shunting charge produced by the electrogenic proton pump. Vesicles were prepared from the osteoclast resorption compartment membranes and have previously been characterized to rapidly acidify in a Mg^{++}-ATP and Cl-dependent fashion independent of Na^+ or K^+. Using this preparation we have explored the influence of ATP-dependent proton transport and Cl^- permeability upon the intra-vesicular pH. pH-gradient dependent accumulation and quenching of the acridine orange is demonstrated by adjustment of extravesicular pH to intra-vesicular pH where acridine orange fluorescence returns to it initial intensity. Using this null point we determined vesicle pH when the pump and the chloride channel are selectively inhibited. The proton pump was inhibited by 1-10 nM concentrations bafilomycin A_1, the chloride channel was reversibly inhibited by 100-250 μM DNDS. Partial inhibition of the proton pump resulted in a reduced rate of acidification but the same final intra-vesicular pH. In contrast partial inhibition of the chloride channel resulted in the same kinetics of acidification but a decreased extent of intra-vesicular acidity. Release from chloride shunting with valinomycin restored full acidification. This result is consistent with a model of intra-vesicular pH control that includes outward rectification of chloride currents. At negative vesicle potentials the channel will be in a low conductance state and therefore limiting to the transport of protons. In this condition any further modification of chloride conductance will influence vesicle pH and therefore exert regulatory effects on vesicle function.

W-Pos238

HETEROGENEOUS HALOTHANE BINDING IN THE SR Ca^{2+}-ATPase. ((**I. Fomitcheva**,[a] R.G. Eckenhoff,[b] M. Lopez,[a] and D. Kosk-Kosicka[a])), Johns Hopkins University, Dept. Anesthesiology, Baltimore, MD 21287[a] and University of Pennsylvania Medical Center, Dept. Anesthesia and Physiology, Philadelphia, PA 19104.[b]

The activity of various Ca^{2+}-ATPases is affected by volatile anesthetics, such as halothane, commonly used in clinical practice. The effect on the enzyme in skeletal muscle sarcoplasmic reticulum (SR) is biphasic, including stimulation at clinical anesthetic concentrations and subsequent inhibition at higher concentrations. We have previously proposed that the action of a volatile anesthetic on Ca^{2+}-ATPases results from its binding in the interior of the enzyme molecule [1]. Presently we investigated whether the anesthetic interacts directly with the SR Ca^{2+}-ATPase as evidenced by binding. Photoaffinity labeling with [^{14}C]halothane demonstrated that the anesthetic binds saturably to SR membranes, and that ≈80% of the binding is specific, with a K_i of 0.6 mM. The K_i value agrees well with the concentration at which halothane half-maximally activates the Ca^{2+}-ATPase in SR. SDS gel electrophoresis of labelled membranes indicates that 40-50% of [^{14}C]halothane incorporates into Ca^{2+}-ATPase, and 38-53% in lipids. Distribution of label among the three fragments produced by controlled tryptic digestion of the Ca^{2+}-ATPase suggests heterogenous halothane binding presumably in discrete sites in the enzyme. The results provide the first direct evidence that halothane binds to the Ca^{2+}-ATPase, potentially being related to anesthetic effect on enzyme's function. NIH GM 447130 (DKK) and 51595 (RE).

W-Pos239

THE MEMBRANE TOPOLOGY OF THE AMINO TERMINAL DOMAIN OF THE RED CELL Ca^{2+}-PUMP.((**Juan Pablo F.C. Rossi**, Pablo R. Castello, F. Luis González Flecha, Ariel J. Caride, and José M. Delfino)). Instituto de Química y Fisicoquímica Biológicas, Facultad de Farmacia y Bioquímica, Junín 956, 1113 Buenos Aires, ARGENTINA.

We have employed hydrophobic photoactivatable probes to localize regions of the erythrocyte Ca^{2+} pump inserted in the plasmic membrane. Using this experimental approach, we have identified three domains that include transmembrane regions (*Biochem. Biophys. Res. Commun.* **201**, 194, 1994) and particularly, two transmembrane segments in the N-terminal domain (*Biophys. J.* **70**, A328, 1996). This result is in agreement with predictions based on the primary structure employing three independent algorithms: Kyte and Doolittle, PHD and TMAP; and alignment with other P-ATPases. The consensus result from these analyses identified two sectors embedded into the membrane for the N-terminal domain, namely, between residues 102-119 (M1) and 150-168 (M2). Algorithms which predict secondary structures (Chou-Fasman, GOR and PHD) estimated a high proportion of alpha helix for M1 and similar probabilities of α and β structures for M2. In addition, we studied the periodic behavior of functions that evaluate hydrophobicity and variability at each position on the sequence of the calcium pump and its homologous family (hydrophobic and variability moments). Both functions maximize when rotation between neighboring residues is close to 100°, for either M1 or M2. With this value for the angle, the resulting vectors were calculated. These vectors were shown to point approximately in the same direction. This behavior is characteristic of transmembranic amphiphilic helices, where the face that shows the greatest variability is coincident with the most hydrophobic one, which is exposed to the lipidic face of the membrane.

Supported by grants from CONICET, UBACyT and Fundación Antorchas

W-Pos240

PROPERTIES OF THE UNIQUE CALMODULIN BINDING DOMAIN OF rPMCA3f ((**A.G. Filoteo**, A. Enyedi, A.K. Verma and J.T. Penniston)) Mayo Clinic, Rochester, MN 55905, USA and National Institute of Haematology and Immunology, Budapest, Hungary

Four genes and alternative splicings at two different sites produce more than twenty isoforms of the plasma membrane calcium pump. A special type of alternative splice that involves the calmodulin binding domain of rPMCA3 produced a unique carboxyl terminus which is much shorter than the corresponding region of the other isoforms, extending only five residues beyond its calmodulin binding domain. This isoform has been called rPMCA3f. A synthetic peptide (c28R3f) representing the calmodulin binding domain of rPMCA3f had a much lower calmodulin affinity (with a Ki of 10 nM) than the corresponding peptides of the "a" and "b" forms of rPMCA3 (Ki values are 0.1 and 1 nM, respectively). The characteristics of this domain were further analyzed by making chimeras of hPMCA4 with the carboxyl terminus of rPMCA3f. Although this chimera bound to calmodulin Sepharose, it was fully active without calmodulin and was not stimulated by calmodulin. These data indicate that the carboxyl terminus of rPMCA3f may not serve as an autoinhibitor of the enzyme. Subsequently, we raised a specific antibody against a peptide representing the carboxyl terminus of rPMCA3f. We will use this antibody to test the existence of this isoform at the protein level. (Supported by NIH grant GM 28835 and by the Howard Hughes Medical Institute.)

W-Pos241

DIFFERENCE INFRARED SPECTROSCOPY OF THE H^+,K^+-ATPASE. ((V. Raussens, J.M. Ruysschaert and **E. Goormaghtigh**)) Department of Physical Chemistry of Macromolecules at Interfaces, Free University of Brussels CP206/2, 1050 Brussels, Belgium.

The gastric H^+,K^+-ATPase is an α, β heterodimer which belongs to the P-type ATPase family. These ATPases are thought to share a common mechanism and a common structure. However, little is known about their structure and reactions which couple ATP hydrolysis to cation transport. We recently used infrared spectroscopy in combination with a proteolytic approach to gain information on the structure of the membrane embedded part of the H^+,K^+-ATPase. In the present work we prepared oriented membrane multilayers on a germanium internal reflection element by drying tubulovesicle membranes. Linear infrared dichroism indicates a preferential transmembrane organisation of the helices. UV circular dichroism obtained on multilayers deposited on quartz plates confirms this preferential orientation of the helices. In order to investigate the changes occurring in the ATPase molecule upon ligand binding (K^+, Mg^{++}, ATP, vanadate and Mg-vanadate), we designed an experimental method to overlay the membrane multilayer system with a flowing buffer. Switching between two buffers containing two different ligands allowed us to record ATR infrared spectra of structural and chemical intermediates relevant of the catalytic cycle of the enzyme. With a noise level in these measurements less than 10^{-4} absorbance units, changes at the level of 1 or 2 amino acid residues should be observed. Our measurements indicate that changes are of very small amplitude with however some reproducible features emerging from the noise level in difference spectra. The origin of these features remains to be assigned. At this stage of the study, we demonstrate that the amplitude changes occurring in the ATPase molecule bound to the ligands tested so far are extremely small, ruling out any major structural change.

W-Pos242

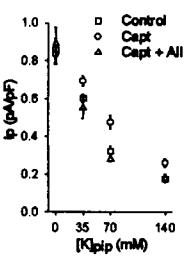
FAST TRANSIENT CURRENTS IN THE Na,K-ATPASE INDUCED BY ATP CONCENTRATION JUMP EXPERIMENTS FROM DMB-CAGED ATP. ((V.S. Sokolov, **H.-J. Apell**, J.E.T. Corrie* and D.R. Trentham*)) Dept. of Biology, Univ. Konstanz, D-78434 Konstanz, Germany and *Natl. Inst. for Med. Res., London NW7 1AA, U.K.

Electrogenic ion transport by Na,K-ATPase was investigated in a model system of protein-containing membrane fragments adsorbed to planar lipid bilayers. Sodium transport was triggered by ATP-concentration jump experiments in which ATP was released from an inactive precursor by an intense UV-light flash. Previous kinetic studies using the P^3-1-(2-nitrophenylethyl) ester of ATP (NPE-caged ATP) have been limited by the rate of photolytic release of ATP (~ 100 s^{-1} at pH 7 and 20°C). This is important for determination of the reaction sequence: Na_3E_1 + ATP → Na_3E_1·ATP → $(Na_3)E_1$-P + ADP (where $(Na_3)E_1$-P is the sodium-occluded form of the phosphorylated enzyme), which may have rates in this time domain. Therefore an alternative caged compound, DMB-caged ATP, the P^3-[1-(3,5-dimethoxyphenyl)-2-phenyl-2-oxo]ethyl ester of ATP (Thirlwell *et al.*, Biophys J. **67**, 2436-2447, 1994) was used which photolyses at > 10^5 s^{-1}. Under otherwise identical conditions ATP released from the DMB compound showed a significantly faster kinetics of the current transient compared to NPE experiments. In the presence of DMB-caged ATP the Na,K-ATPase showed kinetics independent of buffer pH range 6 to 8 and a current rise time independent of the concentration of the released ATP, in contrast to experiments with NPE-caged ATP. The rate constant of enzyme phosphorylation, subsequent to ATP binding (3.5×10^6 $M^{-1}s^{-1}$), was found to be 2000 s^{-1} at pH 7.2 and 20°C in buffer containing 150 mM NaCl.

W-Pos243

TREATMENT OF RABBITS WITH CAPTOPRIL ENHANCES THE NA^+:K^+ SELECTIVITY RATIO OF THE SARCOLEMMAL NA^+-K^+ PUMP. ((**K.A.Buhagiar**, D.F. Gray, P.S. Hansen, A.S. Mihailidou, H.H. Rasmussen.)) Cardiology Department, Royal North Shore Hospital, St Leonards, NSW, AUSTRALIA 2065 (Spon. by H. Rasmussen)

Treatment of rabbits with angiotensin converting enzyme inhibitors increases the apparent affinity of the Na^+-K^+ pump for intracellular Na^+ (Na^+_i) in cardiac myocytes. This increase could be due to an increase in the intrinsic affinity of binding sites for Na^+ or an increase in the Na^+_i:K^+_i selectivity ratio. Myocytes from control rabbits and rabbits treated with captopril (Capt) for 8 days were voltage clamped at -40 mV with ~1 MΩ patch pipettes containing 10 mM Na^+ and a concentration of K^+ ($[K]_{pip}$) of 0, 35, 70 or 140 mM (osmolality maintained constant with TMA.Cl). Pump current (I_p) was identified by the shift in holding current induced by 100 μM ouabain. Mean I_p (±SE) was significantly ($P < 0.05$) larger in myocytes from Capt treated rabbits than in myocytes from controls when $[K]_{pip}$ was 35, 70 or 140 mM (see figure). This difference was eliminated when myocytes from capt treated rabbits were exposed to 10 nM angiotensin II (AII) *in vitro*. A similar effect of exposure to 160 nM PMA was observed. Taken together these results suggest that background levels of AII regulate the Na^+_i:K^+_i selectivity ratio of the pump. The mechanism for this may involve protein kinase C.



INTERCELLULAR COMMUNICATION

W-Pos244

THREE-DIMENSIONAL STRUCTURE OF A C-TERMINAL TRUNCATED CARDIAC GAP JUNCTION CHANNEL AT 7.5Å IN PLANE RESOLUTION *((V.M. Unger, N.M. Kumar, N.B. Gilula and M. Yeager))*
The Scripps Research Institute, La Jolla, CA 92037 (Spon. by M. Yeager)

Gap junction membrane channels directly connect the cytoplasms of adjacent cells and thereby play an important role in tissue homeostasis. We recently utilized electron cryo-microscopy and image analysis to examine frozen-hydrated two-dimensional (2D) crystals of a recombinant, truncated α_1-connexin (α_1Cx263T). The projection map at 7Å resolution revealed a ring of transmembrane α–helices that lines the aqueous pore and a second ring of α–helices in close contact with the membrane lipids. The distribution of densities allowed us to propose a model in which the two apposing connexons that form the channel are staggered by ~30°. Furthermore, apparent non-crystallographic twofold axes predicted that the two apposing connexons adopt identical conformations. We have now recorded images of tilted, frozen-hydrated 2D crystals, and our current 3D map has been computed at an in plane resolution of ~7.5Å and a vertical resolution of ~35Å. As predicted by our model, the two apposing connexons that form the channel are staggered with respect to each other. The two connexons are related by non-crystallographic two-fold axes and the central channel is unobstructed, implying that both connexons adopt identical open conformations. Extensive surface interactions between connexin subunits within each hexamer presumably account for the stability of the connexon oligomer. Within the membrane interior each connexin subunit displays four rods of density, which are consistent with an α-helical conformation for the four transmembrane domains. Furthermore, arcs of density in the extracellular domain are suggestive of β–sheet structures. The stacking of putative extracellular β–sheets between apposed connexons may account for the stability of the dodecameric channel. The structural details revealed by our analysis will be essential for delineating the functional properties of this important class of channel proteins.

W-Pos245

ELECTRON CRYO-CRYSTALLOGRAPHY REVEALS THAT OLEAMIDE, A SLEEP INDUCING COMPOUND, CAUSES STRUCTURAL CHANGES OF A GAP JUNCTION CHANNEL *((V.M. Unger., **D.W. Entrikin**, X. Guan, B. Cravatt, N.M. Kumar, R.A. Lerner, N.B. Gilula and M. Yeager))*
The Scripps Research Institute, La Jolla, CA 92037 (Spon. by A. Cheng)

Gap junction membrane channels mediate the electrical and metabolic coupling between cells. Detailed structural information is essential to delineate the molecular basis for channel gating. We recently utilized electron cryo-microscopy and image analysis to examine frozen-hydrated two-dimensional (2D) crystals of a recombinant, truncated α_1-connexin (α_1Cx263T). The projection map at 7Å resolution revealed a ring of transmembrane α-helices that lines the aqueous pore and a second ring of α-helices in close contact with the membrane lipids. However, it was not possible to discern whether the channel was in the open or closed conformation. Functional studies of BHK cells that express α_1Cx263T demonstrated that oleamide, a sleep inducing compound, blocks *in vivo* dye transfer. As previously demonstrated for other connexins, this behavior is an indication that oleamide causes closure of α_1Cx263T. These results encouraged us to examine whether oleamide affects the structure of the channel. 2D crystals of α_1Cx263T grown in the presence of oleamide displayed reflections to ~11Å resolution by optical diffraction and in many cases to better than 7Å resolution after correction for lattice distortions. The oleamide treated crystals exhibit p6 symmetry with unit cell dimensions of a=b=76.8±1.2Å and γ=120.3±0.5°. A projection density map at 7Å resolution derived from 9 images displayed differences compared with our previous map of the untreated channel. Based on the density distribution in the projected structure of the closed α_1Cx263T, channel closure does not appear to involve an overall change in the tilt of the connexin subunits.