Tu-PM-H7

RANDOM MUTAGENESIS OF A MECHANOSENSITIVE CHANNEL IDENTIFIES REGIONS OF THE PROTEIN CRUCIAL FOR NORMAL FUNCTION ((**Xiaorong Ou**, Paul Blount, Robert Hoffman, Ayumi Kusano, and Ching Kung)) Laboratory of Molecular Biology, University of Wisconsin-Madison, Madison, WI 53706

The *mscL* gene from *E. coli* encodes a peptide of 136 amino acids which forms a homohexameric complex that constitutes a functional mechanosensitive channel (MscL). This channel is thought to sense rapid decreases in environmental osmolarity and respond by release of intracellular small molecules and ions. To study the relationship between the structure and function of this channel, we have randomly mutagenized the *mscL* gene and isolated mutants that, when expressed, slow or halt growth. These are gain-of-function mutants since the *mscL* knockout shows no detectable plate phenotype. Thus far, over 20 mutants with single amino acid substitutions have been isolated. The majority and most severe mutations occur between amino acids 13 and 31, indicating the importance of this region in forming a proper channel. This region contains the first part of the first of two predicted transmembrane domains. Characterization of these mutants by whole cell physiology is now allowing us to sort them into different classes. For example, one class of mutants has lost the ability to retain the major osmolyte K^+ when the mutated gene is expressed, while another class increases K^+ efflux in response to osmotic shock. Preliminary electrophysiological data of mutants tested indicate changes in channel kinetics. Our results identify amino acid residues that are crucial for the proper functioning of this mechanosensitive channel. These data may also provide helpful information in elucidating the physiological role of the channel. (This study is supported by NIH GM47856)

Tu-PM-H8

BACTERIAL POLY(3-HYDROXYBUTYRATE) / POLYPHOSPHATE COMPLEXES FORM LARGE CONDUCTANCE CALCIUM CHANNELS. ((**Sudipto Das and Rosetta N. Reusch**)). Department of Microbiology, Michigan State University, East Lansing, MI 48824.

Complexes of short-chain poly(3-hydroxybutyrate) (PHB; MW~12,000) and inorganic polyphosphate (PPi; MW~5000), extracted from *Escherichia coli* plasma membranes, form ion channels in planar lipid bilayers that display many of the signal characteristics of calcium channels: voltage-activation, selectivity for divalent over monovalent cations, permeant to Ca^{+2}, Sr^{+2}, Ba^{+2}, and block by La^{+3}, Co^{+2}, Cd^{+2}, and Mg^{+2} in that order (Reusch et al. 1995. *Biophys. J.* **69**:754). The channel complexes have also been reconstituted from synthetic PHB_{128} and calcium $polyphosphate_{65}$ (manuscript in preparation).

The channels show a multiple number of substates and unique gating properties. Conductance of the most frequently observed open state is ~104 pS with frequent long openings of the order of several seconds. The channel complexes are impermeable to sodium and potassium. Pure synthetic PHB can form non-selective ion channels only at 100-1000 fold higher weight ratio in lipid (Seebach et al. 1996. *Helv. Chim. Acta* **79**:507). Analysis of open and closed time distributions reveal complex gating kinetics of the channel with multiple open and closed states. The single-channel characteristics in planar lipid bilayers of the biological and synthetic complexes are nearly indistinguishable.

Tu-PM-H9

HIGH RESOLUTION IMAGES OF MEMBRANE-ASSOCIATED STAPHYLOCOCCAL α-HEMOLYSIN OLIGOMERS BY ATOMIC FORCE MICROSCOPY. ((**Daniel M. Czajkowsky** and Zhifeng Shao))* Department of Molecular Physiology & Biological Physics and Biophysics Program, University of Virginia, Box 449, Charlottesville, VA 22908.

Staphylococcal α-Hemolysin (αHL) is a 34 kDa water soluble protein that forms pores in native and artificial membranes. The pores are believed to form when membrane-bound monomers convert into membrane-embedded oligomers during a multistep process. The oligomers, long thought to be hexamers, were recently shown to be heptamers on the basis of X-ray diffraction and gel shift experiments [Gouaux et al. *PNAS* 91, 12828 (1994)]. We have adapted a procedure which is frequently used to grow 2D crystals in electron microscopy to prepare αHL-loaded supported bilayers suitable for high resolution Atomic Force Microscopy in solution. Although Fourier transforms of the samples indicate better than 1 nm resolution, the size, shape, and subunit stoichiometry of the membrane-associated oligomers can be readily discerned even without applying image analysis techniques.
* This study was supported by grants from NIH.

Tu-PM-H10

STRUCTURE-BASED DESIGN OF A HETEROMERIC TRANSMEMBRANE PROTEIN PORE. ((**Orit Braha**[1], Barbara Walker[1], Stephen Cheley[1], John Kasianowicz[2], Michael R. Hobaugh[3], Langzhou Song[3], J. Eric Gouaux[3] and Hagan Bayley[1])) [1]Worcester Foundation, Shrewsbury, MA 01545; [2]NIST, Gaithersburg, MD 20899; [3]Columbia University, NY 10032. (Spon. C.N. Pace)

The ability to assemble, purify and reconstitute a heteromeric pore of known three-dimensional structure would be helpful in studies of the properties of transmembrane channels including permeation and gating. α-Hemolysin (α-toxin, αHL) is a 293-residue polypeptide secreted by *Staphylococcus aureus* as a water soluble monomer, which assembles into lipid bilayers to form a heptameric pore. Structure-based design and a separation method based on targeted chemical modification have been used to obtain a heteromeric form of αHL, $WT_6{}4H_1$. As modeled from the three-dimensional structure of WT_7, the 4H subunit contains an cluster of histidinyl residues that constitute a Zn(II)-binding site in the lumen of the transmembrane channel. The $WT_6{}4H_1$ heteromer was reconstituted into planar bilayers. Single channel currents through the heteromer are modulated by Zn(II), while currents through WT_7 are unaffected. The K_d for Zn(II) is ~ 200 nM and the k_{on} approaches the diffusion limit.

K CHANNELS AND DISEASE

Tu-Pos1

VOLTAGE-DEPENDENT KINETICS OF KVLQT1, A NOVEL DELAYED RECTIFIER K^+ CHANNEL. ((**M. Tristani-Firouzi**, P.S. Spector, A. Zou, M.T. Keating, M.C. Sanguinetti.)) Divisions of Cardiology and Pediatric Cardiology, University of Utah, Salt Lake City, UT 84113

KVLQT1 encodes a novel delayed rectifier K^+ channel. Mutations in *KVLQT1* cause an inherited arrhythmia, long QT syndrome. We recently demonstrated that KVLQT1 and minK coassemble to form I_{Ks} channels. KVLQT1 alone forms functional homomeric channels. The electrophysiological properties of KVLQT1 were studied in *Xenopus* oocytes. Activation of KVLQT1 current was best described by a 3 exponential function, accounting for an initial delay in current and fast and slow components of activation. The rates of activation were voltage dependent ranging from tau_f=154±9 and tau_s=762±57 msec at -10 mV to tau_f=47±2 ms and tau_s=272±10 ms at +40 mV. The relative amplitudes of the fast and slow components varied with membrane potential (fast: 0.22 at -20mV and 0.60 at +40mV; slow: 0.70 at -20mV and 0.26 at +40mV). Deactivating current was hooked when elicited after a pulse to membrane potentials > -30mV. This hook represents recovery of channels from an inactivated state at a rate faster than deactivation. The rate of recovery from inactivation and deactivation increased with increasing membrane potential: Deactivation: tau=70±4 ms at -130 mV; 775±53 ms at -50 mV (n=6). Recovery from inactivation: tau=14±2 ms at -130 mV; 70±5 ms at -50 mV (n=6). The voltage dependence of inactivation, estimated from the relative amplitudes of initial and extrapolated tail currents, was half-maximal at about -15 mV. These properties do not match any known human cardiac K^+ current, suggesting that KVLQT1 usually exists as a heteromultimeric channel in cardiac myocytes.

Tu-Pos2

OXIDATIVE STRESS MODULATES THE HUMAN ETHER-A-GOGO-RELATED GENE (HERG) K^+ CHANNELS

((P. Castaldo, F. Morra, S. Jossa, L. Annunziato and **M. Taglialatela**)) Section of Pharmacology, Department of Neurosciences, Faculty of Medicine, University of Naples Federico II, Via S. Pansini 5, 80131, Naples, Italy.

Oxygen radicals play an important role in arrhythmogenesis and contractile dysfunction in ischemia-reperfusion phenomena. The K^+ channel encoded by the human ether-a-gogo-related gene (HERG) appears to be a crucial molecular determinant for repolarization of the human heart. The aim of the present study was to investigate the modulation by oxidative stress of HERG K^+ channels expressed in Xenopus oocytes. Oxidative stress was induced by perfusion with a solution containing ferrous sulphate (100 μM) plus ascorbate (200 μM) (F/A solution). Oocytes perfused with ND88 (10 mM K^+) plus F/A showed a 10-fold increase in lipid peroxidation. Oxidative stress induced a 30-40% increase in outward K^+ current carried by HERG K^+ channels, without significant changes in inward current, thereby reducing inward rectification. The increase in both lipid peroxidation and HERG outward current started 2-3 min after the beginning of the F/A perfusion, reached a plateau within 6-8 minutes, and lasted for over one hour. Outward currents of uninjected oocytes were not affected by ND88 plus F/A perfusion. The increase in HERG outward currents was completely reversible upon removal of the ND88 plus F/A solution; a second exposure to F/A-containing ND88 induced another increase in outward currents, although slightly less pronounced than with the first application. Other K^+ channels were completely insensitive to oxidative stress (such as Kv 2.1) or only very slightly sensitive (<10% increase in outward currents, such as EAG). In conclusion, the inward rectification of HERG K^+ channels seems to be modulated by changes in the oxidative state of the cell.

Tu-Pos3

A KINETIC MODEL OF THE ACTIVATION AND INACTIVATION OF H-ERG EXPRESSED IN *XENOPUS* OOCYTES. ((Shimin Wang, Shuguang Liu, Michael J. Morales, Harold C. Strauss and Randall L. Rasmusson)) Duke University Medical Center, Durham, NC 27710.

H-erg encodes a K^+ channel that mediates a component of the delayed rectifier current, I_{kr}, in cardiac muscle. Understanding its kinetic properties is critical to elucidating its role in repolarization. We studied h-*erg* expressed in *Xenopus* oocytes using the two electrode and cut-open oocyte clamp techniques. Currents were studied with $[K^+]_o$ of 2 and 98 mM. We measured the activation process of h-*erg* using an envelope of tails protocol. In 98 mM $[K^+]_o$ cells were pulsed to the test potential from a potential of -120 mV for varying periods of time, Δt, upon repolarization to -80 mV a peak tail current was recorded. These peak tail currents were normalized by maximal tail current and plotted against Δt. These experiments demonstrated that activation of h-*erg* was sigmoid in onset. A test based on runs of the best fit to the data indicated that at least 3 closed states were required to reproduce the sigmoid time-course. The voltage dependence of the activation process suggested the existence of at least one relatively voltage insensitive step. A 3 closed state activation model with a single intermediate closed state was able to reproduce the time and voltage dependence of activation, deactivation and steady-state activation. Activation was insensitive to changes in $[K^+]_o$ but inactivation was sensitive to changes in $[K^+]_o$. The time constants of inactivation and recovery formed a smooth bell shaped curve. Both inactivation and recover time constants increased with a change of $[K^+]_o$ from 2 to 98 mM. Steady-state inactivation shifted by ~30 mV in the hyperpolarized direction with a change from 2 to 98 mM $[K^+]_o$. The inactivation kinetics in both cases were well-described by single first order gating variables. Coupling of the activation and inactivation models demonstrated all of the kinetic characteristics of the expressed current, including a transient peak early on in records in 98 mM $[K^+]_o$. Simulations suggested that modulation of inactivation is not the sole mechanism underlying activation of this current by $[K^+]_o$, an increase in conductance may also occur.

Tu-Pos4

MOLECULAR DETERMINANTS OF DOFETILIDE BINDING TO HERG ((E. Ficker [1], W. Jarolimek [1], J. Kiehn [1], A. Baumann [2], and A.M. Brown [1])) [1]Rammelkamp Center, MetroHealth Medical Center, CWRU, Cleveland, OH and [2] Forschungszentrum Juelich, Juelich, Germany

The human ether-a-go-go related gene, HERG, encodes a K^+ channel with biophysical properties nearly identical to the cardiac delayed rectifier K^+ current, I_{kr}. HERG K^+ channel subunits are the molecular target of a variety of K^+ channel blocking antiarrhythmic drugs, including the methansulfonanilides dofetilide and E4031 which are thought to prolong the cardiac action potential by selective block of I_{kr}.

We used a chimeric approach to identify the domains responsible for high affinity dofetilide block. The closely related bovine ether-a-go-go channel (bEAG) was used to engineer large scale chimeras with HERG. Compared to HERG, bEAG is about 100-fold less sensitive to dofetilide. All chimeras which preserved the HERG pore maintained the high affinity block of dofetilide with the slow onset and washout typical for wild type HERG. By contrast, transfer of the bEAG pore into HERG resulted in low affinity dofetilide block with the fast onset and washout characteristic of bEAG channels.

Since the chimeric experiments pointed to dofetilide binding in H5, we introduced point mutations into this region of HERG. Two point mutations, S631A and S620T, greatly reduced the affinity of dofetilide. Both point mutations also strongly reduced the rapid, voltage-dependent C-type inactivation present in wild type HERG. A conformation linked to the inactivated state may contribute to the binding site for dofetilide or access to the binding site may be restricted by conformational changes produced by the point mutations. (Supported by HL55404)

Tu-Pos5

NAB_{HERG}, A SUBUNIT INTERACTION DOMAIN IN *HERG* POTASSIUM CHANNEL.

((**Xiaodong Li**, Jia Xu, Min Li. Departments of Physiology and Neuroscience, Johns Hopkins University School of Medicine, 725 N. Wolfe Street, Baltimore, MD 21205))

HERG (human *eag*-related gene) encodes an inward-rectifier potassium channel that is thought to be formed by the assembly of four subunits. Since the truncated *HERG* protein in patients with long QT syndrome induces a dominant phenotype — that is, cardiac sudden death, the nonfunctional assembly between wild-type and mutated subunits was implicated in causing the disease. To understand *HERG*-mediated cardiac sudden death at the molecular level, it is important to determine which regions in the *HERG* protein participate in subunit interaction. We therefore report the identification of a subunit interaction domain, NAB_{HERG}, that is localized at the hydrophilic cytoplasmic N-terminus and can form a tetramer in the absence of the rest of the *HERG* protein. Truncated *HERG* proteins containing NAB_{HERG}, including one that resulted from the Δ1261 human mutation, inhibit the functional expression of the *HERG* channel in transfected cells. Together, these results support the notion that the functional expression of *HERG* in the human heart may be decreased in the presence of the truncated subunit. Such a decrease of potassium channel expression can contribute to the longer QT intervals observed in the patients with the *HERG* mutation.

Tu-Pos6

MITOSIS PROMOTING FACTOR-MEDIATED SUPPRESSION OF *REAG* POTASSIUM CHANNELS EXPRESSED IN *XENOPUS* OOCYTES

((**Andrea Brüggemann**, Walter Stühmer, Luis A. Pardo))
MPI exp. Medizin, Hermann-Rein-Str. 3, D-37075 Göttingen Germany. (Spon. by Walter Stühmer)

The cell cycle is the crucial process that leads to mitosis in all cell types. The dramatic redirectioning of many cellular processes during the cycle is known to involve ion channels, either changing their level of expression or their voltage dependence, as in the case of inward rectifiers. Here we describe the specific inhibition of heterologously expressed ionic channels at the onset of maturation in *Xenopus* oocytes. In cells expressing rat *eag* (*Reag*[1]) potassium channels, maturation induces a dramatic reduction in the current amplitude, which is almost complete in most cases. The key molecule in oocyte maturation, the mitosis promoting factor (MPF, a complex of cyclin B and p34cdc2), is able to induce similar changes when injected into the oocytes. The mechanism of this reduction in current amplitude is most likely a voltage-dependent block of the channel that appears to be related to inactivation, as it is the case for another member of the *eag* family (*Herg*[2]).

1. Ludwig, J., Terlau, H., Wunder, F., Brüggemann, A., Pardo, L.A., Marquardt, A., Stühmer, W. & Pongs, O. (1994) *EMBO J.* **13**, 4451-4458
2. Smith, P.L., Baukrowitz, T. & Yellen, G. (1996) *Nature* **379**, 833-836 .

Tu-Pos7

A POSSIBLE LINK BETWEEN PROZAC®, THE 5-HT_3 SYNDROME AND Kv1.1 CHANNELS. ((**Ch. Maertens**, P. Daenens and J. Tytgat)) Laboratory of Toxicology, University of Leuven, Van Evenstraat 4, B-3000 Leuven, Belgium.

Prozac® (fluoxetine.HCl) is commonly prescribed for treating depression in humans. It is thought to work by blocking the 5-HT_3 re-uptake and is designated as selective serotonin re-uptake inhibitor. Literature evidence can be found that Prozac® may have other effects, like inhibition of α-adrenergic, muscarinic, dopamine D_2, and histamine H_1 receptors, which may lead to the 5-HT_3 syndrome. Although different doses of Prozac® may be needed to observe these effects, it is clear that the actions of the drug can be numerous and complicated. Here we report the effects of Prozac® on a neuronal, 'delayed rectifier' type, voltage-dependent potassium channel (Kv1.1). Using 2-microelectrode, voltage clamp experiments on *Xenopus laevis* oocytes, expressing RCK1 channels after injection with the corresponding mRNA, a concentration-, voltage-, and pronounced time-dependent inhibitory effect induced by Prozac® was observed ($IC50_{0mV,5min} \approx 500\mu M$). The drug dramatically altered voltage-dependent gating parameters of this potassium channel, leading to cross-over of tail currents, and it showed an irreversible mechanism of action. In connection with the use/abuse of this compound and the corresponding clinical symptoms, a tentative working model is proposed.

Tu-Pos8

FUNCTIONAL CONSEQUENCES OF POTASSIUM CHANNEL MUTATIONS IDENTIFIED IN FAMILIES WITH INHERITED EPISODIC ATAXIA. ((**L.M. Boland**, D.L. Price, and K.A. Jackson)) Department of Physiology and Neuroscience Program, University of Minnesota Medical School, Minneapolis, MN 55455.

Episodic ataxia (EA) is an autosomal dominant neurological disorder characterized by periodic attacks of motor imbalance, incoordination, and involuntary muscle tremor, often precipitated by emotional or physical stress. EA is genetically linked with mutations of the Kv1.1 gene from the *Shaker* K channel subfamily. We used overlap extension PCR to introduce the human mutations into corresponding, conserved regions of *ShakerH4* cDNA. Mutants were sequenced, expressed in Xenopus oocytes, and currents recorded by electrophysiology. To study activation gating, mutations were prepared in an N-terminal deletion mutant. Five of the six EA mutants showed changes in the voltage-dependence of activation, with 10-15 mV hyperpolarizing shifts of $V_{1/2}$ for F307I, and similar, depolarizing shifts for F244C and E395D. To study the effect of an EA mutation when co-expressed with inactivating K channel subunits, mutations were expressed in *ShakerH4* with intact N-terminal inactivation particles. Several mutants showed significant changes in steady-state inactivation, with a -20 mV shift of $V_{1/2}$ for F307I and smaller, depolarizing shifts for V234F, R297S, and V478A. These alterations in K channel activation and inactivation gating are due to single amino acid substitutions, represented in four-fold symmetry in homomeric K channels.

Tu-Pos9

PROPERTIES OF HERG STABLY TRANSFECTED IN L929 CELLS

((H. Musa, S. Taffet, M. Delmar, J.M.B. Anumonwo and J. Jalife)) Department of Pharmacology, Department of Microbiology and Immunology, SUNY Health Science Center, Syracuse NY 13210.

We have stably transfected HERG (*human ether-a-go-go-related gene*) into the mouse fibroblast (L929) cell line. The whole-cell patch-clamp technique was used to characterize properties of the induced K^+ current and recordings were done at 35 °C. Transfected cells had a resting membrane potential of -33.0 ± 1.5 mV (mean,SEM), a membrane capacitance of 21.1 ± 1.5 pF and an input resistance of 1.1 ± 0.4 GΩ (n = 5). In these cells, depolarizing pulses elicited a rectifying current with properties similar to the HERG-induced delayed rectifier current (I_{Kr}) previously studied in other expression systems. Similar depolarizing pulses activated only a time-independent background current in non-transfected cells. HERG-induced current had a half maximum activation voltage of -6.2 ± 0.6 mV and a slope factor of 5.6 ± 0.54 mV (n=5). A single exponential fit to current activation at +20 mV had a time constant of 261 ± 40 ms, while current deactivation decayed biexponentially at -60 mV with time constants of 394 ± 75 ms and 1478 ± 141 ms (n=4). The HERG channels were K^+-selective; tail currents reversed at -113 ± 3 mV, -76 ± 2 mV and -63 ± 3 mV respectively in 1, 5.4 and 10 mM external $[K^+]$ (n = 12). The fully activated current-voltage (I-V) relationship showed the characteristic inward rectification of the channel (n=4) as described elsewhere (Nature 379:833, 1996; J.Gen Physiol 107:611, 1996). Fast inactivation, which underlies this rectification, could be removed by a 25 ms interpulse to -120 mV, revealing an ohmic I-V relationship as previously described. Stable transfection of HERG in L929 cells is important because it will reduce problems associated with variability in levels of channel expression or of oocyte quality. It will also enable the study of HERG channels in a cellular environment similar to that in native cardiac cells.

K CHANNELS: PHARMACOLOGY AND MODULATION

Tu-Pos10

THREONINE 505 DETERMINES STEREOSELECTIVE BUPIVACAINE BLOCK OF hKv1.5 CHANNELS.

((J. Vicente, L. Franqueza, E. Delpón, M.M. Tamkun, J. Tamargo, D.J. Snyders and **C. Valenzuela**)) Institute of Pharmacology and Toxicology, CSIC/UCM, 28040 Madrid, SPAIN.

Block of WT hKv1.5 channels by bupivacaine is stereoselective with R(+)-bupivacaine (RB) being 7-fold more potent than S(-)-bupivacaine (SB). Both enantiomers act as open channel blockers in a voltage-dependent manner consistent with a fractional electrical distance (δ) of 0.16 (referenced from the cytoplasmic side). We used site-directed mutagenesis and thermodynamic mutant cycle analysis to test how amino acid substitutions in the inner mouth of the channel pore affect stereoselective block. Replacement of T505 by hydrophobic amino acids (isoleucine or valine) preserved open channel block, but completely abolished the stereoselectivity of block (δ range 0.15-0.21, K_D range 14-22 μM). In contrast, a serine substitution (T505S) preserved and enhanced stereoselective of block (K_D=247±47 μM and 7.4±1.6 μM, for SB and RB), which in comparison to WT was largely due to changes in the association rate constants for SB and RB in opposite directions. Both enantiomers were equipotent open channel blockers of Kv2.1 channels which carry a valine at the T505 equivalent site with K_D values of 8.3 μM (SB) and 13.1 μM (RB). In energetic terms, the coupling energies ($RT\ln\Omega$) for the Kv1.5 mutations at position 505 and RB correspond to 4.43 kJ mol^{-1} for T505I and 3.96 kJ mol^{-1} for T505V and T505S. For T505I and T505S an additional higher affinity component of block was observed (accounting for 30-50% of total block) which may reflect the existence of a second binding site. The results indicate subfamily selectivity for open channel block at the inner mouth of the pore and suggest that the hydroxyl group of threonine 505 is a molecular determinant of stereoselective block by bupivacaine. Supported by grants FIS 95/0318, CICYT SAF96-0042 and NIH-HL46681.

Tu-Pos11

NIFEDIPINE BLOCK OF hKv1.5 CHANNELS.

((**X. Zhang**, J. Anderson and D. Fedida)) Department of Physiology, Queen's University, Kingston, ON, Canada K7L 3N6

Nifedipine is an antagonist of L-type Ca^{2+} channels, and part of its binding site is located at the extracellular end of the interface between domains III and IV of the α1 subunit. Nifedipine also blocks Kv channels, which are members of the same supergene family. Here, we have examined the effects of nifedipine on a rapidly activating delayed rectifier K^+ channel (hKv1.5) cloned from human heart. In the presence of nifedipine, the current was blocked in a concentration-dependent manner with a K_D value of 6.3μM at +40mV. The effect of nifedipine was to diminish the peak current and accelerate the rate of inactivation of the current. Block increased rapidly between -10mV and +10mV, coinciding with the voltage range of hKv1.5 channel opening. Positive to +20mV, block was slightly relieved with larger depolarizations. The effects of nifedipine block of hKv1.5 were concentration and voltage dependent with a decay process that was well fitted to biexponential functions. The mean fast and slow time constants in the presence of 5μM, 10μM and 50μM nifedipine at +40mV were 16.7 ± 1.20 (S.E.), 11.6 ± 0.76, 4.8 ± 0.61ms and 232.5 ± 23.65, 205.9 ± 15.20, 151.6 ± 14.92ms, respectively (n=4-8). At potentials between 0mV to +80mV, the fast and slow time constants of current decay in the presence of 10μM nifedipine increased from 27.9 ± 6.79ms to 10.5 ± 0.58ms and 205.2 ± 15.23ms to 185.3 ± 40.97ms, respectively (n=4-8). Crossover of tail currents in the presence of nifedipine suggested that nifedipine block of hKv1.5 may be caused by open-channel block. To investigate the location of the binding site of nifedipine in hKv1.5, different macropatch configurations were used. The rank order of block was whole-cell ≈ outside-out > inside-out > > cell-attached macropatch, which suggested that the binding site of nifedipine in hKv1.5 was at the extracellular surface or within the lipid bilayer at a site that is more accessible from the extracellular side. We concluded that nifedipine acts as an open channel blocker of hKv1.5 from the extracellular surface likely at a hydrophobic domain in the channel pore.

Tu-Pos12

BLOCKADE BY IBUTILIDE OF VARIOUS CLONED POTASSIUM CHANNELS.

((**T.D. Tsai**, L.S. Wood, J.K. Gibson and G. Vogeli)) Cardiovascular Pharmacology, Pharmacia & Upjohn, Kalamazoo, MI 49001.

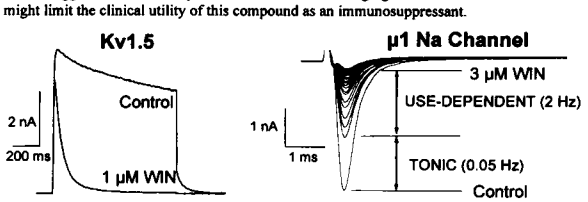
Ibutilide is a Class III antiarrhythmic agent. The main action of ibutilide is to facilitate the slow inward sodium current. In addition, several studies have reported that higher concentrations of ibutilide also block potassium currents. In this work we tested the effect of ibutilide on various cloned potassium channels including voltage-dependent (Kv1.5, Kv1.4, Kv1.3), a Ca^{2+}-activated (hBK) and an inward rectifier (hIRK1) potassium channels . In 300 μM ibutilide the Kv1.5 and hIRK1 currents were decreased to 55.5±2.8% (n=5) and 66.5±8.4% (n=3) respectively. Ibutilide has little effect on the hBK; in 300 μM ibutilide the current decreased less than 1% (n=9). The effect of ibutilide on Kv1.4 was very interesting. At 300 μM it inhibited the peak current (82.7±7.7% (n=3)), slowed down the inactivation process and increased the current after 100 msec (139.13±1.4% (n=3)), leading to a "crossover" of the current. Our finding may partially explain the different observations of the effects of ibutilide on potassium channels from different authors.

Tu-Pos13

NON-SELECTIVE INHIBITION OF Na^+ AND Kv1.x K^+ CHANNELS BY THE IMMUNO-SUPPRESSANT AGENT WIN 17317-3.

((**N.A. Castle**, W. Yu, G. Amato and P.K.Wagoner)) ICAgen Inc. Durham, NC 27703

The immunosuppressant agent WIN 17317-3 (WIN) has been reported to selectively block the lymphocyte potassium channel Kv1.3 with a selectivity factor of about 100 fold over other homologous members of the Kv1.x family of channels (Biophys J. 70 A446, 1996). We have examined more closely the channel selectivity of WIN against human Kv1.3 and Kv1.5 channels heterologously expressed in CHO cells as well voltage-gated Na channels. In contrast to previous reports, we have observed that the potency of block (at +10 mV) of WIN against Kv1.3 and Kv1.5 is similar with IC_{50} s of 0.1 µM. Block of both channel types is time dependent suggesting a dependence on channel activation. WIN is also a potent inhibitor of tetrodotoxin-sensitive voltage-gated sodium currents in GH_3 cells and CHO cells expressing cloned µ1 Na channels. WIN produces both tonic and use-dependent block (at +10 mV) exceeding 90% at 3 µM. Following treatment with chloramine-T to remove inactivation, 3 µM WIN was found to produce a time dependent block (>90%) of Na currents in GH_3 cells. The results suggest that WIN is a potent blocker of both voltage-gated K^+ and Na^+ channels which might limit the clinical utility of this compound as an immunosuppressant.



Tu-Pos14

CHARACTERIZATION AND LOCALIZATION OF THE PHENYLALKYLAMINE ACTION ON THE VOLTAGE-GATED POTASSIUM CHANNEL Kv1.3.

((**H. Rauer** and S. Grissmer)) Department of Applied Physiology, University of Ulm, 89081 Ulm, Germany (Spon. by A. K. Kleinschmidt)

We investigated the action of verapamil and N-methyl-verapamil (D575) on the *m*Kv1.3 channel and a mutation derived from the S5/S6 linker, H404T, to obtain structural and functional information about voltage-gated potassium channels. The whole-cell and inside-out configuration of the patch-clamp technique was used to examine the current properties obtained by injection of *in vitro* transcribed *m*RNA in RBL cells. Measurements in the whole-cell configuration were done in mammalian Ringer's solution with patch-pipettes containing KF. In inside-out patches the bath solution consists of K-aspartate solution with pipettes containing mammalian Ringer's solution. The action of extra- or intracellularly applied verapamil on wildtype *m*Kv1.3 currents had two different properties: 1) acceleration of the rate of current decay during depolarizing pulses, and 2) reduction of steady-state peak current. The membrane impermeable D575 affected current through *m*Kv1.3 wildtype channels similar to verapamil only if applied to the intracellular side of the membrane. The H404T mutation in the outer vestibule decreased the ability of extra- and intracellularly applied verapamil and intracellularly applied N-met-verapamil to reduce steady-state peak current about 25-fold, whereas the acceleration of current decay caused by these compounds was nearly unaffected. Substances known to interact with the extracellular site of the channel, like extracellularly applied tetraethylammonium, $[TEA^+]_o$ or kaliotoxin did not compete with extracellularly applied verapamil on blocking steady-state peak current, whereas intracellularly applied $[TEA^+]_i$, known to interact with the intracellular site of the channel, was able to reduce the effect of extracellularly applied verapamil to block steady-state peak current suggesting competition for a common binding site between $[TEA^+]_i$ and verapamil. The results from the competition experiments as well as from the mutation in the outer vestibule of *m*Kv1.3 are compatible with the idea that verapamil, if applied extracellularly, pass through the membrane to reach its internal binding site on the *m*Kv1.3 channel. (Supported by a grant from Pfizer Inc, CT and the DFG (Gr 848/4-1)).

Tu-Pos15

BLOCKADE OF HERG AND Kv1.5 POTASSIUM CHANNELS BY KETOCONAZOLE.

((R. Dumaine, **M.-L. Roy**, and A.M. Brown.)) Rammelkamp Center, MetroHealth Campus, C.W.R.U. Sch. of Med., Cleveland, OH 44109.

Ketoconazole, a widely-used fungicide, has been associated with QT prolongation and *torsades de pointes* when co-administered with terfenadine (Seldane), a compound which uses the same CYP3A4-P450 metabolic pathway. The common explanation for this cardiotoxicity is that an inhibition of terfenadine's metabolism in the presence of ketoconazole results in toxic plasma concentrations which block I_K. Actions of ketoconazole alone on HERG and Kv1.5 currents were analyzed using heterologous expression in *Xenopus* oocytes. HERG and Kv1.5 currents were reduced to comparable extents by ketoconazole with apparent K_D values of 30 μM and 48 μM, respectively. These values are physiologically significant, as patient plasma concentrations have been reported in the low micromolar range. We did not observe use-or voltage-dependent block for either channel, but a time-dependent extra-block in the open states was observed with HERG. Our results indicate that ketoconazole acts using both pharmacokinetic and pharmacodynamic mechanisms to potentiate the acquired long QT induced by terfenadine.

Supported by Le Fonds de la Recherche en Santé du Québec of Canada to R.D., NIH Fellowship to M.L.R., and NIH NS23877 to A.M.B..

Tu-Pos16

EFFECT OF DIVALENT CATIONS ON THE E-4031 SENSITIVE REPOLARIZATION CURRENT, I_{Kr}, IN RABBIT VENTRICULAR MYOCYTES.

((**Tyna Paquette***, John R. Clay+, Azieb Ogbaghebriel*#, Betty I. Sasyniuk#, and Alvin Shrier*)) *Physiology and #Pharmacology Depts., McGill Univ., Montreal, Canada, H3G-1Y6 and +NIH, Bethesda, MD 20892.

The effects of divalent cations on I_{Kr} in rabbit ventricular myocytes were studied using standard, whole-cell patch clamp recording techniques. Outward currents were evoked by holding the membrane voltage at -40mV and then stepping to test voltages ranging from -30mV to +60mV. The calcium current was blocked using 10μM nifedipine and the various divalent cations were added to the perfusion solution at concentrations ranging between 1μM and 5mM. The amplitude of I_{Kr} was determined from the tail current observed upon return to -40mV from the test voltages. The divalent cations could be separated into three different classes based upon their effects on I_{Kr}. The first class is comprised of Zn^{2+}, which is the only cation we found that blocked I_{Kr}; this blockade was not associated with any shift in the activation curve. The second class consisted of Mg^{2+} and Ca^{2+} which produced slight positive shifts in the activation curve and associated increases in the amplitude of I_{Kr}. The largest class of divalents produced a significant increase in the amplitude of I_{Kr} (Cd^{2+}>>Ni^{2+}~Co^{2+}~Mn^{2+}~Sr^{2+}) and a marked positive shift in the activation curve (Cd^{2+}>Co^{2+}~Mn^{2+}~Ni^{2+}~Sr^{2+}). The effects of the divalent cations on another inwardly rectifying potassium current, I_{K1}, differed from the effects on I_{Kr}. The I_{K1} channel was blocked in a voltage-independent manner (Cd^{2+}>Co^{2+}~Mn^{2+}>Ni^{2+}~Sr^{2+}[no effect]). These findings demonstrate that divalent cations do not operate by a single mechanism associated with a simple surface charge screening. We suggest that divalent cations interact with specific binding sites on the I_{Kr} channel that are related to the inactivation gating mechanism.

Tu-Pos17

EFFECTS OF RP 58866 AND RP 62719 ON TRANSMEMBRANE K^+ CURRENTS IN MAMMALIAN VENTRICULAR MYOCYTES

((B.-F. Yang, G.-R. Li, and S. Nattel)), Montreal Heart Institute and University of Montreal, Montreal, Quebec, Canada

Novel class III antiarrhythmic compounds RP58866 and RP62719 are known to block inward rectifier K^+ current (I_{K1}), and have been used as "specific" probes for the physiologic role of I_{K1}; however, the specificity is not completely established. The present study was designed to determine effects of RP 58866 and RP 62719 on I_{K1}, transient outward K^+ current (I_{to}) and delayed outward rectifier K^+ current (I_K) in isolated cardiac myocytes with the whole cell voltage clamp technique. RP 58866 and RP 62719 significantly decreased I_{K1} in a concentration-dependent manner, with an IC_{50} of 3.4 $\pm$ 0.8 (M $\pm$ SE) and 5.0 $\pm$ 1.0 μM respectively at -100 mV in guinea pig ventricular cells. In dog ventricular myocytes, both RP 58866 and RP 62719 significantly inhibited I_{to} (decreased by 87 $\pm$ 2.1% and 84 $\pm$ 4.4% at 100 μM) with IC_{50}'s of 2.3 $\pm$ 0.5 and 1.2 $\pm$ 0.5 μM. In guinea pig ventricular cells, RP 58866 and RP 62719 at 100 μM decreased I_K: I_{Kstep} by 58 $\pm$ 13 and 59 $\pm$ 5.4 % at +40 mV, and I_{Ktail} by 86 $\pm$ 17 and 63 $\pm$16%, respectively. RP 58866 inhibited I_{Kstep} with IC_{50} 7.5 $\pm$ 0.8 μM, and I_{Ktail} with IC_{50} 3.5 $\pm$ 0.9 μM, while RP 62719 decreased I_{Kstep} with IC_{50} 4.2 $\pm$ 0.8 μM, and I_{Ktail} 3.3 $\pm$ 0.75 μM. The envelope of tail analysis suggested that both I_{Kr} and I_{Ks} were inhibited. Our results indicate that RP 58866 and 62719 inhibit I_{K1}, I_{to} and I_K in cardiac myocytes with similar potency, and that these compounds are not specific I_{K1} inhibitors.

Tu-Pos18

AZIMILIDE BLOCKS hERG AND hIsK IN THE OPEN STATE FROM THE EXTRACELLULAR SIDE OF THE CELL MEMBRANE.

((**J-S Fan**, J-A Yao, M Jiang, G-N Tseng)) Dept of Pharmacology, Columbia University, New York, NY 10032.

We have shown that azimilide (AZ) is a potent blocker of cardiac I_{Kr} and I_{Ks}. The voltage- and time-dependence of its action is consistent with that of an open channel blocker. Since I_{Kr} and I_{Ks} have different structures and gating mechanisms that may impact on drug-channel interactions, we compared AZ action on these two channels using hERG and hIsK expressed in oocytes and HEK 293 cells as a model system. IC_{50} of AZ suppression of hIsK in oocytes was 3.7+0.7 uM (+20 mV, n=4, mean+SE). IC_{50} of AZ suppression of hERG was 0.6+0.1 uM in HEK cells (n=6, -10 mV), but ~10 times higher in oocytes (5.4+ 0.7 uM, n=4). The mechanism of AZ action on hERG was similar in HEK and oocytes. For both hIsK and hERG, block occurred when AZ was applied extracellularly, but not intracellularly. Block was enhanced by membrane depolarization corresponding to the voltage range of channel activation. Strong depolarization that induced inward rectification of hERG reduced AZ block. The apparent τ of block development was 1-2 s for hIsK, vs 0.1-0.2 s for hERG. AZ slowed hIsK deactivation, causing a "cross over" of its tail currents, while accelerated hERG deactivation. At low concentrations and voltages close to the activation threshold, AZ accelerated the activation of both hIsK and hERG. We suggest that AZ binds to the extracellular domain of slow delayed rectifier channel in its subunit-assembled (activated) form. Binding stabilizes the channel in its activated form. AZ binds to the outer mouth region of hERG channel when its binding site becomes available upon channel activation, and conformational changes in the outer mouth region of hERG associated with inward rectification reduce binding affinity.

Tu-Pos19

A NOVEL POTENT AGONIST OF I_{Ks} THAT ACTS VIA A NEGATIVE SHIFT IN THE VOLTAGE DEPENDENCE OF ACTIVATION.

((**J.J. Salata**, N.K. Jurkiewicz, J.Wang, P.K.S. Siegl, M.C. Sanguinetti*)) Merck Research Laboratories, West Point, PA 19446 and *University of Utah, Salt Lake City, UT 84112

The delayed rectifier K^+ current, I_K, has both rapidly (I_{Kr}), and slowly (I_{Ks}) activating components. We have identified 1,4 benzodiazepines that are the first potent and selective blockers of I_{Ks}, and one, L-364,373 [L3; (3-R) 1,3-dihydro-5-(2-fluorophenyl)-3-(1H-indol-3-ylmethyl)-1-methyl-2H-1,4-benzodiazepin-2-one] that is an agonist of I_{Ks}. We studied the effects of L3 on I_{Ks} in guinea pig ventricular myocytes with whole cell voltage-clamp. Holding potential was -50 or -40mV and 1μM dofetilide was used to block I_{Kr}. During 500-ms test voltage steps (V_t) to +20mV, 1μM L3 increased I_{Ks} by ~72%. Addition of 1μM isoproterenol increased I_{Ks} an additional 53%. Using 3-sec V_t to -10mV, 0.03, 0.1, 0.3 and 1μM L3 increased I_{Ks} 106±19, 431±123, 631±105 and 1703±507% (n≥6), respectively. Agonism of I_{Ks} was concentration-, time-, and voltage-dependent, but not mediated by ß-adrenoreceptors and was stereoselective. L3 increases in I_{Ks} were maximal at 1μM and diminished at ≥3μM; block of I_{Ks} occurred with both enantiomers at ≥3μM, but the S enantiomer showed no agonism. Isochronal activation was measured from tail current amplitudes following 7.5-s V_t between -40 and +60mV. I_{Ks} reached half-maximal activation at 19, 3, and -5 mV for control, 0.1 and 1μM L3, respectively (n=3-8), but the slope factor and maximally activated I_{Ks} were unchanged. L3 increased the rate of I_{Ks} activation and decreased the fast (τ_f) and slow (τ_s) time constants of I_{Ks} deactivation, e.g. at -40 mV, τ_f = 97±13, 173±15 ms and τ_s = 354±70, 1115±96 in control and 1μM L3, respectively. L3 at 1μM decreased APD_{90} from 241±11 to 181±6 ms (25%Δ) at 0.2 Hz, consistent with an increase in I_{Ks} and its role in repolarization. These 1,4 benzodiazepines represent the first potent and selective modulators of I_{Ks} and will aid in defining its physiological role in the heart and other organs.

Tu-Pos20

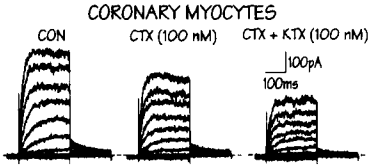
DIFFERENTIAL EFFECTS OF ANESTHETICS ON THE SLOW CARDIAC DELAYED-RECTIFIER POTASSIUM CURRENT. ((**A.T. Martinelli***, Z.J. Bosnjak, W.M. Kwok)) Dept. of Anesthesiology, Medical College of Wisconsin, Milwaukee, WI 53226 and *Dept. of Biology, Marquette University, Milwaukee, WI 53233.

Volatile anesthetics depress cardiac function, but their specific membrane targets and the underlying mechanisms of action at the cellular level have not been elucidated. We investigated the effects of three anesthetics - isoflurane, sevoflurane, and halothane - on the slow cardiac delayed-rectifier potassium current, I_{Ks}, in ventricular myocytes isolated from guinea pig hearts. Using the whole-cell configuration of the patch-clamp technique, we found these anesthetics to differentially and reversibly depress I_{Ks}. The K_d's obtained from the concentration-response curve (in the range of 0.17 to 1.52 mM) fitted with a modified Hill equation were 0.16±0.01 and 0.31±0.05 mM for isoflurane and sevoflurane, respectively. The Hill coefficients were 1.0±0.1 and 1.4±0.4, respectively, suggesting non-cooperativity. In contrast, the depressant effect of halothane was constant in the clinically relevant range, inhibiting I_{Ks} by 59±5% at 0.2 mM and by 52± 10% at 0.9 mM. In all cases, block was voltage-independent and no shifts in the activation curve were observed. All three anesthetics altered the kinetics of I_{Ks} activation to varying degrees at a concentration where approximately 60-80% of I_{Ks} was inhibited. In response to a 1 second depolarizing pulse to +90 mV from a -40 mV holding potential, isoflurane (0.56 mM), sevoflurane (0.68 mM), and halothane (0.2 mM) decreased the slow time constant of current activation, τ_s, by 20±13, 56±1, and 27±9%, respectively. Isoflurane and sevoflurane also decreased the fast time constant, τ_f, by 45±22 and 62±1%. Halothane, on the other hand, increased τ_f by 46±17%. On return to -40 mV from +90 mV, the time constant for deactivation decreased by 42±6, 39±3, and 33±4% in isoflurane, sevoflurane, and halothane, respectively. The differential effects of anesthetics on the activation kinetics may indicate modulation of the voltage-dependent activation of I_{Ks}.

Tu-Pos21

PHARMACOLOGICAL DIFFERENTIATION OF DELAYED RECTIFIER K^+ CURRENTS OF RABBIT CORONARY ARTERIAL AND PORTAL VEIN MYOCYTES. ((**D.P. Sontag**, A.H Safertal, K.A. Loutzenhiser, and W.C. Cole)) The Smooth Muscle Research Group, University of Calgary, Calgary, Alberta, Canada.

Voltage-gated, delayed rectifier K^+ current (I_{KDR}) is an important component of K^+ conductance of rabbit coronary arterial (CA) and portal vein (PV) myocytes. However, the molecular basis of I_{KDR} in these cells is poorly defined: Kv1.5 is thought to be a major component but other Kv channel α subunit proteins may also be expressed, including Kv1.1 and Kv1.2 (Roberds and Tamkum, PNAS 88: 1798-1802 (1991); Mays et al., J. Clin. Invest. 96: 282-292 (1995)). We studied the effects of 4-aminopyridine (4AP; 0.01-10 mM), charybdotoxin (CTX, 0.05-0.1 μM) and kaliotoxin (KTX, 0.05-0.1 μM), on I_{KDR} of CA and PV to test for differences between the two cell types. Isolated myocytes were obtained by a collagenase digestion procedure and studied via whole-cell voltage clamp technique. I_{KDR} of PV was inhibited by 4AP at micromolar concentrations (IC_{50} ~200 μM) but was unaffected by CTX or KTX. In contrast, I_{KDR} of CA was blocked by 4AP and was also sensitive to CTX and KTX, declining by ~25 and 10% in the presence of the toxins, respectively. This indicates that the molecular basis of I_{KDR} in CA and PV smooth muscle cells is different. Support: AHFMR (AHS) and MRC (WCC).



Tu-Pos22

TETHERING A VOLTAGE-GATED CHANNEL TO THE MEMBRANE SKELETON: A MODEL FOR STUDYING THE IMPACT OF MECHANICAL INPUTS ON GATING ((**X.C. Gu**, P. F. Juranka & C.E. Morris)) Neurosciences, Loeb Institute, Ottawa Civic Hospital, Ottawa, Canada K1Y4E9 (Sponsored by Danielle Carrier)

Mechanosensitive (MS) channels are those channels whose open probability changes in response to mechanical stimuli. Most patch-clamped cells show one or more types of MS channel. While some are surely true mechanotransducers, we hypothesize that others exhibit mechanosensitivity only when damage to the cortical cytoskeleton (as produced by patch clamp) allows mechanical loads to be transferred from the spectrin-based membrane skeleton to channels. Normally, "shock absorption" by the intact cortical cytoskeleton would protect channels from mechanical inputs.

We are testing this notion directly by constructing fusion proteins in which a voltage-gated channel (*Shaker*) is supplied with a spectrin-binding moiety from ankyrin. The entire spectrin-binding domain (~62 kDa) of *Xenopus* or human ankyrin is fused to the C-terminus of an "inactivation-removed" *Shaker* peptide. The resulting recombinant proteins express in both *Xenopus* oocytes and mammalian (HEK) cells and exhibit voltage-dependent potassium currents. *Shaker* channels are homotetramers. We can therefore say that 4 x 62 kDa of cytoplasmic globular protein does not prevent assembly of the channel or disable its fundamental gating behavior, but neither does the putative tethering create a frank MS potassium channel comparable to the "stretch channels" in, say, molluscan neurons or *Drosophila* muscle.

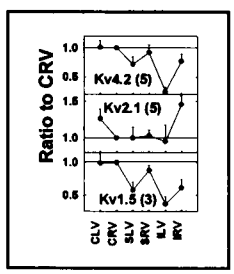
Detailed characterization of the voltage-dependent gating properties of *Shaker* and the ankyrin-*Shaker* fusion proteins without mechanical stimulation or with mechanical stimulation (pressure applied via a patch pipette) is ongoing.

(Supported by MRC, Canada).

Tu-Pos23

DIFFERENTIAL EFFECTS OF MYOCARDIAL INFARCTION ON K CHANNEL EXPRESSION IN RAT VENTRICLE ((**M Jiang**, X Yang, J-A Yao, G-N Tseng)) Dept of Pharm., Columbia U., New York, NY 10032.

Long-term changes in K channel function induced by myocardial infarction may involve alterations in protein expression. We examined the effects of ligating the main left coronary artery on protein level of K channel α-subunits in rat ventricle. 3 days after surgery, membrane proteins were extracted from left (LV) and right (RV) ventricles of infarcted (I), sham-operated (S) and control (C) hearts. Each Western blot contained 6 samples from three animals: LV and RV from I, S and C. Densitometer readings were normalized to that of CRV. Fig shows average ratios to CRV (mean±SE) of Western blots probed with antibodies against Kv4.2, Kv2.1 and Kv1.5 (numbers in parentheses denote number of blots). Comparing ILV to SLV, Kv4.2 and Kv1.5 were reduced while Kv2.1 was unaltered. The reduction in Kv4.2 corresponds to the decrease in the transient outward current in myocytes from ILV, while difference in the change of Kv2.1 and Kv1.5 may explain observed variations in the amplitude of delayed rectifier current from the same cell population.



Tu-Pos24

DIFFERENTIAL EFFECTS OF MYOCARDIAL INFARCTION ON TRANSIENT OUTWARD, DELAYED RECTIFIER AND INWARD RECTIFIER CURRENTS IN RAT VENTRICLE ((**J-A Yao**, Y-Y Zhou, M Jiang, J-S Fan, G-N Tseng)) Dept of Pharmacology, Columbia Unversity, New York, NY 10032.

To understand how myocardial infarction (INF) can affect the function of K channels in the heart, we examined the effects of INF created by coronary artery ligation (LIG) on the transient outward (I_{to}), delayed rectifier (I_K) and inward rectifier (I_{K1}) currents in rat ventricular myocytes. Three days after LIG the ligature was removed and cells were isolated from the outer (EPI) and inner (ENDO) halves of infarcted region of left ventricle (I) and compared to cells isolated from the same region of sham-operated heart (S). Whole-cell K currents were recorded in isolation of Ca and Na currents. The table shows current density (pA/pF) of I_{to}, I_K (both at +40 mV) and I_{K1} (-120 mV) of I and S cells (mean±SD, * $p<0.05$ compared with sham). INF did not affect I_{K1} but reduced I_{to} in both EPI and ENDO. I_K was reduced in EPI but not in ENDO. Despite the changes in current amplitude, the gating behavior of I_{to} and I_K was not altered. These data are consistent with our Western blot analysis, which showed that INF reduced Kv4.2 and Kv1.5 but not Kv2.1. Our results suggest that INF has differential effects on various K channels in the heart.

	EPI		ENDO	
	S (n)	I (n)	S (n)	I (n)
I_{to}	13.5±3.0 (11)	**10.0±2.4*(22)**	7.0±3.6 (16)	**4.6±2.5*(22)**
I_K	3.2±1.3 (15)	**2.0±1.1*(9)**	3.2±2.0 (16)	2.3±1.5 (12)
I_{K1}	-9.8±1.9 (14)	-9.1±3.5 (11)	-11.1±3.0 (15)	-9.4±2.7 (13)

Tu-Pos25

REGULATION OF *SHAKER*-RELATED POTASSIUM CHANNELS BY EXTRACELLULAR POTASSIUM AND pH. ((**H. Jäger**, L. McCauley[1)], H. Rauer, J. Aiyar[1)], A.N. Nguyen[1)], S. Grissmer, M.D. Cahalan[1)] and K.G. Chandy[1)])) Dept. Applied Physiology, University of Ulm, D-89081 Ulm, Germany, [1)] Dept. Physiology & Biophysics, University of California, Irvine, CA 92717.

We previously characterized the effect of changing the extracellular concentration of protons and potassium ions on different mammalian *Shaker*-related voltage-gated K^+ channels (Grissmer *et al.*, Biophys. J. 66:A342, 1994). The presence of a protonated histidine (pH 6.0) at position 404 (H404) in the "P-region" of Kv1.3, or a lysine at the homologous position in Kv1.4, rendered the channel conductance sensitive to a reduction in the external concentration of K^+ (4.5 to 0 mM). In contrast, the presence of uncharged residues at the corresponding positions in Kv1.1 (Y) and Kv1.2 (V), made the channel insensitive to this change. To further characterize the nature of this K^+ channel interaction, we replaced H404 in Kv1.3 with amino acids of different character, size, and charge. We injected *in vitro* transcribed cRNA encoding mutant Kv1.3 channels in RBL cells and investigated whole-cell currents, elicited with 200 ms depolarizing voltage steps from a holding potential of -80 to 40 mV in the presence (4.5 mM) and absence of $[K^+]_o$. In addition, these measurements were done at different pH values (pH 7.4 or 6.0). We found that currents through mutant Kv1.3 channels with uncharged amino acid substitutions like H404V, H404L, and H404A were insensitive to $[K^+]_o$ (compare Kv1.1 and Kv1.2) whereas currents through Kv1.3 channels with H404R at the homologous position were very sensitive to changes in $[K^+]_o$ as expected from the different *Shaker*-related channels. H404S mutant channels behaved nearly like wt, while H404N substitution showed a higher sensitivity to $[K^+]_o$ than wt, comparable to H404R. In addition, currents through Kv1.3 channel dimer constructs (wt-D402N, and wt-Y400V) were also dependent on the presence of $[K^+]_o$. These results indicate that not only charged residues at the external "P-region" are important in conferring potassium-sensitivity. Supported by grants from the DFG (Gr 848/4-1), from Pfizer Inc, CT, USA, and from Pfizer Ltd., Kent, England.

Tu-Pos26

THE ANDROCTONUS AUSTRALIS SCORPION VENOM CONTAINS K+-CHANNELS SELECTIVE BLOCKING TOXINS.

((M. Pisciotta#, F. Coronas*, L.D. Possani* and G. Prestipino#))
Institute of Cybernetic and Biophysics, C.N.R. Genoa 16149 Italy
* Institute of Biotechnology/UNAM, Cuernavaca 62271 Mexico

The outward potassium currents in rat cerebellum granule cells were studied with the patch-clamp technique in the whole cell configuration. Two voltage dependent components were identified: a) a slow activating current characterized by noninactivating kinetics and blocked by external 20 mM tetraethylammonium ions, with properties similar to the classical delyed-rectifier potassium channels; b) a component characterized by fast activating and inactivating kinetics, blocked by external 4 mM 4-aminopyridine and similar to I_A-type current. We have investigated the modifications of these currents recorded from cultured neurons, caused by external application of the scorpion venom *Androctonus australis hector*. This venom was able to block both the potassium currents but with different affinities: the dose-response curves fitted to Michaelis-Menten equation reveal a K_d=(40±6) μgr/250 μl for the noninactivating current and a K_d=(6±3) μgr/250 μl for the similar to I_A-type current. Work are in progress to purify polypeptide toxins from the venom.

Tu-Pos27

DELAYED RECTIFIER K^+ CURRENT OF CANINE BRONCHIAL AIRWAY SMOOTH MUSCLE: ALTERED INACTIVATION WITH RAGWEED POLLEN-SENSITIZATION AND INHIBITION BY PKC ACTIVATION. ((**S.B. Sigurdsson**, G.J. Waldron, E.A. Aiello[1], A.J. Halayko[2], N.L. Stephens[2] and W.C. Cole)) The Smooth Muscle Research Group, University of Calgary, Calgary, Canada, [1]Centro de Investigaciones Cardiovasculares, UNLP, La Plata, Argentina, and [2]Dept. of Physiology, University of Manitoba, Winnipeg, Canada.

The pathophysiology of asthma includes, a depolarized resting membrane potential, enhanced reactivity to constrictor agonists and elevated levels of histamine in bronchial airway smooth muscle (BSM). Recent findings indicate: i) delayed rectifier K^+ current (I_{KDR}) contributes to RMP of BSM (J. Physiol. 469: 645), ii) histamine activates protein kinase C (PKC) in BSM (Eur. J. Pharm. 275: 283), and iii) I_{KDR} in vascular myocytes is depressed by PKC (Am. J. Physiol. 271: H109). Bronchi (4 to 6th order) were removed from age-matched control (*Con*) and ragweed pollen-sensitized (*Sens*) dogs. Single myocytes were isolated by a collagenase digestion procedure and studied by whole-cell voltage clamp technique. No differences in I_{KDR} density (31 ± 2.6 (*Con*) vs 34 ± 4.0 pA/pF (*Sens*) at +30 mV) or voltage-dependence of activation ($V_{0.5}$ of -16 ± 1.2 (*Con*) and -18 ± 1.3 (*Sens*) mV; n = 13-15) were noted. However, the voltage-dependence of inactivation showed a negative shift in cells from sensitized dogs ($V_{0.5}$ of -26 ± 0.7 (*Con*) to -30 ± 1.2 (*Sens*) mV, $P < 0.05$; n = 9-11). Activation of PKC with a diacylglycerol analog, 1,2-diC_8 (10 μM), depressed I_{KDR} tail current amplitude by ~ 80% in 3-8 min. The inactive analog, 1,3-diC_8 was without effect. Enhanced inactivation of bronchial I_{KDR} and/or its suppression by PKC due to histamine may contribute to the pathophysiology of asthma. Support: MRC (WCC), Centres of Excellence Award (NLS), CHS/MRC (GJW) and AHFMR (SBS).

Tu-Pos28

MODULATION OF TRANSIENT K^+ CURRENTS (Kv4.2 AND Kv4.3) BY PROTEIN KINASE C. ((**T.Y. Nakamura**, W.A. Coetzee, E. Vega*, M. Artman, B. Rudy*)) Pediatrics, and *Physiology and Neurosciences, NYU Medical Center, NY10016.

The cardiac transient outward current (I_{to}) is thought to be composed predominantly of Kv4.2 and Kv4.3 gene products. Since α_1-adrenergic stimulation and activation of protein kinase C (PKC) by phorbol esters depress cardiac I_{to}, we examined the role of PKC on the function of these molecular components. We tested the effects of phorbol-12-myristate-13 acetate (PMA) on Kv4.2 or Kv4.3 currents expressed in Xenopus oocytes using two-microelectrode voltage clamping. PMA (1-100 nM) caused marked suppression of these currents in a time and concentration dependent manner. Preincubation of the oocytes with the PKC-inhibitors (chelerythrine or staurosporine) blocked the effect of PMA whereas the inactive stereoisomer, 4α-phorbol-12,13-didecanoate, had no effect on these currents. α_1-Adrenergic agonist, phenylephrine (10 μM), inhibited Kv4.2 currents only when co-expressed with the α_{1C}-adrenergic receptor and this effect was largely prevented by chelerythrine, indicating that receptor-mediated PKC activation also suppressed these currents. Neither PMA nor α_1-adrenergic stimulation had any effect on the voltage-dependence or inactivation kinetics of Kv4.2 currents. Essentially identical results were observed in native I_{to} of rat ventricular myocytes. These results demonstrate that PKC inhibits I_{to} by direct modulation of its molecular components, Kv4.2 and Kv4.3.

Tu-Pos29

Abstract Withdrawn.

Tu-Pos30

EFFECTS OF THIOL-MODIFYING AGENTS ON A $K(Ca^{2+})$ CHANNEL OF INTERMEDIATE CONDUCTANCE IN BOVINE AORTIC ENDOTHELIAL CELLS. ((R. Sauvé and S. Cai)) Department of Physiology, University of Montreal, Montreal, Quebec, Canada H3C 3J7.

Ca^{2+}-activated K^+ channels ($K(Ca^{2+})$) constitute key regulators of the endothelial cell potential during stimulation. Inside-out and outside-out patch-clamp experiments were thus undertaken to determine if the gating properties of a voltage-insensitive $K(Ca^{2+})$ channel of intermediate conductance present in bovine aortic endothelial cells (BAE) could be modified by specific sulfhydryl (SH) oxidative and/or reducing reagents. The results obtained first indicate that cytosolic application of hydrophillic oxidative reagents such as DTNB (1 mM) or thimerosal (1 mM to 5 mM) leads to a total inhibition of the $K(Ca^{2+})$ channel activity with no modification of the channel unitary conductance. The observed inhibition was not reversed following withdrawal of the oxidative agents, but channel activity could partly be restored in 55% of the experiments by addition of the SH group reducing agents DTT (5 mM) or GS (5 mM). We observed, in addition, that the lipid soluble oxidative agent 4-PDS (1 mM) was less potent in inhibiting the $K(Ca^{2+})$ channel in inside-out experiments than DTNB and thimerosal, suggesting that the critical SH groups involved in channel gating were localized at the membrane inner face. This conclusion was further substantiated in a series of outside-out patch clamp experiments which showed that DTNB (5 mM) and thimerosal (5mM) were ineffective in affecting the $K(Ca^{2+})$ channel activity when applied to the external surface of the excised membrane. These results confirmed that the SH groups responsible for the oxidation-induced $K(Ca^{2+})$ channel inactivation are localized on the cytosolic side of the membrane. Finally, in experiments where the $K(Ca^{2+})$ channels showed significant rundown, the application of either GSH or DTT was found to partly restore channel activity. These observations were interpreted as indicating that rundown in this case was related to the oxidation of critical SH groups.

Tu-Pos31

AUTHENTIC NO ACTIVATES BK_{Ca} CHANNELS IN RAT BASILAR ARTERY MYOCYTES ((M. Holland, N.B. Standen & J.P. Boyle)), Cell Physiology & Pharmacology, University of Leicester, LE1 9HN, UK

Hyperpolarization induced by nitric oxide (NO) has been implicated in vasorelaxation in various blood vessels. However, there is still no consensus as to which K^+ channel is activated by NO with reports of BK_{Ca} activation in cerebral arteries[1] and K_{ATP} activation in mesenteric preparations[2]. We have previously reported that SIN-1, a NO donor, activates BK_{Ca} channels in the rat basilar artery. Here we have examined the effects of authentic NO.

Saturated NO solutions were infused into a recording chamber, in the presence of SOD (50U/ml^{-1}), containing a basilar myocyte held close to its resting membrane potential (conventional whole cell configuration; symmetrical 140mM K^+). The inward current activated developed slowly during infusion and continued to increase after infusion had finished. This inward current was insensitive to glibenclamide (5μM) but was blocked by charybdotoxin or iberiotoxin (both 100nM). The current inhibited by ChTX or IbTX reappeared after washout of the toxins and was not abolished by prolonged washing. The unitary current amplitude activated by NO was -14.97 ±1.1pA which compares with an amplitude of -15±2.6pA in the same cells prior to NO infusion. These values are nearly identical to those obtained using SIN-1 as a NO donor and correspond to a channel conductance of 257pS.

Work is continuing to establish whether this activation of BK_{Ca} channels contributes to vasorelaxation in this tissue; as has been suggested in other arteries.

Supported by the MRC & BHF

1. Robertson, BE *et al* (1993) Am.J.Physiol. **265**, C299-C303
2. Murphy, ME & Brayden, JE (1995) J.Physiol. **486**, 47-58

Tu-Pos32

FATTY ACID MODULATION OF THE CLONED Ca^{2+}-ACTIVATED K^+ (CAK) CHANNELS: MSLO, BSLO AND HSLO. ((**Alison L. Clarke,** John V. Walsh, Jr. and Joshua J. Singer)) Department of Physiology, University of Massachusetts Medical School, Worcester, MA 01655.

Previously, we reported that fatty acid modulation of (CAK) channel activity from rabbit pulmonary artery (RPA) smooth muscle cells appears to result from the direct interaction of the fatty acid with a site on the channel protein itself or, alternatively, on some membrane bound channel associated protein (Soc. Neurosci. Abs., 21: 1326, 1995) and not from an indirect or nonspecific mechanism. To determine whether other CAK channels are similarly modulated by fatty acids as well as whether this modulation requires the CAK channel β-subunit, we studied the fatty acid regulation of three cloned CAK channels. The clones mslo, bslo and hslo were expressed in Xenopus oocytes in the presence or absence of the β-subunit RNA and were studied in 100 μM Ca^{2+} containing bathing solutions using the two electrode voltage-clamp technique. The 14 carbon saturated fatty acid, myristic acid, and the 20 carbon polyunsaturated fatty acid, arachidonic acid, caused a 10-30% increase in whole-cell outward current in oocytes expressing each of the three clone variants. Myristic acid and arachidonic acid did not produce such an increase in non-injected or mock injected oocytes and in many cases caused a decrease in outward current. Injection of BAPTA (final oocyte concentration, 5-10 mM) to buffer internal Ca^{2+} did not prevent the increase in current caused by these fatty acids suggesting that this increase was not due to a change in internal Ca^{2+}. Fatty acid regulation of these clones did not require the β-subunit, and co-injection of β-subunit RNA with that of the mslo and hslo α-subunit clones into oocytes did not change the response of these clones to fatty acids. If regulation of these cloned CAK channels occurs via the direct interaction of the fatty acid with a site on the channel protein itself, as appears to be the case for the native CAK channel of the RPA, this site is likely to be a conserved region of the CAK channel family. (Supported by NIH).

Tu-Pos33

CALCIUM INFLUX STRONGLY MODULATES K^+ CURRENTS IN ARTERIAL MYOCYTES. ((Robert H. Cox and Irina Lozinskaya)) Bockus Research Institute, Graduate Hospital, Dept. of Physiology, Univ. of PA, Philadelphia, PA 19146.

Previous studies of K^+ currents (I_K) measured from different holding potentials (HP) and under different conditions of intracellular Ca^{2+} buffering suggest that Ca^{2+} influx through L-type channels (Ca_L) may modulate I_K. To test this hypothesis further, experiments were performed to determine the effects of inhibiting Ca_L with 0.1mM Cd^{2+} on I_K in myocytes isolated from rat small mesenteric arteries. Myocytes were freshly dispersed by collagenase and elastase, and I_K recorded by patch clamp methods with 2mM external Ca^{2+} and 0.2 or 10mM BAPTA in the pipette solution at ≈23°C. When measured with 0.2mM BAPTA from HP = -20mV (only Ca^{2+} activated K^+ current, K_{Ca}, present), Cd^{2+} inhibited I_K by 56±4 at +60mV. When measured with 10mM BAPTA from a HP = -20mV, Cd^{2+} inhibited I_K by 42±8% at +60mV. When measured with 0.2mM BAPTA from a HP = -60mV (both K_{Ca} and voltage-gated I_K, K_V, present), Cd^{2+} inhibited peak I_K by 22±5% at +60mV but increased I_K by 16±8% at -10mV. When measured with 10mM BAPTA from a HP = -60mV, Cd^{2+} had no significant effect on peak I_K at +60mV, but increased peak I_K at -10mV by 35±10%. When measured at -10mV, 500ms after the voltage step from HP = -60mV, Cd^{2+} increased I_K by 46±15% with 10mM BAPTA and by 28±9% with 0.2mM BAPTA. These results suggest that Ca_L influx increases K_{Ca}, and decreases K_V in this preparation. Also, similar results were found under conditions of high or low intracellular Ca^{2+} buffering. This suggests that these effects may be produced by Ca^{2+} influx into a subcellular compartment not in rapid equilibrium with Ca^{2+} buffers (subsarcolemmal space).

CALCIUM CHANNELS I

Tu-Pos34

DETECTION OF α_{1E}-LIKE mRNA IN ATRIAL TISSUE FROM JUVENILE AND OLD RATS. ((**E.S. Piedras-Renteria** and P.M. Best)) Department of Molecular & Integrative Physiology, University of Illinois, Urbana IL 61801.

Atrial tissue expresses two types of voltage-gated calcium currents, L-type and T-type. There is no molecular data on the primary structure of the T-type channel, although when expressed the α_{1E} subunit from rat brain shows properties compatible with low-voltage gated channels (Soong et al., 1993; Bourinet et al., 1996). To find whether an α_{1E}-like subunit is expressed in atrial tissue, we performed RT-PCR using regions of the cDNA sequence for rat brain α_{1E} for primer design. A 908 bp product was obtained, subcloned and sequenced. This clone is highly similar to α_{1E}, except that it is ~10% smaller than expected.The presence of mRNA for this partial clone was determined using RNAse protection assay with a 235 bp antisense probe. Positive signals were detected in mRNA from rat thalamus and atrium; rat ventricle and shark ventricle signals were minimal; and liver was negative. mRNA was quantified using an internal control (cyclophilin); the highest levels of expression were found in thalamus and in atrium from juvenile rats. Atrium from old rats contained considerable lower amounts of α_{1E}-like mRNA. Since previous data from our lab (Xu & Best, 1992) shows larger T-type current density in atrial cells from juvenile rats as compared with aged animals, our present results suggest that our partial clone encodes the cardiac T-type channel.

Tu-Pos35

AN ANTISENSE OLIGONUCLEOTIDE AGAINST THE α1E GENE BLOCKS THE IGF-I-INDUCED INCREASE OF T-CURRENT IN CULTURED ATRIAL MYOCYTES. ((**C.C. Chen,** E. Piedras-Renteria and P.M. Best)) Dept of Molecular and Integrative Physiology and School of Medicine , University of Illinois, Urbana, IL 61801

Previous work in our laboratory shows that IGF-I up-regulates the density of T-type calcium current in cultured atrial myocytes (Chen & Best, 1996). To understand the molecular characteristics of atrial T-type calcium channels, we have used a partial clone from atrial tissuesl that shows significant homology to the α1E gene. Oligonucleotides based on regions of this clone were tested. One of them, a 19mer, had zero homology with that of cardiac L-type α1C gene and is identical with that of α1E gene. One μM of either antisense or sense of this oligonucleotide were added to cultured dishes containing atrial myocytes isolated from the same preparation. The whole-cell configuration of the patch-clamp technique was used to record calcium currents. The antisense oligonucleotide blocked the increase in T-current density normally induced by IGF-I from 88% to 28%. Treatment with the antisense oligonucleotide did not affect L-current density nor the cell capacitance. One μM of sense oligonucleotide had no effect on the increase in T-current density induced by IGF-I, nor on the L-current density or cell capacitance. These results suggest that the partial clone from our laboratory encodes the T-type calcium channel. Supported by AHA, IL Affiliate.

Tu-Pos36

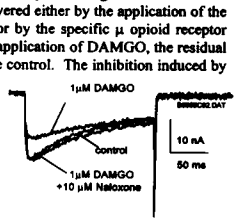
AN EXONS 45/46-LIKE SEQUENCE POINTS TO A NEW HOMOLOGUE OF THE HUMAN Ca^{2+} CHANNEL α_{1C} SUBUNIT GENE ((**Nikolai M. Soldatov***, Terje Raudsepp, and Bhanu P. Chaudhary)) *Department of Pharmacology, Georgetown University Medical Center, Washington, DC 20007, USA and Swedish University of Agricultural Sciences, Uppsala, Sweden (Spon. by R.N. Pittman)

Human L-type Ca^{2+} channel pore-forming α_{1C} subunit gene is composed of 51 exons and is located at p13.3 band of chromosome 12. Exon 45 is proved to be a combinatorial alternative exon. We have studied a 20-kb DNA fragment g8-19 identified in human genomic DNA library by the 300-nt cDNA probe of α_{1C} transcript containing exon 45. It was found that g8-19 bears exons 45/46-like sequence with >85% nucleotide identity to exons 45 and 46 of the α_{1C} subunit gene, the short introns between them being of similar size. Using the multicolored fluorescent *in situ* hybridization technique, it was found that g8-19 hybridizes to previously unknown regions of human chromosome (bands p13.1 and p11.2) which are far away from the location of the α_{1C} gene. DNA probes derived from 5'- and 3'-termini of the α_{1C} subunit gene did not recognize these regions. Thus g8-19 represents a new genomic homologue of the α_{1C} subunit gene.

Tu-Pos37

FUNCTIONAL COUPLING BETWEEN NEURONAL α_{1E} Ca^{2+} CHANNELS AND μ OPIOID RECEPTORS EXPRESSED IN *Xenopus* OOCYTES ((**R. Olcese,** M. Ottolia, N. Qin, D. Platano, M. Birnbaumer, L. Toro, L. Birnbaumer, E. Stefani)) UCLA School of Medicine, Dept. of Anesthesiology, Los Angeles, CA 90095-1778

Voltage dependent Ca^{2+} channels play a fundamental role in neurotransmitter release at the presynaptic terminals. One mechanism for modulating of the synaptic transmission involves a G-protein dependent depression of Ca^{2+} channels activity. We have studied the coupling between μ opioid receptors and neuronal α_{1E} Ca^{2+} channels co-expressed in *Xenopus* oocytes by measuring Ba^{2+} currents with the Cut-open voltage clamp technique. The stimulation of the μ receptor with its specific agonist DAMGO [D-Ala^2,N-Me-Phe^4,Gly-ol^5] (1 μM) produced a ~20% reduction in the ionic current accompanied by a slowing of the inactivation kinetics. The current and overall kinetics were fully recovered either by the application of the universal opioid receptor antagonist naloxone (10 μM) or by the specific μ opioid receptor antagonist β-funaltrexamine (β-FNA) (1 μM). After the application of DAMGO, the residual current had the same activation voltage dependence as the control. The inhibition induced by the μ receptor stimulation was partially relieved by strong depolarizations. Injection of the pertuxis toxin catalytic subunit (25 nl, 100ng/μl) abolished the effects of DAMGO on the ionic current. These results suggest a mechanism involving a direct G-protein effect on the channel after activation of the μ receptor. Co-expression of the α_{1E} subunit with the Ca^{2+} channel regulatory β_{2a} subunit prevented the current inhibition by μ receptor activation. (Supported by NIH grants to LT, ES, LB. LT is an AHA Established Investigator).



α1E + μ opioid receptor

Tu-Pos38

FUNCTIONAL EXPRESSION OF GREEN FLUORESCENT PROTEIN (GFP) FUSED VOLTAGE-DEPENDENT CALCIUM CHANNELS IN DYSGENIC MYOTUBES.
((**M. Grabner**, R.T. Dirksen, C. Proenza and K.G. Beam)) Dept. of Anatomy & Neurobiology, Colorado State University, Fort Collins, CO 80523. (Spon. by N.Lorenzon)

Tagging of heterologously expressed Ca^{2+} channels with the jellyfish green fluorescent protein (GFP) would be extremely useful, enabling the easy identification of transfected cells and the monitoring of temporal and spatial expression. The cDNA encoding each of three classes of Ca^{2+} channel α_1 subunits (α_{1S}, α_{1C} and α_{1A}) was inserted in frame into a CMV promoter-controlled GFP expressing plasmid (kindly provided by Dr. P. Seeburg, and subsequently modified with an S65T mutation), such that the GFP was linked to the amino terminus of the channel (since C-termini are posttranslationally cleaved). GFP-α_1 fusion proteins were expressed in dysgenic myotubes and currents were recorded using the whole-cell patch clamp technique. To allow maturation of GFP-α_1 fusion proteins to the fluorescent form, the culture temperature for the transfected myotubes had to be lowered to 30°C. Fusion with GFP did not appear to alter the kinetic channel properties of α_{1C} and α_{1A} and high current densities could be recorded from every green myotube (average of about 40 pA/pF and 30 pA/pF, respectively). In contrast dysgenic myotubes injected with GFP-α_{1S} cDNA displayed, despite their green color, current densities of only <3 pA/pF instead of the expected ~8 pA/pF. Because the N-terminal region of the skeletal muscle α_{1S} subunit is only a fraction of the length of the N-terminal region of α_{1C} or α_{1A} (33% or 52%, respectively), we are addressing the question of whether the GFP-α_{1S} transition is too short to allow the proper folding of the skeletal Ca^{2+} channel α_{1S} subunit. Specifically, we are extending the N-terminus of α_{1S} to determine whether this increases the yield of functional skeletal Ca^{2+} channels. These results should be useful for the functional expression of other GFP-fused ion channels with short or with functionally essential N-termini (e.g. K^+ channels). Supported by FWF, Austria (J01242-GEN) to MG and NIH (NS24444) to KGB.

Tu-Pos39

BIOPHYSICAL AND PHARMACOLOGICAL PROPERTIES OF HUMAN NEURONAL $\alpha_{1D}\alpha_{2b}\delta\beta_{3a}$ Ca^{2+} CHANNELS STABLY EXPRESSED IN HEK293 CELLS
((**M. Hans**, A. Urrutia, P. Brust, A. Nesterova, A.I. Sacaan, M. Harpold and K. Stauderman)) SIBIA Neurosciences, Inc., La Jolla, CA 92037.

The biophysical and pharmacological properties of human $\alpha_{1D}\alpha_{2b}\delta\beta_{3a}$ channels stably expressed in HEK293 cells were investigated. Whole cell ^{3}H-PN200-110 binding revealed a single class of binding sites with a Kd of ~1 nM and a B_{max} of ~40,000 sites/cell. Whole cell recordings from these cells revealed sustained Ba^{2+} currents with an average current density of 16 pA/pF at +10 mV. With Ca^{2+} as the charge carrier the current density decreased by about 3-fold and currents showed a rapid Ca^{2+}-dependent inactivating component that contributed ~60% of the total current. During two second depolarizations the time constant of this inactivating component was 49 ± 7.6 ms which was ~10-fold faster compared to that obtained in Ba^{2+}. Currents deactivated rapidly with a time constant of ~150 µs (at -80 mV). The dihydropyridine (-)BayK 8644 (10 µM) caused an ~2-fold enhancement in current density, a -10 mV shift of the current voltage relationship, and a slowing in the rate of current deactivation, while nimodipine (5 µM) completely blocked the currents. The single channel slope conductance for human $\alpha_{1D}\alpha_{2b}\delta\beta_{3a}$ Ca^{2+} channels was determined to be 26.9 pS with 110 mM Ba^{2+} as charge carrier. These findings indicate that the human neuronal $\alpha_{1D}\alpha_{2b}\delta\beta_{3a}$ Ca^{2+} channel exhibits a fast Ca^{2+}-dependent inactivation, but also shares many properties generally ascribed to L-type Ca^{2+} channels.

Tu-Pos40

CLONING OF AN L-TYPE Ca CHANNEL HOMOLOG FROM FROG SKELETAL MUSCLE AND FUNCTIONAL EXPRESSION OF A CHIMERIC CHANNEL.
((J.Zhou, L.Cribbs, J.Yi, R.Shirokov, E.Perez-Reyes, E. Rios)). Rush University, Chicago, IL 60612 and Loyola University, Maywood, IL 60153.

The functions of the skeletal muscle DHP receptor have been extensively studied in single fibers of frog skeletal muscle. We cloned an α_1 subunit homolog. A cDNA library was constructed from purified mRNA of skeletal muscle *(Rana catesbeiana)* and screened with specific probes synthesized by RT-PCR with degenerate primers based on well conserved sequences in rabbit skeletal and cardiac α_1 subunits. The positive clones covered a whole length mRNA, including 5' and 3' untranslated regions. Overlapping segments were ligated and subcloned into expression plasmids. The cloned cDNA contains 5600 base pairs. In spite of a 79% homology with α_{1S} and 72% with α_{1C} (rabbit), the frog's sequence has several differences with known DHP receptors. Interestingly, its C terminus is 172 residues shorter than the rabbit's full sequence. The cDNA codes for 1688 residues, or about the same size as the "175 kD" form of the rabbit molecule (Jongh et al., 1991).

In tsA-201 cells we recorded Ca^{2+} currents induced by coexpression (with β_{2a}) of the chimera obtained replacing domain I of rabbit α_{1C} with the corresponding frog's piece. In 10 mM Ca^{2+}, the time to half-peak current was 5.0±0.5 ms (n=7) for the chimeric channels, and 5.5±0.5 ms (n=7) for α_{1C}+β_{2a}. In contrast, in dysgenic murine myotubes the rabbit cardiac channel's activation is slowed when domain I is replaced by its rabbit α_{1S} counterpart (Tanabe et al., 1991). The implications of the present result will be clear when the full frog clone is expressed.

Supported by NIH, MDA and AHA.

Tu-Pos41

ROLE OF GLUTAMATE RESIDUE E1537 IN CALCIUM -DEPENDENT INACTIVATION OF α_{1C} CALCIUM CHANNELS.
((**C. Porter Moore**, D. Talwar, and L. Parent)) Dept Mol Physiol. Biophys., Baylor Coll Med, Houston, TX 77030.

In cardiac cells, calcium-dependent inactivation has been described as a negative feedback mechanism for regulating voltage-dependent calcium influx. Most recent evidence points to the C-terminus of the α_1 subunit, with its EF-hand binding motif, as being critical in this process (DeLeon et al., 1995). The EF-hand motif is relatively conserved among the six Ca^{2+} channel gene families, except for a Glu residue found at the Y coordinate of the putative EF-hand binding motif. The Glu residue (E1537) in α_{1C} is replaced by Ala in brain α_{1B}, α_{1A} and α_{1B} Ca^{2+} channels that lack Ca^{2+}-dependent inactivation. We characterized the role of the Glu residue using a combination of PCR- mutagenesis and electrophysiology techniques. The cardiac α_{1C} subunit (wild-type and point mutations) was expressed in *Xenopus* oocytes with auxiliary β_{2a} and $\alpha_{2b}\delta$ subunits. Whole-cell currents were measured in the presence of 10 mM Ba^{2+} or 10 mM Ca^{2+} after intracellular injection of Bapta. Preliminary results obtained for E1537A showed that only 55 ± 10 % (n=2) of the whole-cell Ca^{2+} current inactivated after a 5 sec prepulse as compared to 88± 6% (n=12) for the wild-type (wt) α_{1C} channel. In contrast, 86 ± 5 % (n=5) of the E1537Q whole-cell Ca^{2+} current inactivated after a 5 s prepulse, with a mean τ_{inact} = 105 ± 20 ms (n=5). The Ca^{2+} inactivation properties of E1537Q were similar to the wt channel, indicating that ion coordination provided by the carbonyl residues, might be important for Ca^{2+}-inactivation. Ba^{2+}-dependent inactivation was similar for all constructs. Altogether, our results confirm the role of the EF-hand binding motif in Ca^{2+}-dependent inactivation and suggest a critical role for a polar residue at the Y position of the EF-hand motif. Supported by training grant NIH (T32)- HL07676 to CPM and NIH-HL54708 to LP.

Tu-Pos42

INTRACELLULAR Ca^{2+} INDUCES INACTIVATION OF EXPRESSED L-TYPE Ca^{2+} CHANNELS WITH A HILL COEFFICIENT CLOSE TO 1 AND A K_i OF 4 µM BY A REDUCTION OF CHANNELS OPEN PROBABILITY RATHER THAN AVAILABILITY.
((G.F. Hößer, K. Hohenthanner, W. Baumgartner, K. Groschner*, N. Klugbauer*, F. Hofmann* and C. Romanin)) + Institute of Pharmacology and Toxicology, Univ. of Graz, Austria; *Institute for Pharmacology and Toxicology, Techn. Univ. of Munich, Germany; Institute for Biophysics, Univ. of Linz, Austria.

The patch-clamp technique was employed to characterize the mechanism of Ca^{2+}-induced inactivation of cardiac L-type Ca^{2+} channel α_{1C-a} + β_3 subunits stably expressed in CHO cells. Single Ca^{2+} channel activity was monitored with 96 mM Ba^{2+} as charge carrier in the presence of 2.5 µM (-) BAYK 8644 and calpastatin plus ATP. This enabled stabilization of channel activity in the inside-out patch and allowed for application of steady-state Ca^{2+} concentrations to the intracellular face of excised membrane patches in an attempt to provoke Ca^{2+}-induced inactivation. Inactivation was found to occur specifically with Ca^{2+} since it was not observed upon application of Ba^{2+}. Ca^{2+}-dependent inhibition of mean Ca^{2+} channel activity was characterized by a Hill coefficient close to 1 and a K_i of ~4 µM. This inhibition manifested predominantly in a reduction of channel's open probability whereas availability remained almost unchanged. The reduction in open probability was achieved by an increase in first latencies and a decrease in channel opening frequency as well as channel open times. At high (12-28 µM) Ca^{2+} concentrations, 72 % of inhibition occurred due to a stabilization of the closed state and the remaining 28% by a destabilization of the open state. Our results suggest that binding of one calcium ion to a regulatory domain induces a complex alteration in the kinetic properties of the Ca^{2+} channel. (supported by Austrian Research Funds S06605, S06606, S06607)

Tu-Pos43

A 80-AMINO ACIDS SEGMENT IN THE C-TERMINUS OF ALTERNATIVELY SPLICED HUMAN α_{1C} Ca^{2+} CHANNEL SUBUNITS IS INVOLVED IN THE REGULATION OF THE KINETICS AND Ca^{2+}-DEPENDENCE OF INACTIVATION
((**Roger D. Zühlke**, Nikolai M. Soldatov, Alexandre Bouron, and Harald Reuter)) Department of Pharmacology, University of Berne, CH-3010 Berne, Switzerland

The pore-forming α_{1C} subunit is the principal component of the voltage-sensitive L-type Ca^{2+} channel. It has a long cytoplasmic C-terminal tail playing a critical role in channel gating. The expression of α_{1C} subunits is characterized by alternative splicing which generates its multiple isoforms. cDNA cloning points to a diversity of human hippocampus α_{1C} transcripts in the region of exons 40-43 that encode a part of the 662-amino acids C terminus. We compared electrophysiological properties of $\alpha_{1C.77}$ and $\alpha_{1C.86}$, two alternatively spliced isoforms of α_{1C}. They contain alterations in the C-terminus due to alternative splicing of exons 40-42. The 2139-amino acids $\alpha_{1C.86}$ has 80 amino acids replaced in positions 1572-1651 of $\alpha_{1C.77}$ by a non-identical sequence of 81 amino acids. When expressed in *Xenopus* oocytes, isoforms retained high sensitivity towards dihydropyridine blockers but showed large differences in gating properties. In contrast to $\alpha_{1C.77}$, Ba^{2+} currents (I_{Ba}) through $\alpha_{1C.86}$ inactivated 8-10 times faster at +20 mV, and its inactivation rate was strongly voltage-dependent. The inactivation curve of I_{Ba} through $\alpha_{1C.86}$ channels was shifted towards more negative voltages by 11 mV. Most strikingly the $\alpha_{1C.86}$ channel lacks a Ca^{2+}-dependent component of inactivation, which is common to all known class C L-type Ca^{2+} channels. The putative transmembrane domains as well as the EF-hand motif, which is located at the beginning of the cytoplasmic C-terminus and which is required for Ca^{2+}-dependent inactivation, are identical in both α_{1C} isoforms. Thus, we could identify a new modulatory 81-amino acid segment in the cytoplasmic tail of α_{1C}, which is critical for the kinetics as well as for the Ca^{2+}- and voltage-dependence of L-type Ca^{2+} channel gating. (Supported by grants from the Swiss National Science Foundation (31-45093.95) and from the Sandoz Foundation)

Tu-Pos44

SINGLE CHANNEL AMPLITUDE AND OPEN PROBABILITY AFFECT RATES OF CA^{2+} DEPENDENT INACTIVATION: A HYPOTHETICAL CA^{2+} SITE IN CLOSE PROXIMITY TO INTERNAL PORE OPENING. ((F. Noceti, R. Olcese, N. Qin, L. Birnbaumer and E. Stefani)) Depts. of Anesthesiology and Physiology, UCLA School of Medicine, Los Angeles, CA 90095-1778.

L-type Ca^{2+} channels are modulated by both voltage and Ca^{2+}. Here we studied the cloned α_{1C} subunit of the cardiac Ca^{2+} channel expressed alone and co-expressed with the auxiliary β_{2a} subunit. Ca^{2+} currents strongly inactivate as opposed to Ba^{2+} currents. The time course of the $\alpha_{1C}\beta_{2a}$ currents can be fitted to a double exponential (fast and slow). Ca^{2+} currents recorded from oocytes expressing the α_{1C} subunit alone have only the slow component. Both inactivation rates present a bell shaped voltage dependence. The peak of inactivation occurs at more negative potentials than the peak of the macroscopic current. Increasing external Ca^{2+} concentration, as well as adding Bay K 8644(-) to the external solution, increases the inactivation rates. This suggests that the open probability has a role in determining the inactivation process. Saturating Bay K 8644(-) concentrations (ca. 500 nM) induces the appearance of the fast rate of inactivation when applied to the α_{1C} subunit alone. This fast rate was undetectable in α_{1C} in control conditions. Oocytes internally perfused with 50 mM BAPTA show a decrease in the rates of inactivation. This finding is in agreement with a mechanism depending on the build-up of Ca^{2+} in proximity to the inner mouth of the pore. The voltage difference between the peaks of inactivation rates and macroscopic currents, implies a non-linear dependence of the inactivation process on single channel amplitude. Altogether these results suggest the presence of a Ca^{2+} site for inactivation located in close proximity to the internal pore opening, thus sensing both ion flux through the channel itself (single channel amplitude) and accumulation of Ca^{2+} ions in a local domain close to the pore (time integral of macroscopic currents). (Supp. by grants: NIH AR 38970; AHA Grant-in-Aid 1113-GI1).

Tu-Pos45

POSSIBLE VOLTAGE-DEPENDENCE OF THE CALCIUM DEPENDENT INACTIVATION PHASE OF I_{Ca} IN ISOLATED GUINEA PIG VENTRICULAR MYOCYTES. ((F. Brette, A. Lacampagne & J-Y. Le Guennec)) Labo. de Physio. des Cell. Card. et Vasc., CNRS EP21, Fac. des Sciences 37200 Tours, France. (Spons. V. JACQUEMOND)

It is known that the inactivation of I_{CaL} depends on voltage and calcium which are two parameters closely linked. Recently, a voltage step has been found in the calcium dependent inactivation phase (Höfer *et al*, 1995, *Biophys J*, 70 :A186). L-type calcium current was measured in the whole cell configuration of the patch clamp technique in conditions where no contaminant sodium (TEA instead of Na^+), sodium-calcium exchange and potassium (Cs^+ instead of K^+) currents were present. The bulk calcium was buffered using 10 mM EGTA but the subsarcolemmal calcium was allowed to vary. In such conditions, we already showed that a hump might appear during the inactivation phase of currents. This hump was linked to the functional integrity of the sarcoplasmic reticulum since treatments which altered the normal SR function (ryanodine 100 µM, caffeine 10 mM, cyclopiazonic acid 100 µM) removed the hump (Lacampagne *et al*, 1995, *Biophys. J.*, 70 : A271). Surprisingly, we observed that this hump, due to the liberation of calcium by the SR which induced a calcium dependent inactivation, was more pronounced at negative voltages. More over, the presence of this hump depends on the holding potential : with more depolarized potentials, the hump disappeared. To explain our results, we hypothesized that a step into the inactivation induced by calcium is voltage dependent. Computation to test this hypothesis was performed.

Tu-Pos46

EXPRESSION, LOCALIZATION AND ASSOCIATION OF L-TYPE Ca CHANNEL SUBUNITS IN CARDIAC MYOCYTES.
((T. Gao, T. Puri, B. Gerhardstein and M. M. Hosey)) Northwestern Univ. Medical School, Chicago, IL 60611.

Cardiac L-type Ca channels (CaChs) play important roles in controlling intracellular Ca^{2+} concentration and excitation contraction coupling in cardiac muscle. CaChs are multisubunit complexes. In heart, molecular cloning studies and Northern analysis have identified the α_{1C} isoform as the major pore-forming subunit, while at least four different mRNAs for β subunits were found. It is uncertain which β isoform is associated with the α_{1C} subunits to form functional L-type channels in cardiac myocytes. Another important question concerning the composition of native cardiac L-type CaCh is whether the full-length vs. truncated α_{1C} subunits are part of the native L-type channels. To address these issues, freshly dissociated rabbit cardiac myocytes were used in our studies. The α_{1C} and the β_{2a} subunits of CaChs were isolated by either WGA purification or direct immunoprecipitation using subunit-specific antibodies. Furthermore, we used immunocytochemistry techniques to visualize channel subunits by immunostaining rabbit cardiac myocytes. The results revealed that the β_{2a} isoform is expressed in cardiac myocytes along T-tubule membranes. The full length α_{1C} subunit exists in cardiac myocytes and co-localizes with other CaCh subunits. We also observed that the expression of Ca^{2+}-sensitive adenylate cyclases is on T-tubule membranes and closely associated with L-type CaChs in myocytes. Taken together, our results provide new insights into the structure of native L-type channels. Moreover, the fact that the CaChs are co-localized with the adenylate cyclases provides a structural basis for understanding receptor-mediated regulation of CaChs and Ca^{2+}-mediated regulation of adenylate cyclases.

Tu-Pos47

CALCIUM CHANNEL β AND G PROTEIN βγ INTERACT WITH DISCRETE AND SEPARATE REGIONS OF A NEURONAL Ca^{2+} CHANNEL α_{1E} SUBUNIT ((N. Qin, D. Platano, R. Olcese, J. Zhou, E. Stefani and L. Birnbaumer)) UCLA School of Med. Los Angeles, CA 90095

Voltage-gated calcium channels are multimeric protein complexes including the pore forming and voltage sensing α_1 subunit, and β and $\alpha_2\delta$ regulatory subunits. In neuronal and endocrine cells, the response of calcium channels to voltage is under a secondary regulatory control imposed by the activation of receptors that are coupled by the Gi/Go class of G proteins, which is of a negative type, leading to the so-called kinetic slowdown of channel activation. The receptor mediated inhibition is reduced by coexpression Ca^{2+} channel β subunit. It has been proposed that the G protein mediated inhibition is due to direct interaction of Gβγ with Ca^{2+} channel α_1, and Gβγ and Ca^{2+} channel β subunits act by functionally opposing each other. A segment of α_1 loop1 as a site of interaction with β has been identified by Campbell and collaborators. By using the yeast two-hybrid system and the GST pulldown technique, we have identified a second segment of α_{1E} that interacts with β. Also, we characterized two motifs on α_{1E} that directly interact with Gβγ, but not Go trimmer. These results support the hypothesis that receptor mediated inhibition is due to direct interaction of α_1 with Gβγ. Ca^{2+} channel β may modulate this effect by either interfering with the action or the binding of Gβγ.

Tu-Pos48

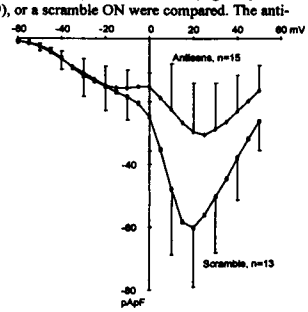
THE L-TYPE CA^{2+} CURRENT OF β_1-NULL MYOTUBES IS NOT A DYSGENIC CURRENT ((C. Strube, M. Sukhareva, P.A. Powers*, R.G. Gregg*, R. Coronado)) Department of Physiology and *Waisman Center, University of Wisconsin, Madison, WI 53706, USA.

We previously characterized Ca^{2+} currents and EC coupling in β_1-null myotubes from mutant mice carrying a null mutation in the β_1 (cchb1) gene (Strube et al., Biophys. J., Nov. 1996). The β_1-null cells are characterized by a reduced L-type Ca^{2+} current, reduced charge movement, and an EC uncoupling. Here, we compare $I_{\beta null}$, the L-type Ca^{2+} current of β_1-null myotubes and I_{dys}, the L-type Ca^{2+} current of *mdg* (α_{1S} mutant) myotubes. I_{dys} and $I_{\beta null}$ had a similar density (0.69±0.18 and 0.65±0.04 pA/pF for I_{dys} and $I_{\beta null}$ respectively). However, I_{dys} activated much faster than $I_{\beta null}$. At +40 mV, the activation time constant for $I_{\beta null}$ was 28±6 ms and for I_{dys} was 6±1 ms. In addition, I_{dys} activated at more negative potentials and the peak of the I_{dys}-V curve was more negative than that of the $I_{\beta null}$-V curve. Bay K 8644 stimulated I_{dys} more strongly than $I_{\beta null}$ (2.7 vs 2 fold) and shifted the peak of the I_{dys} -V curve more strongly than that of the $I_{\beta null}$-V curve. These results indicate that $I_{\beta null}$ is different from I_{dys} and therefore is unlikely that the bulk of $I_{\beta null}$ originates from a non-α_{1S} isoform, as suggested for I_{dys}. Instead, the bulk of $I_{\beta null}$ may originate from a reduced density of α_{1S} complexes in the membrane of β_1-null cells. *(Supported by NSF, NIH, MDA).*

Tu-Pos49

LOW-THRESHOLD Ca CURRENT (T-TYPE) IS NOT AFFECTED BY BLOCK OF β SUBUNIT SYNTHESIS IN NODOSUS GANGLION SENSORY NEURONS ((R.C. Lambert, Y. Maulet, M. De Waard#, R. Beattie*, S. Volsen*, A. Feltz)) UPR 9009-CNRS, 5 rue B. Pascal, F-67084, Strasbourg; # U 374-INSERM, Bvd P. Dramard, 13916 Marseille, FRANCE; *Lilly Res. Centre Ltd, Windlesham, Surrey, GU20 6PH, U.K..

At least 3 subunits (α1, β, α2δ) form the high-voltage activated (HVA) Ca channels but the structure of the T-type channels is still unknown. Here an antisense oligonucleotide (ON) strategy was used to test if a β subunit contributes to T-type channel properties in nodosus ganglion neurons (Bossu et al., 1985, Pflügers Arch. 403:360). Ca currents recorded in neurons transfected with either an antisense ON shown to disrupt the synthesis of all 4 β gene products (Lambert et al., 1996, Mol. Cell. Neurosc., 7:239), or a scramble ON were compared. The antisense treatment clearly modified the HVA current characteristics without affecting the T-type current. Four days after transfection, HVA current densities (in 10mM Ca) were reduced from -52.7±18.2 to -22.6±17.3 pA/pF (±sd, n=13-15) in antisense ON treated neurons, whereas in the same cells, T-type current densities were respectively -17.0±8.0 and -15.6±6.1 pA/pF. A shift in the voltage dependence of the HVA was also induced by the antisense (see Fig.) but no modification of the I/V relationships nor of the current kinetics were observed for the T-type channels. Therefore known β subunits do not participate in the T-type current properties and are probably not part of T-channels in sensory neurons.



Tu-Pos50

MUSCARINIC INHIBITION OF THE NEURONAL α_{1E} CALCIUM CHANNEL AND ITS MODULATION BY THE REGULATORY β SUBUNITS. ((**D. Platano**, N. Qin, R. Olcese, L. Birnbaumer and E. Stefani)) Dept. of Anesthesiology, UCLA School of Medicine, Los Angeles, CA 90095-1778.

Muscarinic receptors are involved in many complex phenomena such as memory and learning. Alteration of the mechanism underlying this process seems to be related to several neurological diseases. We studied muscarinic regulation of the human neuronal α_{1E} calcium channel expressed in *Xenopus laevis* oocytes. We co-expressed α_{1E} alone or in combination with the regulatory beta (β_{2a} and β_{1b}) subunits, in conjunction with the m_2 receptors. Ionic currents were recorded using the Cut-open Oocyte Vaseline Gap Technique and an external solution containing 10mM Ba^{2+}. The oocyte was maintained at -90mV and pulses to +10mV were delivered. External application of the agonist carbachol (CCh) (5-50μM) slowed the current inactivation and reduced the peak current amplitude by ~ 25-30%. These effects were antagonized by atropine (500nM) which reversed or prevented them. As a signature for G-protein mediated regulation, strong depolarizations preceding the test pulse released the CCh induced current inhibition. In agreement with a G-protein mediated effect, the injection of 25nl of the pertuxis toxin catalytic subunit (0.1ng/nl) prevented the effect of CCh. Co-expression of α_{1E} with the β_{1b} or β_{2a} regulatory subunits, dramatically reduced the extent of muscarinic modulation. These data support the direct involvement of G-proteins in coupling receptor activation and channel modulation. The expression of this regulation is functionally operated by the accessory β subunit. (Supported by NIH grants to ES and LB).

Tu-Pos51

MODULATION OF CARDIAC Ca^{2+} CHANNEL (Cach) BY DIFFERENT β SUBUNITS ((**A. Neely** and A. Ba)) Texas Tech U. HSC. Lubbock TX 79430.

In *Xenopus* oocytes, ionic currents through Cach are increased by the β_{2a} subunit without changes in gating currents indicating a functional modulation of the α_{1C} subunit[1]. Effects of β_{2a} on whole-cell and single channel currents are consistent with two activation paths to either short or long channel openings, the latter being promoted by the interaction of α_{1C} with β_{2a}[2]. However this view is being questioned by recent experiments in mammalian expression systems showing that cotransfeccion of α_{1E} and β_3 increases expression with no change in function[3]. Also recently, an endogenous subunit homologous to β_3 was cloned from *Xenopus* oocytes and found to be expressed in variable amount[4]. It would then appear plausible that the two activation paths and the changes observed in coexpression experiments stems from the coexistence of Cach with different subunit compositions rather than functional modulation. As an alternative strategy to study modulation of Cach by auxiliary subunits, we compared currents from *Xenopus* oocytes expressing $\alpha_{1C}\beta_{2a}$ and $\alpha_{1C}\beta_{1a}$. Contribution of Cach either lacking β or combined with endogenous β was minimized by increasing β:α_{1C} cRNA ratios until functional changes were no longer observed. The voltage-dependence of channel opening were measured from tail currents at -40 mV in 10 mM Ba^{2+} by extrapolating to time zero double exponential functions that best fitted the tail currents. The amplitudes of this instantaneous current were then fitted by the sum of two Boltzmann distributions. Only the voltages for half activation ($V_{1/2}$) for the first component and its relative contribution (%G_1) were significantly different in the two conditions ($P < 0.01$). $V_{1/2}$ values were 8.3 ± 1.6 and 19.0 ± 1.8 and %G_1 were 53 ± 2 and 32± 5 for β_{2a} and β_{1a} respectively. A shift was also observed in the voltage dependency of activation time constants. Together these result suggest that interaction with the β subunits stabilize some late closed conformation of the α_1 subunit rather than driving the channel through a more efficient activation path. Moreover they confirm that the functional changes associated with coexpression of β arise from the modulation of intrinsic properties of the channel protein rather than reflecting heterogeneity and variation in the subunit composition of expressed Cach.

1.Neely et al (1993) *Science* 262:575; 2.Neely et al. (1995) *Am. J. Physiol.* **268**: C732, 3.Josephson & Varadi (1996). *Biophys. J.* 70:1285;4 Quin et al, (1995) *Biophys. J.*70:A143

Tu-Pos52

CHARACTERIZATION OF α1C AND A NEW PUTATIVE ß SUBUNIT OF THE L-TYPE Ca^{2+} CHANNEL IN HUMAN HEART AND IN ANIMAL MODELS. ((**H. Haase**, I. Morano)) Max Delbrück Center for Molecular Medicine, Robert-Rössle-Straße 10, D-13122 Berlin, Germany.

The α1C- and the ß2 subunits of cardiac Ca^{2+} channels are known to be sensitive to proteolytic degradation. We, therefore, tried to identify these subunits without purification in cardiac tissue homogenates by affinity-purified, sequence-directed antibodies. The anti-α1C antibody (directed against the cytoplasmic linker between repeat II and III of α1C) detected specifically a 240-kDa protein in human heart and in heart samples of several animal models (pig, dog, rat). In cardiac tissue of patients with hypertrophic obstructive cardiomyopathy immunostaining of the 240-kDa protein was stronger than in normal human hearts resembling the DHP binding data (Haase et al., [1996] J Mol Med 74: 99-104). During Ca^{2+} channel purification (pig heart) the 240-kDa form disappeared subsequently in favour of the 190-kDa form. These data suggest that the 240-kDa α1C is the unique channel forming subunit in native cardiac Ca^{2+} channels while the 190-kDa form is a degradation product. The ß2 subunit showed a more heterogenous expression pattern. Beside some species-specific differences two cross-reacting proteins of 83- and 95-kDa were detected by antibodies directed either against specific (ß2) or common sequences of ß subunit isoforms. Both proteins copurified on heparin sepharose and were in-vitro phosphorylated by PKA. In accordance with previous results the 83-kDa protein is considered to represent the cardiac ß2 subunit. The 95-kDa protein appears as a hitherto undescribed ß-like subunit.

Tu-Pos53

THE β SUBUNIT OF THE DHP RECEPTOR REQUIRES INTERACTIONS WITH α_1 FOR ITS MEMBRANE ASSOCIATION

((**B. Neuhuber**, J. Mitterdorfer, H. Glossmann, and B.E. Flucher)) Institute of Biochemical Pharmacology, University of Innsbruck, Austria.

The β subunit of the DHP receptor interacts with a specific binding site on the cytoplasmic loop between repeats I and II of the α_1 subunit. We studied the role of this interaction in the formation of the multi-subunit channel complex in a mammalian non-muscle cell line. tsA201 cells were transiently transfected with expression plasmids encoding the cDNAs for α_{1S} alone or in combination with the β_{1a} and $\alpha_2\delta$ subunits. The subunits were visualized with immunofluorescence labeling, or in the case of the β_{1a} subunit, with a fluorescent tag (GFP) linked to its C-terminus. α_{1S} was expressed primarily in a tubular cytoplasmic membrane system (presumably ER) and in the nuclear envelope of tsA201 cells. This localization remained unchanged when α_{1S} was coexpressed with $\alpha_2\delta$ or β_{1a}, or with both. βGFP expressed alone gave a diffuse cytoplasmic labeling pattern. However, when βGFP was coexpressed with α_{1S} the β_{1a} subunit became localized in the internal membrane systems, together with α_{1S}. To verify the role of the α_1-β interaction in the translocation of βGFP from the cytoplasm to the membranes, we coexpressed β_{1a} with a α_{1S} in which the β-interaction domain was deleted (aa 351-380) or mutated (aa Y366S). In both mutants the α_{1S} subunit was localized in the internal membranes; however, βGFP failed to associate with the α_{1S} subunit and was diffusely distributed in the cytoplasm. These results suggest that the α_{1S} subunit is necessary for the association of the β_{1a} subunit with membranes and that the essential interaction required for the formation of the DHP receptor α_1-β complex is with the β-binding domain in the I-II linker. (Supported by FWF grants S06601, S06612)

Tu-Pos54

MAPPING FUNCTIONAL DOMAINS OF THE β SUBUNIT NECESSARY FOR REGULATION OF CA^{2+} CHANNEL FUNCTIONS

((T. Cens, A. Vallentin, J. Nargeot and P. Charnet)) CRBM, CNRS ERS155, Montpellier FR 34033.

Ca^{2+} channels are composed of a central α1 subunit associated with several auxiliary subunits, of which β seems to have the most pronounced effects on channel properties. Each of the 4 cloned β subunits specifically modulates channel inactivation and facilitation (Bourinet et al., 1994, EMBO J., 13 : 5032-5039 ; Cens et al., 1996, Pflügers Arch., 431 :771-774). These subunits are composed of 2 conserved regions surrounded by variable N-terminal, central and C-terminal domains. Recent data indicate that (i) the 2nd conserved domain is involved in the interaction with α1 (Dewaard et al., 1994, Neuron, 13 : 495-503) and (ii) the N-terminal domain is important for the regulation of the voltage-dependent inactivation of the class E Ca^{2+} channel (Olcese et al., 1994, Neuron, 13 : 1433-1438).

We have constructed deleted and chimeric forms of the β1 and β_2 subunits, where the 2nd conserved domain was always preserved, and analysed their effects on the voltage-dependent inactivation, the Ca^{2+}-dependent inactivation and current facilitation of the α_{1A} and α_{1C} subunits. Our data show that the N-terminal domain of the β subunit play an important role in these 3 processes and give some clues on their underlying mechanisms.

(Supported by CNRS, MESR and Association pour la Recherche contre le Cancer (N° 1154))

Tu-Pos55

BETA-SUBUNIT HETEROGENEITY IN NEURONAL L-TYPE CALCIUM CHANNELS. ((M. Pichler, T. N. Cassidy, D. Reimer, H. Haase, R. Kraus, D. Ostler, H. Glossmann and **J. Striessnig**)) *Institut für Biochemische Pharmakologie, Universität Innsbruck, Austria and Max-Delbrück Centrum für Molekulare Medizin, Berlin, Germany.*

α1C and α1D polypeptides are the pore-forming subunits of L-type Ca^{2+}-channels (LTCCs) in mammalian brain. However, it is unknown which of the four β-subunit isoforms (β_1 - β_4) participate in the formation of these hetero-oligomeric channel complexes. To investigate this question we raised sequence-directed antibodies selective for the different β-subunit isoforms and an antibody recognizing all β-subunits (anti-β_{conn}). For sensitive detection in Western blots β-subunits from different regions of rabbit or guinea-pig brain were solubilized in CHAPS and purified by affinity chromatography on GST-AIDA-Sepharose in the presence of protease inhibitors. In cerebral cortex (CTX), hippocampus (HIP) and cerebellum (CER) β_1 (~85 kda), β_3 (~67 kda) and β_4 (~67 and ~64 kda) immunoreactivity was detected and together accounted for the β_{conn}-immunostaining. β_1 and β_3 immunoreactivities were expressed at high levels in CTX and HIP but at lower levels in CER. β_{conn} staining revealed that β_1 was the most abundant isoform in HIP. β_4 was expressed at similar levels in all three brain regions and was the most abundant isoform in CER. β_2 immunoreactivity was not detected in these regions.

β-subunit association with LTCC complexes was determined by immunoprecipitation of crude solubilized or partially purified channels prelabeled with the L-type channel selective ligand (+)-[^{3}H]isradipine. As expected for β-associated LTCCs 72-89% of the solubilized binding activity were specifically recognized by anti-α1C and anti-β_{conn} in rabbit CTX and guinea-pig HIP. In all three regions anti-β_4 recognized more of the β-subunit-associated channel complexes (CTX 42%; HIP 29%; CER 44%) than anti-β_3 (CTX 42%; HIP 19%, CER 21%) or anti-β_1 (CTX 21%; HIP 13%; CER < 10%). β_2 recognized 13% in CTX but less than 10% in the other regions. The β-subunit association profile for [^{125}I]-ω-CTx-GVIA labeled N-type channels in CTX and CER was found to be very similar to LTCCs (CTX:β_4 44%, β_3 42%, β_1 10%, β_2 4%; CER: β_4 40%, β_3 18%, β_1 18%, β_2 <10%). Our results demonstrate that several β-subunit isoforms, preferentially β_4-subunits, participate in the formation of LTCCs in different regions of the mammalian brain. This β-subunit heterogeneity may yield LTCCs differing with respect to their biophysical properties and biochemical modulation.*Supported by a grant from the Austrian Science Foundation (S6602 to J.S.) and a fullbright fellowship (to TNC).*

Tu-Pos56

EXPRESSION OF THE DIHYDROPYRIDINE RECEPTOR ALPHA-1 SUBUNIT ISOFORMS IN ADULT RAT AND MOUSE SKELETAL MUSCLE.

((Y. Péréon, J. Navarro, and **P. Palade**)) Dept. Physiology & Biophysics, Univ. Texas Medical Branch, Galveston, TX 77555-0641

We have investigated the expression of the genes for the skeletal and cardiac isoforms of the dihydropyridine receptor (DHPR) $\alpha 1$ subunit in adult rat extensor digitorum longus (EDL), tibialis anterior (TA), soleus (SOL) and diaphragm (DIA) muscles as well as in *mdx* and control mouse TA and DIA. RNase protection assays were carried out with a rat DHPR cDNA probe specific for skeletal muscle and a mouse DHPR cDNA probe specific for cardiac muscle. Expression levels for the gene of the skeletal DHPR isoform were lower in SOL and DIA than in EDL or TA muscle. To our surprise we found substantial expression of the gene for the cardiac DHPR isoform in both DIA and SOL (8% that of cardiac muscle), with significantly less expression in EDL or TA. Immunolocalization with a cardiac-specific DHPR antibody (graciously provided by Dr. Masami Takahashi, Tokyo) indicated that the protein is expressed in adult skeletal muscle fibers. The presence of the cardiac isoform of the DHPR in SOL and DIA suggests that it may serve some hitherto unsuspected function, particularly in slow-twitch and continuously active skeletal muscles. In mice, there was also more of the cardiac isoform in DIA than TA, but there was no difference between *mdx* and control mice. However, there was nearly twice as much message for the skeletal DHPR isoform in *mdx* DIA than control DIA, whereas *mdx* and control TA levels were similar. This may correlate with the enhanced severity of the disease in DIA.

Tu-Pos57

THE INTRACELLULAR LOOP BETWEEN DOMAINS I AND II OF THE B TYPE CALCIUM CHANNEL CONFERS ASPECTS OF G PROTEIN SENSITIVITY TO THE E TYPE CALCIUM CHANNEL

((K. Page, G.J. Stephens, N.S. Berrow and **A.C. Dolphin**)) Dept. Pharmacology, Royal Free Hospital School of Medicine London NW32PF, UK

It is a characteristic of neuronal voltage-dependent calcium channels that they undergo inhibitory modulation by G protein activation, and this process generally involves both kinetic slowing of the current and a steady-state inhibition. We have previously shown that the β subunit of neuronal calcium channels plays an important role in this process, since when it is absent, greater receptor-mediated inhibition is observed (Campbell et al., 1995). We therefore hypothesised that the calcium channel β subunits may normally occlude G protein mediated inhibition. Calcium channel β subunits bind to the cytoplasmic loop between transmembrane domains I and II of the $\alpha 1$ subunits. We have examined the hypothesis that this loop is involved in G protein-mediated inhibition by making chimeras containing the I-II loop of $\alpha 1B$ or $\alpha 1A$ inserted into $\alpha 1E$ ($\alpha 1EBE$ and $\alpha 1EAE$ respectively). This strategy was adopted because $\alpha 1B$ (the molecular counterpart of N type channels) and to a lesser extent $\alpha 1A$ (P/Q type) are G protein modulated, whereas $\alpha 1E$ is not. Whereas $\alpha 1B$, co-expressed with $\alpha 2$-δ and $\beta 1b$ transiently expressed in COS-7 cells showed both kinetic slowing and steady-state inhibition, when recorded with GTPγS in the patch pipette, both of which were reversed with a depolarising prepulse, the chimera $\alpha 1EBE$ (and to a smaller extent $\alpha 1EAE$) showed only kinetic slowing in the presence of GTPγS, and this was also reversed by a depolarising prepulse. These results indicate that the I-II loop may be the molecular substrate of kinetic slowing, possibly involving direct interaction with G protein $\beta\gamma$ subunits, but that the steady-state inhibition shown by $\alpha 1B$ involves a separate site on this calcium channel.

In biochemical studies, using GST fusion proteins of the I-II loop of $\alpha 1E$ and $\alpha 1B$, we have found that both are able to bind to G$\beta\gamma$. Therefore the basis for the differential modulation of $\alpha 1B$ remains to be determined.

Campbell V., Berrow N.S., Fitzgerald E.M., Brickley K., and Dolphin A.C. (1995) *J. Physiol. (Lond.)*, **485**, 365-372.

Tu-Pos58

SUBCELLULAR DISTRIBUTION OF CALCIUM CHANNEL ALPHA1 AND BETA SUBUNITS DURING RAT BRAIN ONTOGENY.

((D.J. Suciu*, C.M. **Begg***, **C.L. Vance***, L.K. Zimmermann*, T.D. Copeland#, and M.W. McEnery*)) *Dept. of Physiology and Biophysics, Case Western Reserve Univ. Sch. of Med., Cleveland, OH 44106, and #ABL-Basic Research Program, NCI-Frederick Cancer Research and Development Center, Frederick, MD 21702

Understanding the expression, intracellular trafficking and assembly of multisubunit complexes in developing neurons remains a challenging problem. We are interested in the trafficking and assembly of neuronal calcium channels during rat brain ontogeny. Voltage dependent calcium channels are comprised of a membrane-spanning alpha1 subunit and a hydrophilic beta subunit. We are interested in determining the intracellular localization of the alpha1B subunit, the pore-containing subunit which comprises the N-type VDCC, relative to the pool of beta subunits during postnatal development. Subcellular fractionation of neonatal and developing rat brain was carried out with attention towards identifying the alpha1B and beta subunits present in synaptosomal and growth cone particle (GCP) fractions. Radioligand binding was used to evaluate the density and pharmacological properties of the resident N-type VDCC and a panel of antibodies specific for a variety of marker proteins was used to characterize the various fractions (growth cones: GAP-43: mitochondria: VDAC; active zones: synaptotagmin; plasma membrane: Na/K-ATPase). The alpha1B subunit of calcium channels was detected by western blotting in GCPs and was highly enriched in mature synaptosomes. The density of the alpha1B protein paralleled the density of [^{125}I]conotoxin GVIA binding sites. Interestingly, the complement of beta subunits detected in adult, neonatal, or embryonic rat brain homogenates persisted in both the synaptosomal and GCP fractions.

Tu-Pos59

CHANGES IN THE LEVEL AND PATTERN OF EXPRESSION OF VOLTAGE-DEPENDENT CALCIUM CHANNEL BETA SUBUNITS DURING NEURONAL DIFFERENTIATION AND MATURATION.

((**C. M. Begg***, L.C. Williamson^, E. A. Neale^, C. L. Vance*, T.D. Copeland#, and M.W. McEnery*)) *Dept. of Physiology and Biophysics, Case Western Reserve Univ. Sch. of Med., Cleveland, OH 44106,^Lab. Dev. Neurobiol., NICHD, NIH and #ABL-Basic Research Program, NCI-Frederick Cancer Research and Development Center, Frederick, MD 21702

Voltage-dependent calcium channels (VDCC) are critical to the process of calcium-dependent excitation-secretion. We and others have proposed that VDCC may be expressed during neuronal maturation in parallel with the acquisition of functional synapses. The objective of these experiments is to identify the VDCC subunits whose pattern of expression most closely correlates with synapse formation. Given the variety of alpha1 subunit and beta subunit isoforms which might comprise a neuronal VDCC, we have focused upon the alpha1B isoform, the alpha1 subunit known to form the N-type VDCC, a neuron-specific VDCC implicated in neurotransmitter release. Identifying the beta subunit which assembles with the alpha1B during development is the goal of these studies. To pursue this at the biochemical level, we have produced and characterized antibodies to both the pore-forming alpha1B subunit and the auxiliary beta subunits. The focus of this study is the expression of alpha1B and beta subunits in developing rat brain, differentiating human neuroblastoma cell lines (IMR32 cells) and primary spinal cord neurons obtained from embryonic mice. The results of these experiments indicate an increase in the expression of the alpha1B subunit in all neuronal samples as a function of differentiation and maturation. The pattern of expression of beta subunit during development appears to be cell-type specific with dramatic increases in density .

ACETYLCHOLINE RECEPTORS

Tu-Pos60

PHARMACOLOGICAL PROPERTIES OF IONOTROPIC ACETYLCHOLINE RECEPTORS IN DEVELOPING INSECT NEURONES IN PRIMARY CULTURE.

(I. Van Eyseren, F. Tihao, J. Le Guen and **Y. Pichon**.) C.R.M., CNRS URA 256, F-35042 RENNES-Cedex, France.

Acetylcholine (ACh) is the main excitatory neurotransmitter in the central nervous system of insects. We have shown (Beadle *et al.*, **142**, 337-355,1989) that acetylcholine and carbamylcholine (CCh) induced the opening of two categories of non-selective cationic channels in embryonic cockroach neurones grown in culture for two weeks and suggested that the low conductance channel (15 pS with CCh) could correspond to an embryonic channel and the high conductance channel (52 pS with CCh) to an adult channel. In the present study, we have followed the development of the sensitivity to Ach of these neurones during growth and differentiation in culture. Special attention was paid to the pharmacological profile of the receptors. The "whole-cell" configuration of the "patch-clamp" technique (Hamill *et al.*,Plügers Arch. ges. Physiol., **391**, 85-100, 1981) was used to measure the currents induced by 15 s superfusion of the patched cell with a wide range of concentrations of cholinergic agonists (ACh, nicotine (Nic), CCh, suberyldicholine (SCh) and oxotremorine (Oxo)) and their inhibition by the following antagonists (d-tubocurarine (d-TC), α-Bungarotoxin (α-BGT), mecamylamine (Mec), atropine (Atr) and methyllycaconitine (MLA). The sensitivity of the neurones was found to increase progressively during the first 20 days in culture, paralleling the formation of a dense network between the neurones in culture. The sequence of efficacy of the five agonists remained the same throughout the culture (i.e.: ACh>Nic>CCh>SCh>Oxo) but the relative efficacy of ACh decreased significantly during the first two weeks. The five antagonists were all efficient in reducing the response to ACh or the other agonists. MLA was by far the most efficient antagonist (100 % block for 10 nM). These results are in agreement with previous biochemical observations and suggest that, in this preparation, the binding site of the (ionotropic) ACh receptor(s) is of mixed nicotinic-muscarinic type.

Tu-Pos61

"REVERTANT" PHARMACOLOGY OF A NON-DESENSITIZING $\alpha 7$ NICOTINIC ACETYLCHOLINE RECEPTOR MUTANT EXPRESSED IN *XENOPUS* OOCYTES.

((**L.K. Lyford**[1], J.W. Lee[1], S. Desai[2], C. Labarca[3], and R.L. Rosenberg[1])) [1]Dept. of Pharmacology, University of North Carolina, Chapel Hill, NC 27599-7365, [2]Dept. of Medicine, Duke University Medical Center, Durham, NC 27710, [3]Div. of Biology, Caltech, Pasadena, CA 91125.

Mutation of the conserved leucine in the M2 domain of $\alpha 7$ neuronal nAChRs causes a reduction in receptor desensitization. In addition, these L9' mutations cause an extreme alteration in receptor pharmacology, such that antagonists can activate the receptor (Revah *et al. Nature*, 353:846-849, 1991). We have discovered that the addition of a second mutation in the M2 domain at a position homologous to the selectivity filter of the muscle nAChR generates a "double mutant" receptor that does not desensitize in the continual presence of acetylcholine, yet has a pharmacology similar to that of the wild type receptor. Specifically, tubocurarine and DHβE were antagonists of the double mutant, with IC_{50}s of 0.1 μM and 3 μM, respectively, similar to the IC_{50}s of these agents on wild type $\alpha 7$. Hexamethonium was also an antagonist with an IC_{50} of 17 μM. The EC_{50} for acetylcholine was 20 μM, intermediate between the EC_{50}s for the wild type and L9'T $\alpha 7$ nAChRs. QX-222 caused a voltage dependent block, and Ca^{2+} could readily permeate, suggesting that the pore of the double mutant nAChR was similar to the pore of wild type nAChRs. These results showing normal pharmacology and pore properties in a non-desensitizing receptor argue against the "desensitized, conducting" model for the L9'T $\alpha 7$ nAChR mutant described by Bertrand *et al.* (*Proc.Nat.Acad.Sci.*, 89:1261-1265, 1992).

Tu-Pos62

A MUTATION ASSOCIATED WITH EPILEPSY ENHANCES DESENSITIZATION OF THE α4β2 NEURONAL NICOTINIC RECEPTOR.

((**A. Figl**, N. Viseshakul, J. Forsayeth, and B. N. Cohen)) Division of Biomedical Sciences, University of California, Riverside, CA 92521-0121.

We have investigated the properties of a point mutation that is associated with a form of congenital epilepsy called ADNFLE. This single residue change (α4:S6'F) occurs in the second transmembrane region of the neuronal nicotinic α4 subunit. We made the α4:S6'F mutation in a rat α4 neuronal nicotinic subunit and co-expressed the mutated α4 subunits with wild-type rat β2 subunits in *Xenopus* oocytes. The results show that the α4:S6'F mutation did not affect the EC_{50} (3.6-3.7 μM) and the apparent Hill coefficient (0.6-0.7) of the whole-oocyte ACh current. The mutation also did not affect the apparent affinity of the desensitized receptor for the agonist ^{3}H-epibatidine (1.8-2.0 nM), or the level of receptor expression in the oocytes as measured by maximum ^{3}H-epibatidine binding (~9 fmol/oocyte). However, the mutant did dramatically increase steady-state desensitization of the receptor at all the [ACh]'s we tested and altered the kinetics of the ACh voltage-jump relaxation current. The desensitization data suggest that the mutant reduces the rate constant for returning to the open state from one of the desensitized conformations. This enhanced desensitization may produce a decline in nicotinic receptor activity during high-frequency synaptic activity and a decrease in cortical feedback inhibition produced by ACh-evoked GABA release. Support from the AHA (96-112,96-254) and the UC CRCC.

Tu-Pos63

ACETYLCHOLINE RECEPTORS ARE ALLOSTERICALLY BLOCKED BY HYDROCORTISONE.

((**C. Bouzat**, I. Garbus, A. Roccamo and F. J. Barrantes)). Instituto de Investigaciones Bioquímicas. CC857, 8000 Bahía Blanca, Argentina.

Mechanisms of ion channel blockade by low-affinity inhibitors of the nicotinic acetylcholine receptor (AChR) have been particularly difficult to elucidate. We have combined here transient expression of embryonic, adult and a mutated muscle AChR associated with a slow-channel syndrome (*Ohno et al. 1995; Proc. Natl. Acad. Sci. U.S.A. 92,758-762*) with single-channel recordings to determine subunit specificity and mechanisms of action of hydrocortisone (HC). We demonstrate that this glucocorticoid acts as a noncompetitive inhibitor of the AChR. HC affects in a similar manner all types of AChRs, producing briefer openings with normal amplitudes. The forward rate constant for the blocking process is similar for all channel types, indicating that the structural differences between them are not involved in HC interaction. The reduction in the channel open time is not dependent on agonist concentration and it is slightly voltage-dependent. Competition studies with the open-channel blocker QX-222 suggest that the two act at different sites. Taken together the results support the existence of specific sites located in nonluminal domains probably at the lipid-protein interface from where HC allosterically mediates channel blockade. We also show that the HC sites, and its effects, are different from those of ethanol. Current studies are aimed at testing whether mutations at M4 residues labeled by hydrophobic probes modify the interaction of HC with the AChR.

Tu-Pos64

SUBUNIT INTERACTIONS WITHIN THE PORE, AND BETWEEN THE PORE AND TRANSMITTER BINDING SITE, OF MOUSE RECOMBINANT NICOTINIC ACETYLCHOLINE RECEPTORS

((**J. Chen** and A. Auerbach.)) Department of Biophysical Sciences, SUNY at Buffalo, NY 14214.

Residues from all five AChR subunits come into close contact in the channel pore, presumably formed by M2 segments. In order to understand the nature of intersubunit interactions during the concerted channel gating reaction, we have studied ACh-activated single channel currents of adult mouse AChR (expressed in HEK cells) bearing one or more P mutations at the 12' position of the M2 segment (3 residues above the central leucine). The open channel lifetime was greatly prolonged in receptors with mutated α, β, δ, ε, ε+β, ε+δ, β+δ, or ε+β+δ subunits, suggesting that all 12' residues participate in the gating reaction. The channel opening rate constants is fast in these receptors (i.e., the effective opening rate did not saturate up to 1 mM ACh), thus we could not determine to what extent the lifetime was prolonged by a slower closing rate (k_C) and/or a higher ratio of the opening/dissociation rates. In order to make this separation, the above mutants and mutant combinations were expressed with α subunits having a D200N mutation, which lowers the opening rate ~100-fold and effectively reduces the opening/dissociation rate ratio to zero. The 12' mutations slowed k_C, but to different extents in each subunit (s^{-1}): wt=4200, ε=740, β=610, α=390 (2 residues), and δ=1690. This suggests that there is a functional asymmetry at this pore position, especially with regard to the δ subunit.. The effects of pairwise combinations of 12' mutations were not independent (ΔΔG values were not additive), with a strong interaction between δ and both ε and β apparent (k_C in s^{-1}): ε+β=195, ε+δ=1030, and β+δ=1040. Interestingly, while the M2 mutations in β and ε did not effect binding or channel opening rate constants, the δ mutation increased both the equilibrium dissociation constant and the channel opening rate constant of αD200N receptors. This suggests that there is energetic coupling between the 12' M2 residue of the δ subunit, and the transmitter binding site near the α200 residue. (supported by NS-23513 from NIH)

Tu-Pos65

RELATIONSHIP BETWEEN AGONIST STRUCTURE AND THE NICOTINIC ACETYLCHOLINE RECEPTOR CHANNEL OPENING RATE CONSTANT

((F. Salamone, M. Zhou, G. Akk, and A. Auerbach.)) Department of Biophysical Sciences, SUNY at Buffalo, Buffalo, NY 14214.

The channel opening rate constant of nicotinic AChR is strongly agonist-dependent, while the channel closing rate constant is not. We are investigating mechanistic interpretations of this fact by measuring gating rate constants of doubly-liganded AChR activated by different agonists, and correlating these values with structural aspects of the ligand. Classic allosteric theory holds that protein conformational changes are driven by the higher 'affinity' of the activated complex. The ligand is usually treated as a static structure, but many molecules, including ACh (Behling et al, PNAS, 85:6721-6725, 1988), assume an energetically unfavorable, folded conformation upon binding to their target protein. We are exploring the possiblity that, in addition to ligand-protein interactions, changes in the torsion energies of the ligand bonds contribute to the lower free energy of the activated ligand-protein complex. In adult mouse receptors (wild type and/or αG153S) expressed in HEK cells, the opening rate constants (ms^{-1}) determined by single-channel kinetics were: ACh (55), CCh (12), TMA (7), AtCh (3), and choline (0.02). An agonist that has a methylene in place of ACh's ether oxygen, 4KPTMA, elicited clusters with heterogeneous kinetics, and appeared to support an opening rate constant similar to that of TMA. Ramachandran plots of terminal rotors were constructed using the MM+ forcefield. For ACh and CCh, the 'bound' configuration energy is significantly above the global minimum, while for 4KPTMA the energy surface is relatively flat. *Ab initio* quantum mechanics calculations (3-21G* basis set) were also used to estimate the energy differences between folded and extended configurations.

TMA and 4KPTMA lack a high energy state, and support a slow opening rate constant. CCh may not adopt a fully folded conformer due to the like charges at either end of the molecule. We are continuing to explore the possibility that in addition to steric and electrostatic factors, potent agonists may be 'spring loaded', i.e. may store conformational energy that is released upon gating. (supported by NS-23513 from NIH and UB School of Medicine)

Tu-Pos66

BINDING OF *d*-TUBOCURARINE ANALOGS TO THE NICOTINIC ACETYLCHOLINE RECEPTOR (AChR): EVIDENCE FOR SPECIFIC AMINO ACID INTERACTIONS

((**Rao V. L. Papineni** and Steen E. Pedersen)) Dept of Molecular Physiology and Biophysics, Baylor College of Medicine, TX 77030.

Analogs of *d*-tubocurarine that differ in methylation at the 2N and 2'N ammoniums show affinity changes in binding to the acetylcholine (ACh) binding sites of mouse AChR, but not to the *Torpedo* AChR. This result suggests the presence of interactions not accounted by the amino acids presently thought to stabilize the ammonium moiety, because such amino acids are conserved among the mouse and *Torpedo* sequences. Binding of 13'-iodo- and the 13'-bromo-*d*-tubocurarine to the αγ-subunit ACh binding site differed dramatically when the mouse and *Torpedo* AChR were compared: binding affinity for the mouse AChR was ~100 fold lower than binding to the *Torpedo* AChR. Binding of the iodo-*d*-tubocurarine is independent of the receptor conformation in the mouse AChR. This result demonstrates that the affinity changes are due to the intrinsic differences at the αγ binding sites of the mouse and *Torpedo* AChR residues rather than changes in the conformational equilibrium. Comparison of the mouse and *Torpedo* residues previously mapped to the ACh binding sites reveals mouse γ Ile 116 (*Torpedo* γ Met 116) as a likely candidate for direct interacton with the 13'-position on *d*-tubocurarine.

(Supported by PHS NS35212)

Tu-Pos67

SECONDARY STRUCTURE ANALYSIS OF INDIVIDUAL TRANSMEMBRANE SEGMENTS OF THE NICOTINIC ACETYLCHOLINE RECEPTOR: CD SPECTROSCOPY

((John Corbin[1], Howard H. Wang[1] and Michael P. Blanton[2]))
[1]Department of Biology, University of California, Santa Cruz, CA 95064. [2]Department of Pharmacology, Texas Tech University Health Sciences Center, Lubbock, TX 79430.

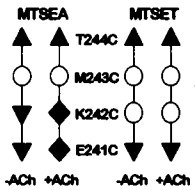
The AChR serves as the prototypical model for the superfamily of ligand-gated ion channels (LGIC). In spite of extensive characterization, the secondary structure of the AChR transmembrane (TM) segments have not been resolved. Recent reports suggest that the four TM segments (M1-M4) present in each receptor subunit may be entirely α-helical or may consist of a mixture of α-helical and β-sheet type structures. In this study, CD spectroscopy was used to examine the secondary structure of individual TM segments of the *Torpedo. calif.* AChR. Proteolytic fragments containing one or more of the TM segments were isolated from digests of receptor subunits and the purified peptides reconstituted into either detergent micelles (2% β-octylglucoside) or lipid (asolectin) vesicles. Based on their CD spectra, peptides containing the M4 segment of the α- and γ-subunit, are estimated to be predominately α-helical. Similarly, the CD spectra of a peptide containing the M2 and M3 segments of the δ-subunit is also consistent with a predominantly α-helical secondary structure. In contrast, for a peptide containing the M1 segment of the α-subunit, the secondary structure is less certain, at least in 2% β-OG. Further analysis of the α-M1 peptide reconstituted into lipid vesicles is currently underway.

Tu-Pos68

THE GATE OF THE MOUSE MUSCLE ACETYLCHOLINE RECEPTOR.

((Gary G. Wilson and Arthur Karlin)) Center for Molecular Recognition, Columbia University, College of Physicians & Surgeons, New York, NY 10032. (Spon. by I. Edelman)

The permeation pathway of the nicotinic ACh receptor is partly lined by the M2 membrane-spanning segment. We used the substituted-cysteine accessibility method to determine the sidedness of four residues close to the cytoplasmic end of αM2. The mutants αT244C, αM243C, αK242C, and αE241C were expressed with wild-type β, γ, and δ subunits in HEK 293 cells. Under whole-cell patch-clamp, all mutants gave near wild-type current amplitudes in response to ACh. Methanethiosulfonate ethylammonium (MTSEA) or methanethiosulfonate ethyltrimethylammonium (MTSET), in the presence or absence of ACh or d-tubocurarine, was applied extracellularly via the bath or intracellularly via the recording pipet. The effect of each reagent was determined by comparing a final test response to ACh, following reagent application, to an initial test response. Significant inhibition is indicated in the Figure by closed symbols, pointing upward if due to extracellular application, and pointing downward if due to intracellular application. In the closed state, there is a permeability barrier to both MTSEA and MTSET around M243. Since there is no other barrier in the channel to MTSET or MTSEA from either side, the gate must lie around M243. MTSEA appears to permeate the open channel. Supported by NS07065, MDA and the McKnight Foundation.



Tu-Pos69

BINDING SITE FOR QX-314, A QUATERNARY LIDOCAINE DERIVATIVE, IN THE CHANNEL OF THE ACETYLCHOLINE RECEPTOR.

((Juan M. Pascual and Arthur Karlin)) Center for Molecular Recognition, Columbia University, College of Physicians & Surgeons, New York, NY 10032.

QX-314 inhibits ACh receptor currents in a noncompetitive, voltage-dependent manner. Several cysteine-substituted residues in the M2 segment of mouse α subunit react with extracellularly applied methanethiosulfonate ethylammonium (MTSEA), causing an irreversible modification of ACh-elicited current. We tested whether QX-314 protected these engineered cysteines from reaction with MTSEA. When the channel was open, QX-314 retarded the reaction of MTSEA at residues located towards the cytoplasmic end of M2 but not towards the extracellular end (Table). For T244C and S248C, the degree of protection, determined by the reduction in the rate constant for the reaction, was equal to the occupation by QX-314, as calculated from its IC_{50}, and thus the protection was complete. Towards the extracellular end of M2, there was no protection. In the middle, QX-314 gave partial protection. Since the rates of reaction of MTSEA with V255C and L251C are much greater in the open state than in the closed state, the protection by QX-314 is not likely to be due to an induction of the closed state. Rather, the protection is likely to be due to steric and electrostatic effects of QX-314 binding in the open channel below L251. In the closed channel, QX-314 affords no protection against the reaction of MTSEA.

Mutant	Protection
E262C	no
L258C	no
V255C	partial
L251C	partial
S248C	complete
T244C	complete

Supported by NIH NS07065, MDA, and McKnight Foundation

Tu-Pos70

METOCURINE AND PANCURONIUM SHOW COMPETITIVE ANTAGONISM FOR INHIBITING CURRENT THROUGH MOUSE FETAL ACH RECEPTORS

((I.L. Wennlngmann, J.P. Dilger)) Depts. of Anesthesiology, Physiology & Biophysics, SUNY, Stony Brook, NY 11794.

Clinical studies on patients show a synergistic effect from the combination of certain pairs of nondepolarizing muscle relaxants (NDMR), for example pancuronium (Panc) and metocurine (Met). NDMRs have different affinities for the two ACh binding sites of the AChR. One way to explain this synergy would be that the high-affinity binding site for Panc is the low-affinity site for Met. We are testing this hypothesis by measuring the potency of these drugs in inhibiting ACh-induced current flow through the AChR.

We used patch clamp techniques to study the inhibitory effects of Panc and Met and the combination of both on nAChR-channels from BC3H-1 cells. Macroscopic currents were activated by rapid perfusion of 100μM ACh to outside-out patches. Control currents were compared with currents obtained during constant exposure of the patch to the NDMR. For Panc, the IC50 is 5.2±0.4 nM (mean ± SD), the Hill coefficient (n) is 1.1± 0.08. For Met, IC50=109±11 nM, n= 0.82 ± 0.06. In the presence of 10 nM Panc, the IC50 for Met is 265±30 nM, n = 0.9 ± 0.1. This is 2.4±0.3 times greater than the IC50 for Met alone and is inconsistent with the synergistic hypothesis. If Panc and Met compete for the same binding site, the IC50 for Met in the presence of 10 nM Panc should be 10/5.2=1.9 times greater than the IC50 for Met alone. Our results suggest that Panc and Met share the same high-affinity binding site on the fetal mouse AChR. Future experiments will be done with adult and human AChRs to look for synergy between NDMRs. Supported by GM42095.

Tu-Pos71

THE ROLE OF HYDROPHOBIC RESIDUES IN THE M2 DOMAIN OF THE ACHR IN CHANNEL GATING.

((C.G. Labarca, H. Zhang, P. Deshpande and H.A. Lester)) Division of Biology, Caltech, Pasadena, CA 91125

The M2 transmembrane domain of the AChR has numerous hydrophobic residues, mainly Leu and Val. We previously showed that mutation of Leu 9' to Ser altered the gating of the channel. The presence of each mutated subunit decreased the EC50 by roughly a factor of 10, and the effect was nearly independent of the subunit mutated. The frequency of spontaneous openings increased with the number of mutated subunits. We have now examined the effect of mutating hydrophobic residues to Ser in M2 at positions other than 9'. In receptors with α subunit mutations at positions 7', 11', 13', 15', 16' and 17', the dose response relation for ACh shifts to the left; the largest shift occurs at position 13', with an EC50 over three orders of magnitude lower than wild type. In a helical wheel representation, most of these residues cluster on two opposing faces and orthogonal to the polar 6' and 10' residues that face the open lumen, as though these hydrophobic positions face the flanking M2s of neighboring subunits. In contrast, receptors with 8' mutations show a rightward dose-response shift; these residues would face away from the open pore. Thus, gating is influenced by multiple hydrophobic interactions (a) between the M2 transmembrane helices surrounding the ion pore and (b) between the M2 helices and the distal surrounding domains of the receptor. We propose a model for AChR gating. Supported by NIH and CA TRDRP.

Tu-Pos72

CHOLINE: AN EFFICACIOUS ENDOGENOUS AGONIST AT α7-BEARING NEURONAL NICOTINIC RECEPTORS.

((M. Alkondon, E.F.R. Pereira, and E.X. Albuquerque)) Dept. Pharmacol. Exp. Ther., Univ. Maryland Sch. Med., Baltimore, MD 21201, USA; Lab. Mol. Pharmacol., IBCCF, UFRJ; Rio de Janeiro, RJ 21944, Brazil. (Spon. E.X. Albuquerque).

It is well accepted that acetylcholine (ACh) hydrolysis by acetylcholinesterase represents the main mechanism by which ACh actions at the cholinergic synapses are terminated *in vivo*. In fact, numerous studies have shown that at the neuromuscular junction choline, the breakdown product of ACh, acts as a very poor agonist at the muscle nicotinic receptor (nAChR) (reviewed in Swanson *et al.*, "The Toxic Action of Marine and Terrestrial Alkaloids, pp. 191-280, 1995). Here, using the whole-cell mode of the patch-clamp technique, we demonstrate that choline acts as a full agonist at α7-bearing nAChRs expressed on hippocampal neurons. The α7-bearing nAChRs, which mediate the activation by nicotinic agonists of fast-desensitizing, type IA currents, can be activated by choline, which was found to be as efficacious as ACh and approximately 10 times less potent than ACh in evoking type IA currents (EC_{50} for ACh ~ 135 μM; EC_{50} for choline ~ 1.6 mM). Also, similar to ACh (Alkondon *et al.*, *J. Pharmacol. Exp. Ther.* **278:** 1460, 1996), choline activates presynaptic α7-nAChRs that modulate glutamate release from olfactory bulb neurons in culture. A continuous 20-min exposure of hippocampal neurons to choline at concentrations as low as 10 μM resulted in the inhibition of the α7-nAChR activity. In contrast, fast application of choline (0.5 sec; 100 μM - 10 mM) via a U-tube to hippocampal neurons that responded to ACh with slowly desensitizing, type II currents mediated by α4β2 nAChRs did not evoke measurable whole-cell currents, and only at concentrations higher than 100 μM was choline capable of inhibiting the activation by ACh of type II currents; the IC_{50} for choline was about 1.1 mM. Fast application of ACh to most PC12 cells in culture resulted in the activation of slowly desensitizing, mecamylamine-sensitive nicotinic currents, which are likely to be subserved by α3β4 nAChRs. Choline (100 μM-10 mM) acted as a partial agonist at the α3β4 nAChRs, exhibiting only 20% of the efficacy of ACh as an agonist. The finding that choline acts as a full agonist at α7-bearing native nAChRs is important for the understanding of the physiological processes modulated by the α7-nAChR function in the mammalian central nervous system. (USPHS grants NS25296; ES05730)

Tu-Pos73

CHOLESTEROL IS REQUIRED AT THE LIPID-PROTEIN INTERFACE TO PERMIT ACTIVATION OF THE NICOTINIC ACETYLCHOLINE RECEPTOR

((George H. Addona, Heinrich Sandermann and Keith W. Miller)) Department of Anesthesia, Massachusetts General Hospital, and Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02114

The nicotinic acetylcholine receptor requires cholesterol in the surrounding membrane for agonist-induced activation to occur (Fong and McNamee, *Biochemistry* 25, 830, 1986). However, the mechanism of cholesterol's action is not clearly understood. Therefore, we tested three hypotheses that might explain cholesterol's role in receptor activation. The first hypothesis involves relief of transient, conformation change–induced bilayer curvature by unidirectional cholesterol movement across the bilayer. The second hypothesis is that some interaction of cholesterol at the lipid-protein interface is necessary to maintain conditions that permit channel opening. The third hypothesis is that there are interstitial cholesterol binding sites on the acetylcholine receptor, distinct from the lipid-protein interface, that must be occupied for receptor activation (Jones and McNamee, *Biochemistry*, 22, 2364, 1988). Affinity purified nAcChoRs from Torpedo were reconstituted into DOPC:DOPA:steroid bilayers at mole ratios of 58:12:30. Cholesterol analogs with charged groups on the 3–position's hydroxyl were found to support channel opening. This result is contrary to the first hypothesis in which bilayer mechanical strain is relieved by cholesterol flipping from one leaflet to the other. Another cholesterol analogue formed by covalent attachment of lysophosphatidylcholine to cholesterol hemisuccinate was also found to support receptor activation, suggesting that discrete interstitial sites on the receptor do not need to be occupied for channel opening. Thus, either there are cholesterol binding sites on the receptor at the lipid-protein interface, or the physical properties of the lipid-protein interface are important to permit channel opening. (GM15904)

Tu-Pos74

CHARACTERIZATION OF A SOLUBLE 68 AMINO ACID PEPTIDE FRAGMENT FROM THE EXTRACELLULAR DOMAIN OF THE NICOTINIC ACETYLCHOLINE RECEPTOR α-SUBUNIT. ((**M. A. Grant**, Q.-L. Shi, E. Hawrot)) Dept. of Molecular Pharmacology, Physiology, and Biotechnology, Brown University, Providence, RI 02912.

We have constructed a synthetic gene for a 68 amino acid fragment (α143-210) of the N-terminal portion of the nicotinic acetylcholine receptor (nAChR) α-subunit based on the sequence from *Torpedo californica*. This gene was attached to the C-terminus of thioredoxin and expressed as a soluble fusion protein in a modified plasmid pTrxFus where the cysteines in thioredoxin were replaced by serines. The construct was re-designed to include a thrombin cleavage site in the polylinker region and a six-residue Histidine tag C-terminal to the synthetic gene. The fusion protein was over-expressed in *E. coli* GI724 cells, purified by 35% ammonium sulfate precipitation, and treated with thrombin to liberate the desired αT68H_6mer. Following purification by gel filtration chromatography under denaturing and reducing conditions, 12.5 mg of the αT68H_6mer protein were produced per liter of bacterial culture. The fragment is soluble upon removal of the denaturant by dialysis and appears to be monomeric at least to protein concentrations approaching 0.15 mg/ml. Purified αT68H_6 receptor fragment was shown, by ^{125}I-bungarotoxin (BGTX) solid-phase competition binding assays, to have an apparent affinity for BGTX that was identical to that of native intact receptor in *Torpedo* membranes. The slope and shape of the competition curves suggest a structurally homogenous population of binding sites. The CD spectrum of the purified αT68H_6mer indicated significant folded β-sheet structure (~39%). A cooperative, two-state folding transition was observed upon incremental urea denaturation. We have now produced metabolically labeled (^{15}N) αT68H_6mer in order to facilitate a high resolution NMR analysis of the receptor fragment in its stoichiometric complex with BGTX. Supported by NIH-NIGMS-GM32629.

Tu-Pos75

ETHANOL INTERACTIONS WITH NICOTINIC ACH RECEPTOR PROBED IN AN ALCOHOL-SENSITIVE MUTANT, αS10'I+βT10'I.
((**Stuart A. Forman**)) Dept. of Anesthesia, MGH, Boston, MA 02114.

Ethanol may cause CNS depression by interacting directly with synaptic ion channels. A molecular model for studying this hypothesis is the nicotinic acetylcholine receptor (nAChR), which contains a channel site blocked by longer alcohols and general anesthetics (Forman et al, *Mol. Pharm.* 48: 574-81 (1995)). Ethanol does not block wild-type nAChRs, but alters agonist activation, shifting agonist concentration-response curves leftward. Mutant nAChRs with increased side-chain hydrophobicity at two putative channel-forming (M2 domain) residues, αS10'I and βT10'I, have a 10-fold increase in sensitivity to longer alcohols, hexanol and octanol. Ethanol interactions with the αS10'I+βT10'I receptor and each subunit mutation expressed individually were studied. Peak current (I_p) vs. [EtOH] and I_p vs. [ACh] curves (in the presence and absence of ethanol) were measured for both wild-type and mutant receptors. ACh and alcohol effects were assessed by fast perfusion patch-clamp electrophysiology in *Xenopus* oocyte patches expressing nAChRs. Results: 1) ethanol at up to 500 mM does not inhibit wt nAChR currents, but blocks peak current through αS10'I+βT10'I mutant receptors with an IC_{50} of 250 ± 30 mM; 2) αS10'I mutant receptors are also inhibited by ethanol, but βT10'I receptors are not--ethanol enhances current in this mutant; 3) 300 mM ethanol causes an approximately two-fold leftward shift in I_p vs. [ACh] curves for both wt and αS10'I+βT10'I mutant receptors. The data indicate that ethanol-induced agonist affinity modulation in nAChR is mediated by (a) site(s) other than the channel site for hydrophobic blockers. Ethanol inhibition of mutant receptors differs mechanistically from block by longer alcohols that have been shown to interact with both alpha and beta subunits.

(Supported by NIAAA #1-K21-AA00206-01).

Tu-Pos76

MOLECULAR DYNAMICS STUDY OF WATER AND SODIUM IONS IN MODELS OF THE PORE REGION OF THE NICOTINIC ACETYLCHOLINE RECEPTOR.
((**G. R. Smith** and M. S. P. Sansom)), Laboratory of Molecular Biophysics, Rex Richards Building, University of Oxford, South Parks Road, Oxford OX1 3QU, U.K.

The nicotinic acetylcholine receptor forms cation-selective channels in lipid bilayers. The central pore is lined by a bundle of five, approximately parallel, M2 helices. In previous work, we developed candidate structures for the M2 helix bundle in both the open and closed states of the channel using the simulated annealing by restrained molecular dynamics technique. These structures were generated using constraints derived from Unwin's 9-Å resolution electron-diffraction structures, and are also consistent with most mutagenesis data.

Extensive MD simulations of these two pore models solvated with modified TIP3 water have now been conducted. In models containing water alone, we have examined the effects that the water's confinement within the pore and its interactions with the M2 helix protein have on its bulk properties. It is found that the mobility of the water is substantially reduced within the pore, and some is held in long-lived hydrogen-bonding networks. We have also performed short simulations of the pore models containing a sodium ion as well as water, in which the ion is placed at a series of positions along the pore axis and the water and protein relaxed while the ion's position is restrained. The energetics of the ion's interaction with the water and protein side chains, and its effect on the pore water, have been investigated. It is found that the ion perturbs the water only in its immediate neighbourhood; the helix dipoles remain the dominant orienting force. It is also found that the central ring of leucines, which is believed to form a gate in the closed channel, may not produce a sufficiently large energy barrier to ion permeation to fully account for the gating of the channel.

Tu-Pos77

THE STEROID PROMEGESTONE IS A NONCOMPETITIVE ANTAGONIST OF THE *TORPEDO* NICOTINIC ACETYLCHOLINE RECEPTOR WHICH INTERACTS WITH THE LIPID-PROTEIN INTERFACE. ((M.P. Blanton[1], L.J. Dangott[2], Y. Xie[2] and J.B. Cohen[2])) [1]Department of Pharmacology, Texas Tech University Health Sciences Center, Lubbock, Tx 79430. [2]Department of Neurobiology, Harvard Medical School , Boston, Ma. 02115.

The synthetic progestin, promegestone (PG) acts as a noncompetitive antagonist (NCA) of the AChR, reversibly inhibiting acetycholine (ACh) induced currents of *Torpedo* AChRs expressed in *Xenopus* oocytes. PG at 10-100 μM enhances the binding of [^{3}H]Nicotine to *Torpedo* AChR membranes, consistent with stabilization of the AChR desensitized state. In the presence of agonist, PG displaced the binding of the NCA [^{3}H]PCP to the receptor by 90% (IC_{50} = 9μM), but did not inhibit the binding of [^{3}H]HTX (IC_{50} >100 μM). In order to identify the binding loci of this steroid, the AChR was photoaffinity labeled with [^{3}H]Promegestone ([^{3}H]PG). In the AChR α-subunit all measurable incorporation mapped to two large *S. aureus* V8 protease fragments: α-V8-20 (Ser-173-Glu-338), containing the transmembrane segments TM1-TM3 and α-V8-10 (Asn-339-Gly-437), containing TM4. The TM2 and TM4 segments were isolated from proteolytic digests of each of the receptor subunits and subjected to N-terminal sequence analysis. While no evidence of [^{3}H]PG incorporation was detected in sequencing any of the TM2 segments, residues reacting with [^{3}H]PG were identified in the TM4 segments and found to be identical to those previously shown to be in contact with lipid.

Tu-Pos78

SIMULTANEOUS BINDING OF TWO DIFFERENT NONCOMPETITIVE INHIBITORS TO THE OPEN-CHANNEL CONFIGURATION OF THE NICOTINIC ACETYLCHOLINE RECEPTOR. (D.A. Johnson, S. Ayres, and W. Woltman)) Div. of Biomed. Sci., U. C., Riverside, Riverside, CA 92521

Much evidence supports the conclusion that although there are multiple discrete binding sites for 'open-channel' noncompetitive inhibitors (NCIs) both in and out of the lumen of the nicotinic acetylcholine receptor (nAcChR), at equilibrium only one NCI binds per functional receptor. The implications of this conclusion include: (1) that there must exist functional links between binding sites such that NCI binding to one site allosterically inhibits NCI binding to the other sites, and (2) that upon channel opening the various NCI binding sites are all capable of interacting with their respective ligands and thus simultaneous binding of two or more NCIs is momentarily possible. To confirm this latter implication, stopped-flow fluorimetry was used to examine the ability of various nonfluorescent NCIs to block the initial carbamylcholine-induced binding of the fluorescent NCI quinacrine to the open-channel configuration of the nAcChR. Using concentrations of the nonfluorescent NCIs (PCP, 40 μM; tetracaine, 100 μM; QX-314, 40 μM) that block quinacrine (2 μM) binding under equilibrium conditions, the nonfluorescent NCIs either had no effect or enhanced the initial quinacrine binding following simultaneous mixing of quinacrine and carbamylcholine. Because these NCIs bind at or near diffusion controlled rates and are present at ≥20-fold higher concentrations than quinacrine, the nonfluorescent NCIs must be bound before quinacrine binds, thus indicating multiple, simultaneous NCI binding to the nAcChR. (Supported by NSF grant IBN-9515330)

Tu-Pos79

STRUCTURE-FUNCTION STUDIES OF THE RECOMBINANT BRAIN-DERIVED NEUROTROPHIC FACTOR WITH NATIVE AND NON-NATIVE DISULFIDE BONDS. ((**T. Sakamoto**, S. Fukuzono, and N. Shimizu)) Advanced Research Laboratory, Hitachi, Ltd., Hatoyama, Saitama 350-03, Japan.

We have investigated functional brain-derived neurotrophic factor (BDNF) production in *Escherichia coli* and found that *E. coli* cells produced fully biologically active BDNF and less functional products in supernatant and precipitate fractions of disrupted cell lysates, respectively. From the less functional products we further fractionated 6 different BDNF species to homogeneity. To investigate structure-function relationships of these native and less functional recombinant BDNFs, we have performed disulfide bond analyses, circular dichroism measurement, biological activity assay (neurite outgrowth), and receptor-binding experiments. We confirmed that BDNF from the supernatant fraction was identical to the authentic BDNF in the above criteria. In contrast, all of the purified BDNF products from precipitate contained different non-native disulfide linkages and showed lower biological activity (EC_{50} of 2 to 15 ng/ml) than authentic one (30 pg/ml). Native BDNF forms β-sheets, whereas non-native species have lower β-sheet content and some of them appear to contain α-helical structure. BDNF is known to bind two different receptors: high-affinity TrkB tyrosine kinase receptor and low-affinity NGF receptor ($p75^{NGFR}$). We have generated mammalian cell lines expressing chicken *trk*B gene. The results of competitive binding assay between non-native species and TrkB revealed a partial agreement with the biological activity data. Similar binding assay using PC12 cells showed little correlation between TrkB and $p75^{NGFR}$ in regard to the binding affinity to each BDNF species. These results suggest the complex contribution of BDNF receptors to the functional responses in neuronal cells.

Tu-Pos80

INHIBITION OF LIPID-INDEPENDENT PROTEIN KINASE C ACTIVITY BY PHORBOL ESTERS AND DIACYLGLYCEROLS. ((**S.J. Slater**, M.B. Kelly, A. Mazurek and C.D. Stubbs)) Department of Anatomy, Pathology and Cell Biology, Thomas Jefferson University, Philadelphia, PA 19107.

The activity of membrane-lipid associated protein kinase C (PKC) is mediated by discrete low and high affinity activator binding regions [Slater, et al., (1996) J. Biol. Chem. 271, 4672]. PKC activity may also be induced in the absence of lipid by interaction with other proteins, such as, for example, nuclear and cytoskeletal elements. In the present study, using a lipid-free binding assay based on the fluorescent phorbol ester, sapintoxin-D (SAPD), it was shown that PKCα associated with the arginine-rich protein substrate, protamine sulfate, contains a high affinity phorbol ester binding site. The affinity of the interaction with this site was found to be close that for the high affinity SAPD binding site present on membrane-associated PKCα. Interestingly, by contrast to the commonly observed potentiation of membrane-associated activity by phorbol esters, interaction of SAPD with the lipid-independent site on PKCα resulted in a potent *inhibition* of activity. The potency of this inhibition of lipid-independent activity and that of the potentiation of membrane-associated activity were similar. Furthermore, the PKC "activators" TPA, OAG and bryostatin-1, competed with SAPD for the lipid-independent binding site and also *inhibited* the corresponding activity, again contrasting with the effects of these agents on membrane-associated PKCα. The results demonstrate that association of PKC with the membrane is not necessary for high affinity ligand binding and that the cellular effects mediated by these PKC "activators" may therefore be highly dependent on the cellular localization of the enzyme.

Tu-Pos81

PROTEIN KINASE C α AND β HAVE DIFFERING CALCIUM REQUIREMENTS FOR MEMBRANE-ASSOCIATION. ((S.J. Slater, B.A. Aleong, A. Mazurek, M.B. Kelly, C. Ho and **C.D. Stubbs**)) Department of Anatomy, Pathology, and Cell Biology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

Evidence is presented supporting the contention that the membrane-association of the -α and -β isoforms of protein kinase C (PKC) have different Ca^{2+} requirements. Measurements of PKCα binding to unilamellar lipid vesicles showed that the interaction proceeds by two distinct "high" and "low" affinity Ca^{2+}-induced steps. However, PKCα activity was found to be negligible for Ca^{2+} concentrations corresponding to the "high" affinity membrane-interaction ($<10^{-7}$ M) and activity was only observed at Ca^{2+} concentrations greater than 10^{-4} M. From these results we propose that PKCα contains two distinct "classes" of Ca^{2+} binding sites. By contrast, the membrane-binding isotherm for PKCβ only yielded a single binding step which coincided with enzyme activation and occurred at a Ca^{2+} concentration comparable to that observed for the "low" affinity membrane-interaction of PKCα. Thus, by contrast to PKCα, the β-isoform may only contain a single "class" of Ca^{2+}-binding site corresponding to the "low" affinity site on PKCα. The results suggest that, within the resting cellular Ca^{2+} concentration range, PKCβ may reside in the cytosol and translocate to the membrane as a response to a transient increase in Ca^{2+} levels, while PKCα may exist at the inner surface of the plasma membrane in a "primed" inactive state. These differences between PKCα and PKCβ may therefore provide a basis for the divergent cellular processes controlled by these otherwise structurally and enzymatically similar isoforms.

Tu-Pos82

THEORY FOR PROTEIN REBINDING AT CELL MEMBRANE SURFACES. ((**B. Christoffer Lagerholm** and Nancy L. Thompson)) Department of Chemistry, University of North Carolina, Chapel Hill, NC 27599-3290

Conditions for which proteins that have desorbed from cell surfaces rebind to their surface binding sites have been theoretically examined. An analytical solution for the coupled differential equations that describe the reaction at the interface between a plane and three-dimensional solution together with solution and surface diffusion has been described previously (Thompson et al.,1981, *Biophys. J.* **33**, 435). In this work, we use this theoretical formalism to predict the spatial and temporal dependence of the surface concentration of a population of tagged molecules, given initial release at the origin, as a function of the magnitudes of the characteristic rates that describe the process. The surface concentration results are used to predict an apparent lateral diffusion coefficient along the surface. This apparent coefficient results from repeated surface detachment, diffusion in solution, and reattachment (rebinding). Additional calculated parameters are the average time that a molecule is free, and the average distance travelled in solution, between binding events. Methods for experimental verification by using total internal reflection with fluorescence photobleaching recovery or correlation spectroscopy are discussed.

Tu-Pos83

CAPPING OF Fcγ-RECEPTOR IIA IS CONTROLLED BY TYROSINE PHOSPHORYLATION/DEPHOSPHORYLATION OF PROTEINS IN U937 CELLS.

(K. Kwiatkowska, B. Pyrzynska, **A. Sobota**) Nencki Institute of Experimental Biology, Warsaw, PL-02093, Poland. (Spon. by X. Wu)

Cross-linking of Fcγ-receptor IIA (FcRIIA), the first step in capping phenomenon, induces phosphorylation of tyrosine residues of proteins, and of protein tyrosines, and leads to reorganization of actin cytoskeleton. To investigate the role of protein tyrosine kinases (PTKs) and phosphatases (PTPs) in capping of FcRIIA we used genistein, pervanadate and PAO, inhibitors of the enzymes. Genistein arrested the receptors at intermediate stages of their aggregation and FcRIIA did not assemble into the fully formed cap. Capping of FcRIIA was completely blocked by 2 mM pervanadate and by 15 μM PAO, inhibitors of PTPs; the receptors remained uniformly distributed over the cell surface. The effect was reversed and capping occurred when PTPs inhibitors were removed from the medium. Immunoblotting analysis revealed that cross-linking of FcRIIA was accompanied by tyrosine phosphorylation of several proteins: 130-110, 75, 56-52, 48, 40, 28-26 kDa. The phosphorylation was markedly enhanced at the presence of pervanadate. Removing of pervanadate caused dephoshorylation of the proteins followed by formation of FcRIIA caps. We concluded that protein tyrosine phosphorylation/ dephosphorylation controls capping of FcRIIA being involved in regulation of the receptor-actin skeleton interactions.

Tu-Pos84

Pharmacological characterization of angiotensin II induced effect on the guinea-pig circular muscle: desensitization without uncoupling of the signal transduction. ((R.L.Prioste,C.A.Kanashiro,**H.Zalcberg**,S.I.Shimuta)) Universidade Federal de Sao Paulo, SaoPaulo 20388, Brazil. (Spon. by A.T.Ferreira)

The aim of the present study was to investigate the effects of angiotensinII (AngII) on the circular muscle derived from guinea-pig ileum mainly about the mechanism involved in smooth muscle desensitization. AngII (100 nM) induced transient contractile responses which were not inhibited by 100 nM nifedipine. Furthermore it was found that $^{45}Ca^{2+}$ influx was not detected but the removal of this ion from the medium or the incubation of the preparation with Ni^{2+} (0.6 mM), inhibited the contractile responses completely, suggesting that a Ca^{2+} release dependent on Ca^{2+} entry through a channel insensitive to nifedipine was necessary to induce the contraction. AngII induced desensitization of contractile responses, but produced no effects on the $^3HIP_3$ production and $^{24}Na^+$ influx, the opposite effects observed in longitudinal muscle layer described previously. These data led us to conclude that the desensitization of the circular muscle to AngII is probably due to a delay in the replenishment of the intracelular calcium store and that there is no negative feedback mechanism exerted by protein kinase C on the receptor-G protein coupling in striking contrast to that observed in longitudinal muscle. Financial Supports: FAPESP, CNPQ.

Tu-Pos85

DISTINCT DESENSITIZATION BY ANGIOTENSIN II IN CHO CELLS TRANSFECTED WITH AT_1 RECEPTOR. ((S.I.Shimuta, A.T.Ferreira, M.E.M.Oshiro, A.C.M.Paiva, C.A. Kanashiro)) Universidade Federal de São Paulo, São Paulo 20388, Brazil. (Spon. by A.C.M.Paiva)

Angiotensin II (AngII) is an important effector molecule which elicits numerous responses in the cardiovascular and other systems through type 1 (AT_1) receptor. In order to determine the mechanism by which the rapid and specific desensitization to AngII occurs following agonist receptor occupancy, we studied the AT_1 receptor transfected in CHO cells. Binding of ^{125}I-AngII to the permanently transfected receptor was specific and saturable (K_d, 5 nM). AngII induced a transient increase in the intracellular calcium (Ca_i^{2+}) signaling and stimulated 1,4,5-^{3}H-IP_3 production (EC_{50} , 0.5 nM). It was also found that AngII stimulated a rapid and transient influx of Ca^{2+} returning to the basal value within 60 s. Repetitive exposure to the peptide completely inhibited the stimulation of $^{45}Ca^{2+}$ influx as well as the increase in intracellular free Ca^{2+} concentration, reflecting blockade of both, influx and release of calcium from the intracellular stores. This tachyphylaxis was also observed with Lys^{2}AII, an AngII analog, which is known to be unable to induce tachyphylaxis in smooth muscle cells. The finding that AngII did not stimulate Na^+ influx in CHO cells in another striking contrast to that observed in smooth muscle cells suggests that the tachyphylaxis caused by AngII as well as by Lys^{2}AII is due to a differential mechanism from that proposed for the specific tachyphylaxis phenomenon caused by AngII in smooth muscle.

Financial Supports: FAPESP, CNPq.

Tu-Pos86

IN VITRO STUDY OF TWO POTENT FLUORESCENT PKC'S PROBES.
((**A. Janoshazi** and J. de Barry)) Université L. Pasteur, and Cellular Neurobiology Laboratory, CNRS UPR9009, 67084 Strasbourg, France

By using fluorescent probes, we characterized the reversible and irreversible activation of PKC_α and its μ-calpain mediated proteolysis in an *in vitro* system, namely brain lipid extract and PS vesicles. These probes (*i.e.* fluorescein- and BODIPY FL-derivatives of bis-indomaleimide : Fim-1 and Bim respectively) bind to the catalytic domain of PKC and exhibit selectivity for PKC one order of magnitude higher than PKA. The fluorescent spectra of the probes were deconvoluted into three different gaussians and each peak was followed separately during the binding of the probe to PKC_α, the enzyme activation and the proteolysis process.

When the probe-PKC_α complex was incorporated in PS vesicles, the intensity of all the peaks of increased in $[Ca^{2+}]$ dependent manner. The phorbol-ester (TPA) induced activation of PKC_α was measured by the probe's increased fluorescent yield and an $EC_{50} = 49 \pm 1.5$nM was found in brain lipid vesicles.

After addition of μ-calpain we detected the appearance of PKM (*i.e.* soluble catalytic domain generated by calpain mediated cleavage of the regulatory and catalytic PKC domains) by following the fluorescence intensity decay. With saturating concentration of PS, Ca^{2+} and TPA, the fluorescence decay rates were temperature- and concentration-dependent ($\tau_1 = 51 \pm 5\ s^{-1}$ with 2.05 U and $\tau_2 = 118 \pm 3\ s^{-1}$ with 6.15 U of μ-calpain at 25.5°C). Our goal is now to provide a basis for observing by fluorescence microscopy the translocation of PKC isoenzymes in cells in response to stimulating factors.

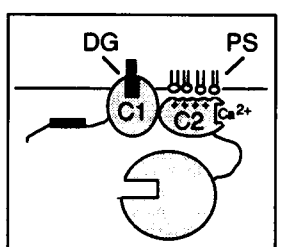
Tu-Pos87

SIGNAL TRANSDUCTION WITHIN G-PROTEIN COUPLED RECEPTORS VIA AN ION TUNNEL? INFERENCE FROM MODELING μ-OPIOID RECEPTOR WITH AGONISTS. ((**B.S. Zhorov** and V.S. Ananthanarayanan)) Biochemistry Dept., McMaster Univ. Canada, L8N 3Z5.

To explain the well-known effects of metal ions on G-protein-coupled receptors, we modeled rat μ-opioid receptor (μR) with ligands using our data on 3D similarity between Ca^{2+}-bound morphine (MP), Ca^{2+} -bound Met-enkephalin (EK) and a polyprotonated agonist KYDRFK (*J.Biomol.Struct.Dynam.* 1995, **13**:1; *ibid*, 1996, *in press*). Monte Carlo-with-energy-minimization (MCEM) method was used as described earlier (*Biophys.J.* 1996, **70**:22). The MCEM-optimized helices H1-H7 were arranged to match the 2D map of rhodopsin and Baldwin's model (*EMBO J.* 1993, **12**:1693). The bundle was MCEM-optimized with the helices allowed to rotate around and shift along their long axes. The extracellular loops (EL) were added and folded randomly. Cationic groups of agonists were restrained to acidic residues of μR, the complexes were MCEM-optimized and then re-optimized without the restraints. In the optimal complexes, Ca^{2+}-MP and Ca^{2+}-EK fit between H2, H3 and H7. The ligands' ammonium group binds to the conservative residue (CR) D114 in H2, a supposed site for Na^+ (Kong et al. *J.Biol.Chem.* 1993, **268**:23055), while ligand-chelated Ca^{2+} binds to CR D147 in H3. The N-end of KYDRFK imitates Ca^{2+}-MP while C-end binds to E229 in EL2 and E310 in EL3. CRs N86, Y106, L110, S154, L158, S162, L324, N328 and N332 form a tunnel between D114 and salt-bridged CRs D164-R165. We suggest that a charged agonist would knock out a metal ion from D114, the ion would move via the tunnel to disengage R165 from D164 such that R165 would be exposed and bind to G-protein. (Supported by MRC, Canada).

Tu-Pos88

REGULATION OF PROTEIN KINASE C'S MEMBRANE INTERACTION BY TWO MEMBRANE-TARGETTING DOMAINS
((**Alexandra C. Newton**)) Department of Pharmacology, University of California at San Diego, La Jolla, CA 92093-0640

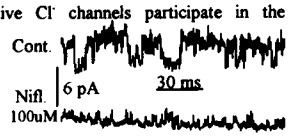


Protein kinase Cs transduce the myriad of signals promoting phospholipid hydrolysis to generate diacylglycerol. Members of this family of kinases "translocate" to membranes upon generation of this lipid second messenger, where they bind a second lipid, phosphatidylserine. Binding to these two lipids results in a conformational change that removes an autoinhibitory (pseudosubstrate) domain from the active site. Translocation is mediated by two separate membrane-targetting domains, the C1 and C2 domains, in the regulatory region of the protein. The C1 domain binds diacylglycerol or phorbol esters, and the C2 domain binds phosphatidylserine and, for conventional protein kinase Cs, Ca^{2+}; each domain, separately, can recruit protein kinase C to membranes via a low-affinity interaction, but both domains must be membrane-bound for the high-affinity interaction that results in release of the pseudosubstrate from the active site and activation. This contribution examines the role of each domain in increasing protein kinase C's affinity for membranes.

Tu-Pos89

SINGLE CHANNEL RECORDING IN MOUSE SPERM. ((F. Espinosa, *R. Delgado, J. L. de la Vega, *P. Labarca and A. Darszon)). Dept. Genética. y Fisiología Celular, Inst. de Biotecnología-UNAM, Cuernavaca, México and *Centro de Estudios Científicos de Santiago and Facultad de Ciencias, Univ. de Chile. (Spon. by A. Liévano)

Ion channels are key elements in the machinery that triggers the sperm acrosome reaction (AR) necessary for fertilization in many species. Previous indirect evidence indicated that Cl^- channels play a role in the AR. Sperm are very small (~3 μm diameter), and this has precluded direct ion channel characterization. Here we present the first single channel recordings obtained from intact mammailian sperm documenting 3 types of ion channels. One of them, a Cl^- channel with subcunductance states (44 and 23 pS, 160 mM Cl^- pipette), is blocked by Niflumic acid (kd~20 μM) (Fig.). Our results also show that the mouse sperm AR induced by zona pellucida, GABA and progesterone is blocked by μM Niflumic acid. These results supply direct evidence of the presence of various ion channels in mammalian sperm, and suggest that Niflumic sensitive Cl^- channels participate in the physiological induction of the AR.



Supported by DGAPA-UNAM, and CONACyT. A. D. is an international Scholar of the Howard Hughes Med. Inst. P.L holds a Catedra Presidencial en Ciencias.

Tu-Pos90

DYNAMIC CHANGES IN MEMBRANE CAPACITANCE IN HUMAN MACROPHAGES DURING PHAGOCYTOSIS ((**K.O. Holevinsky** and D.J. Nelson))University of Chicago, Dept. of Neurology, Chicago, IL 60637.

We report the use of capacitance measurements to monitor particle uptake during phagocytosis. Human monocyte-derived macrophages and cells from the murine macrophage-like cell line J774.1 were exposed to immune complexes or sized latex particles. Changes in capacitance were recorded along with changes in membrane conductance that occurred as a result of particle uptake. In cells exposed to immune complexes, we observed a total decrease in cell capacitance of 7.9 pF (n = 5). Monocytes in which particle uptake was inhibited by cytochalasin B treatment prior to exposure to immune complexes, showed an average decrease of 2.4 pF (n = 4) over the same time period. Exposure of cells to sized latex particles led to a graded, stepwise decrease in membrane capacitance. Monocytes exposed to larger latex particles show a greater step change in capacitance than cells exposed to smaller particles. The average step size for smaller (0.8 μM) particles was 500 fF and the average step size for larger (3.2 μM) particles was 1700 fF. In addition, the dynamic interaction between endocytosis and exocytosis was seen in cells in which a gradual increase in capacitance, indicating degranulation and secretion, was observed along with decreases in capacitance. Supported by NIH R01 GM36823.

Tu-Pos91

THRESHOLD CALCIUM REQUIREMENT FOR EXCESS MEMBRANE RETRIEVAL IN BOVINE ADRENAL CHROMAFFIN CELLS. ((**K.L. Engisch** and M.C. Nowycky)) Dept. of Neurobiol. and Anat., Allegheny University of the Health Sciences., Phila. PA, 19129.

In perforated patch recording mode, bovine adrenal chromaffin cells respond to single prolonged depolarizing voltage pulses with increases in membrane capacitance (C_m), that are followed by rapid but smooth decays in C_m that can be fit with one or two exponentials. For endocytotic responses to 320 ms duration pulses in over 50 cells, a histogram of first time constants (τ_1) has peaks at 225 ms, 1 sec, and a spread of values between 3 and 50 sec. Often, following the first 320 ms pulse of an experiment, retrieval far outstrips the exocytotic response ("excess retrieval"); in 60% (33/53)of the cells, C_m decayed to a baseline that was more than 100 fF below the prestimulus level. Excess retrieval is strongly associated with a brief τ_1; $\tau_1 = 990 \pm 228$ ms for responses with excess retrieval, but is 10.1 ± 3.2 sec for responses with little or no excess retrieval. Significant excess retrieval occurs only after pulses with integrated calcium entry above a threshold value (20×10^7 ions), independent of the amplitude of the exocytotic response. C_m usually does not remain at the level reached during excess retrieval, but recovers slowly to the prestimulus baseline over a period of 8 min. These results suggest an extremely rapid endocytotic process that can be triggered by large amounts of calcium entry, but that is not tightly coupled to exocytotic responses.

Tu-Pos92

ACTIVITY-DEPENDENT CHANGES IN THE Ca^{2+}-SECRETION RELATIONSHIP DURING PULSE TRAINS IN BOVINE CHROMAFFIN CELLS ((**M.C. Nowycky**, K.L. Engisch, N.I. Chernevskaya,)) Dept. Neurobiol. & Anatomy, Allegheny Univ. Health Sci., Philadelphia, PA 19129.

Ca^{2+}-secretion relationships have been examined in isolated bovine chromaffin cells by capacitance detection techniques. If cells are stimulated by single depolarizing pulses in perforated patch recording mode, the amount of secretion is a simple transfer function of the form $\Delta C_m = g * (\Sigma Ca^{2+})^n$, where g is a proportionality constant, ΣCa^{2+} is the integral of the Ca^{2+} current, and $n = 1.5$ on average (Engisch & Nowycky, 1996). Trains of depolarizing pulses evoke 3 types of responses: those that exactly or closely follow the standard probability transfer function obtained by single pulses, those that exhibit increased Ca^{2+} efficacy, and those with decreased efficacy. The probability of obtaining a given response is correlated with pulse protocol. Trains of shorter pulse duration (e.g. 5 ms or 10 ms, 200 ms interval) elicit more responses that follow the curve or have increased efficacy, while the majority of 40 ms pulse trains have diminished Ca^{2+} efficacy. The type of response is also determined by interpulse interval. Decreasing the interpulse interval of 5 ms trains to 100 or 44 ms in cells that responded with enhanced efficacy eliminated the potentiation, and the response followed the standard curve. Increasing the interpulse interval of 40 ms trains that evoked depression to 1000 ms evoked responses that followed the standard curve or even had enhanced efficacy. This demonstrates that efficacy of Ca^{2+}-secretion coupling in undialyzed chromaffin cells is subject to short-term changes.

Tu-Pos93

Ca^{2+} MEASUREMENTS AND THE EFFECT OF Ca^{2+} INDICATORS ON EXOCYTOSIS DURING PULSE TRAINS IN BOVINE CHROMAFFIN CELLS. ((**N.I. Chernevskaya**, M.C. Nowycky)) Allegheny Univ. Health Sci., Philadelphia PA 19129. (Spon. by C.A. Lewis).

In chromaffn cells recorded in perforated patch mode, trains of depolarizing pulses evoke Ca^{2+}-secretion relationships that follow the standard transfer function curve or show increased or decreased efficacy (Nowycky et al., this volume). To determine if changes in global $[Ca^{2+}]_i$ accounted for any of the activity-dependent behaviors, we preloaded cells with Fura Red AM and monitored fluorescence changes with a Gen-III camera. All pulse protocols evoked modest Ca^{2+} increases that reached a plateau by the 1-5th pulse of 40 ms trains, or 7-20th pulse of 5 ms trains. Within individual cells, single 320 ms pulses and 40 ms trains caused identical average $[Ca^{2+}]_i$ increases, while 5 ms trains either evoked the same final level (5/11 cells) or slightly lower levels (6/11). After all stimulus protocols, $[Ca^{2+}]_i$ decayed with $\tau \sim 3$ sec, so that trains of 1 sec intervals showed signifcant $[Ca^{2+}]_i$ drops between pulses. Thus, the only relationship between secretory response patterns and average $[Ca^{2+}]_i$ is that diminished Ca^{2+} -coupling efficacy during 40 ms trains is correlated with slightly higher $[Ca^{2+}]_i$ at the plateau.

Small concentrations of exogenous Ca^{2+} chelators can alter normal Ca^{2+}-secretion relationships in undialyzed cells. Fura Red loading diminished the amount of secretion during some 5 ms trains. On the other hand, the first pulses of trains occasionally evoked large 'docked'-type responses. These 'docked'-type C_m responses were correlated with elevated basal levels of $[Ca^{2+}]_i$.

Tu-Pos94

DEPLETION OF INTRACELLULAR Ca^{2+} STORES POTENTIATES EXOCYTOSIS IN BOVINE CHROMAFFIN CELLS. ((**A.F. Fomina**, M.C. Nowycky)) Dept. Neurobiol. & Anat., Allegheny Univ. Health Sci., Philadelphia, PA, 19129.

A crucial step in agonist-driven secretion in nonexcitable cells is the influx of extracellular Ca^{2+} stimulated by depletion of intracellular stores. It is not known whether such a pathway exists in excitable cells. We employed the capacitance tracking technique in perforated patch voltage-clamp mode to assay single cell exocytosis in bovine chromaffin cells. Simultaneously, intracellular Ca^{2+} ($[Ca^{2+}]_i$) was monitored as changes in the fluorescence of Fluo-3 or Oregon Green (loaded in AM forms) with a confocal laser microscope.

Application of thapsigargin (Tg) at high concentrations (1-5 μM) evoked depolarization-independent exocytosis which was preceded by a rise of $[Ca^{2+}]_i$ and could add up to several pF to the plasma membrane over a few minutes. These effects were not seen in nominally Ca^{2+}-free extracellular solutions. Low concentrations of Tg (0.2-1 μM) did not induce either a detectable rise of $[Ca^{2+}]_i$ or depolarization-independent exocytosis, however, the exocytotic response to depolarizations was enhanced for >10 min. In voltage-clamped cells, application of Ca^{2+}-free solution alone induced capacitative Ca^{2+} influx and potentiation of depolarization-stimulated exocytosis upon reintroduction of extracellular Ca^{2+}. Our data suggest that intracellular store depletion stimulates Ca^{2+} influx which can potentiate secretion in electrically excitable cells.

Tu-Pos95

REGULATION OF THE EXOCYTOTIC FUSION PORE BY CYTOSOLIC CALCIUM AND CONDITIONS AFFECTING THE DISSOCIATION OF SECRETORY PRODUCTS FROM THE GRANULE MATRIX ((E. Alés and G. Alvarez de Toledo)). Dept. Physiology and Biophysics. Univ. Of Seville. E-41009, Sevilla. Spain.

The delay between exocytotic fusion pore formation and serotonin release was determined by simultaneous whole cell capacitance and amperometric measurements in single rat peritoneal mast cell granules. This delay is shortened by increased $[Ca^{2+}]_i$ and represents the time required for fusion pore expansion (Fernández-Chacón and Alvarez de Toledo, *FEBS Let.* 363, 221, 1995). We have further investigated the role of $[Ca^{2+}]_i$ in dilating the fusion pore. The relationship between $[Ca^{2+}]_i$ and delay for release was sigmoidal and half saturation occurred at 300 nM $[Ca^{2+}]_i$. The Hill coefficient for this curve was 2.4, indicating that at least 3 calcium ions must bind to their sites to dilate the fusion pore. This step of exocytosis was unaffected by the absence of Mg-ATP inside the patch pipette. The delay between fusion and release was lengthened under conditions that inhibit swelling of the granule matrix. Bathing the cells in hiperosmotic solutions increased the delay between fusion and release, on average, from 81 ms measured in standard experiments (pH = 7.25, 8.6 μM $[Ca^{2+}]_i$), to 217 ms; to 248 ms in 150 mM histamine chloride and to 169 ms in a regular external saline buffered at pH 5.5. In all of these conditions the delay for release was still calcium dependent but shifted to higher values. These results show that fusion pore expansion is subjected to cytosolic control, acting as the rate limiting step for release. Dissociation of secretory products from the granule matrix become the rate limiting step only when the driving forces for release are hindered by depletion of secretory products or abnormally high extracellular concentration of substances that inhibit secretory granule swelling.

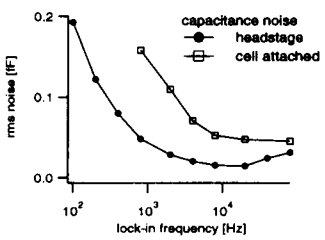
Tu-Pos96

CAPACITANCE CHANGES AND CATECHOLAMINE RELEASE UNRELATED TO EXOCYTOSIS OF DENSE CORE VESICLES IN CHROMAFFIN CELLS. ((**G. Dernick**[1], A. Albillos[1,2], G. Alvarez de Toledo[3] and M. Lindau[1]). [1]Dept. Molecular Cell Research, MPI f. Medical Research, D-69120 Heidelberg, Germany; [2]Dept. Pharmacology & Therapeutics, Autonomous Univ., E-28029 Madrid, Spain; [3]Dept. Physiology & Biophysics, Univ. of Seville, E-41009 Sevilla, Spain.

Exocytosis was studied in bovine chromaffin cells using patch amperometry. Capacitance measurements and amperometric detection of catecholamine release with a carbon fiber electrode were performed simultaneously in the cell attached patch clamp configuration. Patch depolarisation for several seconds induced a continuous capacitance increase during depolarization which persisted after repolarization of the patch. This increase was present in the absence of calcium in both the bath and pipette solution. The slope was voltage dependent increasing with increasing depolarization. Hyperpolarization induced a continuous decrease of patch capacitance. These continuous changes could reflect a voltage dependent equilibrium of constitutive exo-endocytosis of small vesicles. Application of suction leading to patch rupture was followed by amperometric spikes with short duration. The time course of these spikes was very similar to that previously reported for exocytotic spikes measured with extracellular carbon fiber electrodes. The spikes were dependent on continued suction and presumably arise from chromaffin granules bursting at or close to the carbon fibre electrode. These results indicate that during prolonged depolarization capacitance changes unrelated to exocytosis of chromaffin granules may occur. Furthermore, cell damage might lead to liberation of intact granules which upon rupture may generate amperometric signals similar but unrelated to exocytosis. Measurements using only membrane capacitance or only amperometric detection of catecholamine as an assay of exocytosis should thus be interpreted with caution.

Tu-Pos97

FREQUENCY DEPENDENCE OF NOISE LEVELS IN CELL ATTACHED CAPACITANCE RECORDINGS. ((**Knut Debus** and Manfred Lindau)). Dept. Molekulare Zellforschung, MPI für medizinische Forschung, D-69120 Heidelberg, Germany.



Exocytosis of vesicles < 300 nm can not be resolved as stepwise capacitance increases in the whole cell patch-clamp configuration. The cell attached configuration, however, allows the resolution of vesicle steps as small as 0.1 fF, when a lock-in frequency of 8 kHz is used. We investigated the dependence of the capacitance noise on the lock-in frequency between 100 Hz and 80 kHz, using an EPC-9 patch-clamp amplifier and a command sine voltage of 20 mV rms. With an open headstage, the instrumental noise spectrum had a minimum of 15 fF at about 20 kHz and varies only slightly between 4 kHz and 40 kHz. At lower frequencies, the noise increases, being 3 times larger at 800 Hz. In the cell-attached configuration, the noise is about 3 times bigger, depending on the seal, with a similar spectrum which is basically flat above 8 kHz. This wide range of frequencies providing high resolution allows detection of a wide range of fusion pore conductances in vesicles bigger than 200 nm.

Tu-Pos98

SCALING LAWS of AMINE RELEASE: ROLE OF THE EXOCYTOTIC FUSION PORE (**B. Farrell, P. Marszalek and J. M. Fernandez) Mayo Clinic, Rochester, MN 55905**

Release of amines during exocytosis is monitored from different cells by amperometry by use of a carbon fiber electrode. In a previous communication (Marszalek et al. 1996. Soc. Gen. Physiol. Ser. 51: 211-222) we compiled the data obtained from seven laboratories and found that the time course of the amperometric spike scales with the size of the secretory vesicle. Specifically the $t_{1/2}$ of the amperometric spike (the width of the main spike at half of its amplitude) is related to the secretory vesicle radius (r) by a power law $t_{1/2} \alpha r^{1.6}$. We use monte carlo simulations and further experimental measurements to verify and explain this and other scaling relationships. In our monte carlo simulations we assume that the diffusivity of the particles within the vesicles of varying size is the same and the particles can only diffuse out of a vesicle through a pore in the membrane. As expected when the particles escape through a fusion pore of constant radius the time course of release is proportional to r^3 ($t \alpha r^3$). In contrast, when the fusion pore expands at a constant rate so that the surface area exposed is a constant fraction of the surface area of the granule (i.e. the solid angle is constant) then: $t \alpha r^2$ and the efflux is maximum when the pore reaches its maximum expanded state. An exponent of 1.6 suggests either that the diffusion coefficient of the amines within smaller vesicles is less than that measured for the beige mouse mast cell at ~ 3 x 10^{-6} cm^2s^{-1} or that the fusion pore expands at a slower rate in cells with smaller vesicles (e.g. Retzius neuron) than for cells with larger secretory granules (e.g. beige mouse mast cell). We are conducting further simulations and experiments to verify the value of the exponent and to establish if the diffusivity of the amines is similar within secretory cells.

Tu-Pos99

NANOFABRICATED CARBON DETECTORS (NACAD) FOR ATOMIC FORCE MICROSCOPY (AFM) STUDIES OF SINGLE SECRETORY VESICLES. ((**V. Parpura** and J. M. Fernandez)) Mayo Clinic, Rochester, MN 55905.

Secretory products are stored in the lumen of secretory granules and released upon exocytosis. The mechanisms of storage and release from synaptic vesicles are unknown. However, recent experiments using AFM and secretory granules isolated from mast cells have shown that transmitter binds electrostatically to the intragranular gel matrix that serves as an ion-exchanger (Parpura and Fernandez, 1996, Biophys J., In Press). To examine the possibility that neurotransmitters are stored in synaptic vesicles by similar mechanisms, we are attempting to combine AFM techniques with the electrochemical measurements of transmitter release. Towards this end we nanofabricated a carbon detector (NACAD; constructed at the Cornell University Nanofabrication Facility) whose dimensions and properties will permit simultaneous AFM and electrochemical studies. NACAD has a carbon island (5 µm x 5 µm x 180 nm; top surface RMS roughness ~ 4 nm; AFM image shown in figure) in its center which can be located using AFM. This carbon island was able to detect serotonin in physiological buffers as determined by cyclic voltammetry. To determine whether NACAD is capable of detecting secretion of serotonin from single intact secretory granules, we applied isolated intact mast cell granules onto the surface of a NACAD. The adhered granules were exposed to Triton X-100 (0.01%) to brake down their membranes and trigger release. We detected amperometric "spikes" that corresponded to serotonin release. We are currently examining the possibility of using NACAD in conjunction with AFM to determine whether storage/secretion from synaptic vesicles is regulated by an ion-exchange through an intravesicular gel matrix.

Tu-Pos100

THREE-PHOTON EXCITATION IMAGING OF SEROTONIN SECRETION BY RBL-2H3 CELLS. (Rebecca M. Williams[1], Jason B. Shear[2], Warren R Zipfel[1], Sudipta Maiti[1] and Watt W. Webb[1].) [1]Dept. of Physics and [2]School of Applied and Engineering Physics, Cornell University, Ithaca, NY 14853. [3]Dept. of chemistry, University of Texas at Austin, 78712.

Three photon excitation using a femtosecond, mode-locked ti:sapphire laser source enables red light excitation of native serotonin (5-hydroxytryptamine: 5HT) fluorescence, a molecular transition that is normally excited at deep UV wavelengths. Using this non-linear optical process we report imaging measurements of 5HT loaded RBL-2H3 cells undergoing antigen stimulated secretion. Sampling at one frame every ten seconds, we observe each vesicle's contents to disappear suddenly with no evidence for granule motion. Release is frequently spatially coupled, suggesting partial compound exocytosis. We analyze granule distributions by constructing isocontours around regions of intensity that are greater than three times the basal cell photon shot noise. Imaging measurements show faster secretion kinetics than those observed either by bulk specimen ^{3}H-5HT or β-hexosaminidase assays. Preliminary results indicate a correlation between granule volume and granule release time.

This project was supported by NSF (DIR8800278), NIH (RR04224) and NIH (R07719) to the Developmental Resource for Biophysical Imaging and Opto-electronics. Analysis was carried out on the SP2 at the Cornell Theory Center.

Tu-Pos101

OPTIMIZATION OF THE STIMULUS WAVEFORM FOR MEMBRANE CAPACITANCE MEASUREMENTS ((K. D. Gillis)), Max-Planck-Institute for Biophysical Chemistry, 37077 Göttingen, Germany

Membrane capacitance (C_m) measurements have been used widely to study exocytosis and endocytosis in patch-clamped cells. The most common stimulus waveform is a single sinusoid, however, multiple-sinusoidal stimuli have also been used for the case where appreciable changes in membrane resistance (R_m) occur. Stimulus waveforms used to date are far from optimal in minimizing C_m noise. Larger amplitude waveforms give lower C_m noise, but the total voltage excursion (VE) seen by the membrane must be limited in order to avoid activation of voltage-dependent conductances and/or membrane breakdown. Binary (rectangular) waveforms *across the membrane* provide the greatest input power for a given VE. Simple substitution of a square wave for the single sinusoid requires no changes in existing processing algorithms and no increase in VE, but results in 21% lower C_m noise. Other periodic waveforms will be presented emphasizing frequencies which result in lower C_m noise while also containing low frequency components needed for robust estimation of R_m. The maximum rate that independent parameter estimates can be generated is equal to the lowest frequency present in the stimulus. In the whole-cell configuration, high frequency stimuli result in higher C_m noise because a fraction of their amplitude is dropped across the access resistance (R_a). However, since it is the VE across the membrane that is limiting, higher frequencies components can be "boosted" in amplitude in a process analogous to "supercharging" or series resistance compensation. The use of higher frequency stimuli is particularly useful for applications where R_m drops to less than 1 GΩ, because in this case the Johnson noise of R_m substantially increases C_m noise at "typical" stimulus frequencies.

Tu-Pos102

DEPOLARIZATION-INDUCED ASYNCHRONOUS QUANTAL RELEASE FROM RAT ADRENAL CHROMAFFIN CELLS. ((**Stanley Misler**, Jun Liu and David W. Barnett*)) Washington University Medical Center, St. Louis, MO 63110 and *Parks College, Saint Louis University, Cahokia, IL 62206.

We have monitored chromaffin granule exocytosis by simultaneously tracking (i) increases in membrane capacitance (C_m) and (ii) the occurrence of amperometrically detected quantal events (AE). Low frequency, brief depolarizations are usually accompanied by AEs during the depolarization and stepwise increase in C_m immediately thereafter. In contrast, in response to higher frequency, longer depolarizations, an increasing fraction of total exocytosis is seen as a tail of AEs and a creeping increase in C_m lasting up to several seconds after each depolarizing pulse. Using (i) a phase detection method with dual sinusoidal excitation about a baseline of -70 mV, and (ii) appropriate ionic conditions (high Cs^+ internal solution/40 mM TEA Ringer's bath), it is possible to record Ca^{2+} currents during the depolarization and remnant tail conductances and currents after the depolarization. The tail currents are apamin sensitive and have other features of current carried by small conductance, Ca^{2+}-activated K^+ (SK) channels. For a given cell, both the number of events in the AE tail, as well as the rate of C_m creep following a pulse, increase monotonically in tandem with I_{SK} ; this suggests that both delayed, asynchronous release and the SK tail current are related to the "residual Ca^{2+}" from prior entry. Tail currents and asynchronous release persists with repetitive depolarization despite impressive rundown of Ca^{2+} currents, the latter probably resulting from Ca^{2+}-induced Ca^{2+} channel inactivation. If these results are applicable in situ, they suggest that during repetitive electrical activity, adrenal chromaffin cells can utilize asynchronous quantal release promoted by residual Ca^{2+} as a key mode of depolarization-induced exocytosis. This would permit sustained release while reducing the need for pulsatile Ca^{2+} entry.
Support: NIH DK37380 and Barnes-Jewish Hospital Research Foundation.

Tu-Pos103

CAPACITANCE MEASUREMENTS OF VESICLE CYCLING IN A SYNAPTIC TERMINAL. ((**G. Neves** and L. Lagnado)) MRC Laboratory of Molecular Biology, Cambridge CB2 2QH, UK.

The giant synaptic terminal of depolarizing bipolar cells from the goldfish retina releases glutamate from small vesicles. Exocytosis and endocytosis of these vesicles was investigated by making capacitance measurements using permeabilized patch and conventional whole-cell recordings from the cell body of isolated cells. The rise in capacitance measured immediately after a depolarizing pulse from -80 mV to 0 mV increased as the duration of the pulse was increased up to 5 s. The capacitance then recovered to baseline with a time-constant that was independent of pulse duration. Immediately after a 200 ms depolarization the increase in capacitance averaged 70 fF (range 30-125 fF) and the signal fell without delay with a time-constant of 1.6 s. However, in 16 out of 28 cells, pulses lasting 500 ms or more caused the capacitance to continue to rise for up to 3 s after repolarization, after which the capacitance fell with a time-constant that averaged 2.4 s. The rate of capacitance increase immediately after repolarization was up to 40 fF s^{-1}. Thus the capacitance signal represented the net difference between ongoing exocytosis and endocytosis. These results indicate that the bipolar cell terminal can support the maintained release of neurotransmitter through the action of a Ca^{2+}-sensor that does not require the Ca^{2+} microdomains generated by open Ca^{2+} channels (Lagnado et al., *Neuron*, In Press, 1996). Further, residual Ca^{2+} does not delay the fall in capacitance by simply inhibiting endocytosis (von Gersdorff & Matthews, *Nature*, 370, 652, 1994). Supported by the HFSP.

Tu-Pos104

EXOCYTOSIS IN ADRENAL CHROMAFFIN CELLS VIEWED AS AN ELECTROPORATIVE PROCESS ((**K. Rosenheck**)) Department of Membrane Research and Biophysics, The Weizmann Institute of Science, 76100 Rehovot, Israel

Secretion of neurotransmitters occurs by their release from intracellular storage vesicles directly into the extracellular space via a process called exocytosis, that involves the fusion of the vesicle membrane with the plasma membrane of the cell. The detailed mechanism of fusion is at present unknown. It can be divided into, at least, two distinct temporal phases. The first is the close apposition of vesicle and plasma membranes, by activation of specific proteins that exhibit calcium sensitive interactions with other cellular components, proteins as well as lipids. The second one, fusion proper, arises from interactions between the two appositioned bilayers. What causes the rupture of the appositioned bilayers and subsequent stochastic evolution of the fusion pore? This communication attempts to present an answer, based on concepts developed in the course of the experimental work and theoretical modeling of the electroporation effect (Neumann & Rosenheck, 1972; Rosenheck et al. 1975; Lindner et al. 1977). Electric field pulses of short (10 to 40 μsec) duration, ranging in intensity from 20 to 40 kV/cm, were found to cause transient increases in the membranes permeability of chromaffin granules, leading to release of the stored soluble constituents during a time period of several miliseconds. Since the advent of patch-clamp methods, enabling one to monitor release from single vesicles at the cellular level, it has become meaningful to compare the time course of release from chromaffin cells with that of electric field induced membrane perturbation and release in the submillisecond to millisecond range. The time courses of the two processes are similar, suggesting that the natural event is fast enough to be consistent with an electroporative mechanism. Furthermore, it is found from a consideration of a fixed surface-charge membrane model that the local electric fields attained during the mutual approach of the two interacting membranes are sufficiently intense to cause electroporation of the bilayer. This model seems able to account also for "aborted fusion attempts", the presence of which appears to be established in recent experimental work with chromaffin as well as other secretory cells.

Tu-Pos105

Characterization of acidic Ca^{2+} pools in secretory cells
((**Wim JJM Scheenen**, Paola Pizzo, Tullio Pozzan and Cristina Fasolato))
Department of Biomedical Sciences, University of Padova, Via Trieste 75, 35121 Padova, Italy (Spon. by A. Hofer)

By monitoring $[Ca^{2+}]_i$ with fluorescent probes, Ca^{2+} pools have been originally identified through the increase in $[Ca^{2+}]_i$ upon application of Ca^{2+} ionophores, after depletion of the Tg-sensitive pools with a combination, or a sequence, of $InsP_3$-generating agonists, Tg, and caffeine. Using this approach it has been shown that in PC12 cells part of the intracellular stores is inaccessible by ionomycin unless the H^+ gradient over these compartments is broken down. This acidic Ca^{2+} pool has now been demonstrated in a variety of cell types, including GH3 cells from the pituitary gland, Ins-1 cells from an insulinoma, and cerebellar granule cells. In all these cell types the Ca^{2+} content of the acidic pools was significantly reduced by depolarizing the cells with high $[K^+]_e$. In general, depolarization drastically enhances secretion by increasing Ca^{2+} influx across the plasma membrane. We also tested stimulants that release Ca^{2+} from intracellular stores such as the $InsP_3$-generating hormone TRH in the case of GH3 cells and acetylcholine in the case of Ins-1. In contrast to stimuli that enhance Ca^{2+} influx, these factors did not significantly reduce the Ca^{2+} content of the acidic pools. Our results indicate that a large part of the acidic Ca^{2+} pool includes the secretory compartment. Details of the functional coupling between acidic Ca^{2+} pools and the process of secretion monitored by cell capacitance will be discussed for Ins-1 cells.

Tu-Pos106

THE SPECIFIC CELL MEMBRANE CAPACITANCE OF MAST AND CHROMAFFIN CELLS IS 0.5 $\mu F/cm^2$
((**B. Innocenti** and J. M. Fernandez)) Mayo Clinic, Rochester, MN 55905.

We used the ballon patch-clamp technique (C. Solsona and J.M. Fernandez 1996 Biophys. J. 70, A151) to inflate mast cells and chromaffin cells. A positive pressure of 6-12cm of H_2O was delivered to the interior of a cell throughout a patch-pipette in the whole cell configuration. During inflation, we monitored the cell membrane capacitance (Cm). Upon inflation mast cells increased their radius from 5.5±0.39 μm to 7.93±1.09 μm (n=12), however, no significant change in total capacitance (Cm) was observed (3.83±1.11 pF compared to 4.15±1.26 pF). Although the calculated specific capacitance of resting mast cells was 1.0 ± 0.26 $\mu F/cm^2$ (n=12), upon inflation we measured 0.52 ± 0.09 $\mu F/cm^2$. Similarly, resting chromaffin cells had an apparent specific capacitance of 0.81± 0.22 $\mu F/cm^2$ (n=10). However, upon inflation we measured 0.53±0.03 $\mu F/cm^2$, a value that is remarkably close to that found in mast cells. The specific capacitance of individual resting cells varied greatly and ranged from 0.52 to 1.33 $\mu F/cm^2$ (n=22). For all cells, the degree of inflation was linearly related to their specific membrane capacitance calculated at rest. Cells that at rest had a low specific capacitance could not be inflated very much. In contrast, in those cells with a high specific capacitance at rest, the radius increased by up to 176% upon inflation.
We propose that inflation of a cell smoothes out the highly folded surface membrane, allowing an accurate estimate of the total surface membrane area. Hence we propose that the true specific capacitance of mast cells and chromaffin cells is ~0.52 $\mu F/cm^2$, which is far from the well accepted value of 1.0 $\mu F/cm^2$. We speculate that given accurate measurements of cell membrane surface area, other mammalian cells may be shown to have a specific membrane capacitance similar to that measured here for mast cells and chromaffin cells.

Tu-Pos107

INTRACELLULAR MEMBRANE RECORDINGS IN SQUID GIANT TERMINAL AND APLYSIA BAG CELL NEURONS ((**E.A. Jonas**, R.J. Knox, J.E. Moreira, and L.K. Kaczmarek)) Dept. Pharmacology, Yale Univ. Sch. of Med., New Haven, CT, 06520 and NINDS/LNB, Rockville, MD, 20892.

Previous studies of intracellular ion channels have examined ion channel activity in isolated organelles, after reconstitution in lipid bilayers, or by patch clamping nuclear membranes of oocytes. We have used a technique to form giga-ohm seals on intracellular membranes in intact cells. Our goal is to patch clamp organelles that may contribute to the control of neurosecretion, such as secretory organelles, endosomes, or specialized portions of endoplasmic reticulum found at release sites. Squid giant terminals provide a preparation devoid of nuclear membrane, and the bag cell neurons of Aplysia provide a unique system in which to study neuropeptide release. In squid terminals, we found several channels that may be candidates for such regulatory channels. The most commonly seen had conductances of 24, 37 and 80 pS, and had reversal potentials close to 0 mV using pipettes containing normal intracellular solutions (cytoplasmic side). In addition, we detected a very large conductance channel (1.13 nS). We were able to verify the presynaptic location of our pipette by recording action potentials after stimulating the presynaptic nerve. In Aplysia neurons, we were able to record channels with conductances of 7, 13, and 78 pS, some of which may be similar to the squid channels. We attempted to localize the organelles on which these channels reside by video-enhanced and confocal imaging of fluorescent BODIPY-ceramide dyes and DiOC6, and by electron microscopy of immunolabeled BODIPY dyes. The labeled membranes co-localize with Golgi apparatus, endoplasmic reticulum, and large diameter vesicles.

Tu-Pos108

OUTWARD-RECTIFIER K^+ CHANNEL ON THE NUCLEAR ENVELOPE OF NEONATAL MICE ISOLATED NUCLEI.

((R. Tonini and M. Mazzanti)) Dip. Fisiologia e Biochimica Generali, Via Celoria 26, I-20133, Milano Italy.

In the last few years many investigators using the patch-clamp technique have shown the presence of ionic permeabilties on the membrane of several intracellular organelles. Potassium, Chloride and Ca^{2+}- release ionic channels have also been observed on the nuclear envelope in nuclei isolated from different kind of cells. Since the complex structure of the cell nucleus their real location and function is still at a level of hyphotesis. Many authors suggest the presence of different population of nuclear ionic channels, some of which may be ligand gated and some may be dependent from electrical potential across the nuclear envelope. Using on-nucleus patch-clamp technique on isolated nuclei from neonatal mice epatocytes we detect a voltage-dependent K^+ channel with an unitary conductance of 25 pS. The channel needs high voltage steps to be activated (more than 40 mV in the patch pipette) and is able to conduct preferentially outward current (from the nucleus to the recording pipette). The channel is very sensitive to divalents. Removing Ca^{2+} and Mg^{2+} ions from the electrode solution abolishes the rectification. This novel type of channel was never found in adult nuclei. There are other nuclear channels that appear to be transiently expressed as consequence of particular stimuli or cell age. This could represent a characteristic of the nuclear envelope: activate ionic channels only when needed. Temporary ionic fluxes could modulate several nuclear functions such as nuclear envelope break-down and cell activation

Tu-Pos109

EFFECTS OF CYTOPLASMIC Ca^{2+} CONCENTRATION AND Ca^{2+} CHELATORS ON SINGLE-CHANNEL PROPERTIES OF THE IP_3 RECEPTOR IN *XENOPUS* OOCYTE OUTER NUCLEAR MEMBRANE

((D. D. Mak and J. K. Foskett)) Dept. of Physiology, University of Pennsylvania, Philadelphia, PA 19104.

The regulation by cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_{cyto}$) of inositol-1,4,5-trisphosphate (IP_3) mediated Ca^{2+} release is believed to be important in generating complex $[Ca^{2+}]_{cyto}$ signals in many cell types. The $[Ca^{2+}]_{cyto}$ dependence of the IP_3 receptor (IP_3R) in its native membrane environment was studied by patch clamping the outer membrane of nuclei isolated from *Xenopus* oocytes. Although recent studies suggested that IP_3R activity was modified by Ca^{2+} chelators in sub-millimolar concentrations, our study (10 μM IP_3 and 20 mV on the cytoplasmic side; symmetric 140 mM KCl, 10 mM HEPES, 0.5 mM ATP, pH 7.1 on both sides) revealed no effect on open probability (P_o) and single-channel kinetics of IP_3R when the free concentration of the Ca^{2+} chelator BAPTA was varied over a wide range from 35 μM to 1 mM ($[Ca^{2+}]_{cyto}$ =250 nM) on the cytoplasmic side of the channel or when BAPTA was replaced with the high K_d chelator 5,5'-dibromo BAPTA ($[Ca^{2+}]_{cyto}$ =750 nM). At low $[Ca^{2+}]_{cyto}$ ($\leq$100 nM), the IP_3R displayed low P_o (<0.05) and short open durations ($\tau \approx 2$ ms). At $[Ca^{2+}]_{cyto} \geq 50$ μM, IP_3R activity was also inhibited ($P_o < 0.2$). In contrast, the IP_3R exhibited high P_o (>0.6) and long open durations ($\tau \approx 15$ ms) over a wide range of $[Ca^{2+}]_{cyto}$ between 200 nM and 10 μM. This broad $[Ca^{2+}]_{cyto}$ insensitivity may indicate that the $[Ca^{2+}]_{cyto}$ dependence of IP_3R is different in its native membrane environment or that the $[Ca^{2+}]_{cyto}$ dependence is regulated.

Tu-Pos110

PRELIMINARY CHARACTERISATION OF A RYANODINE RECEPTOR-CALCIUM RELEASE CHANNEL FROM THE FLIGHT MUSCLE OF *HELIOTHIS VIRESCENS*.

((**T. Scott-Ward**[1], J.D. Windass[2], S.J. Dunbar[2], A.J. Williams[1])) [1]NHLI, Imperial College, LONDON. [2]ZENECA Agrochemicals, Bracknell, U.K. (Spon. by S.E. Harding)

The plant alkaloid ryanodine was originally identified as an insecticide, however information on the invertebrate RyR is still rudimentary. The aim of this study was characterisation of an insect RyR and comparison of its functional properties with those of a mammalian isoform. An assay was developed to characterise the ^{3}H-ryanodine binding properties of microgram quantities of membrane protein. A simple purification protocol yields membrane fractions from homogenised pre-frozen *Heliothis* thoracic muscle and fresh rabbit skeletal muscle with stable binding activities (1.19$\pm$0.21 and 2.99$\pm$0.49 pmol per mg protein (n=4 ; SEM) respectively). Binding to both isoforms was dependent on ryanodine concentration in a hyperbolic fashion with a K_D of 3.82 nM for insect compared to 2.04 nM for rabbit (n=3). The binding was also highly dependent on calcium concentration (optimal at 200 μM for insect and 500 μM for rabbit ; n=3) and molar concentrations of KCl. Addition of 1 mM ATP stimulated binding to both isoforms by 1.5-fold in the presence of 50 μM calcium, where as millimolar caffeine activated binding to rabbit but not insect fractions in the presence of nanomolar calcium. Binding to both isoforms in 50 μM calcium is inhibited more than 75% by 10 mM magnesium. Density-gradient centrifugation produced membrane vesicles suitable for single-channel studies. Calcium permeable channels from both insect and rabbit preparations were successfully inserted into artificial lipid bilayers. The open probabilities of both were shown to be dependent on calcium and increased in the presence of 1 mM ATP. Both isoforms display a characteristic modification of ion handling and gating in the presence of 100nM ryanodine.

Tu-Pos111

INTRACELLULAR Ca^{2+} RELEASE IN HEK-293 CELLS EXPRESSING THE WILD TYPE CALCIUM RELEASE CHANNEL (RYANODINE RECEPTOR) OF SKELETAL MUSCLE SARCOPLASMIC RETICULUM AND MUTANT FORMS ASSOCIATED WITH CENTRAL CORE DISEASE AND/OR MALIGNANT HYPERTHERMIA.

((**J. Tong‡**, H. Oyamada‡, N. Demaurex@, S. Grinstein@, and D. H. MacLennan‡)) ‡Banting and Best Department of Medical Research, University of Toronto, Toronto, Ontario, Canada M5G 1L6, and @ Division of Cell Biology, the Hospital of Sick Children, University of Toronto, Toronto, Ontario, Canada M5G 1X8.

Malignant hyperthermia (MH) is an autosomal dominant disorder of skeletal muscle which manifests as a potentially fatal hypermetabolic crisis triggered by commonly used anaesthetic agents. To date, eight mutations in the human *RYR*1 gene encoding the Ca^{2+} release channel of skeletal muscle sarcoplasmic reticulum (the ryanodine receptor,) have been linked to or associated with MH and/or central core disease. The corresponding mutations were made in a full length rabbit *RYR*1 cDNA and wild type and mutant cDNAs were transfected into HEK-293 cells. After 48 hours, intact cells were infused with the fluorescent calcium indicator fura-2 and intracellular calcium release, induced by caffeine or halothane, was measured by photometry. Ca^{2+} release in cells expressing mutant ryanodine receptors was invariably sensitive to lower concentrations of caffeine than Ca^{2+} release in cells expressing wild type receptor and the amplitude of Ca^{2+} release in response to 1 mM halothane in cells expressing mutant ryanodine receptors was invariably higher than Ca^{2+} release in cells expressing wild-type receptor. These results provide supporting evidence that each of these single amino acid mutations in the ryanodine receptor are causative of malignant hyperthermia.

(Supported by the Medical Research Council of Canada)

Tu-Pos112

INTERACTION OF THE CHEMICAL MODIFIER EDC AND THE RYANODINE MODIFIED SR Ca^{2+}-RELEASE CHANNEL.

((**Andrew Griffin** and Alan J. Williams)). N.H.L.I. Imperial College, University of London, London.UK. (Spon.by R. Sitsapesan).

The single channel conductance of the sarcoplasmic reticulum (SR) Ca^{2+}-release channel is extremely high, a phenomenon which maybe dependent on a high density of negative charge in the conduction pathway of the channel (Tinker *et al.*, J.Gen.Physiol., 1992; 100, 495-517). Chemical modification of specific amino acid residues by EDC permits the investigation of the importance of this negative charge. Purified sheep cardiac SR Ca^{2+}-release channels were incorporated into planar phospholipid bilayers and modified to a characteristic high-*Po* reduced conductance state with 100nM ryanodine. Experiments were performed under voltage-clamp conditions, with K^+ (100mM) as the charge carrier. The application of EDC to the cis chamber (cytosolic face of the channel) caused a concentration dependent block of the ryanodine modified channel. At +60mV the *Po* was significantly ($p<0.05$) decreased from approximately 1.0 to values of 0.2$\pm$0.3 (n=4) and 0.05$\pm$0.02 (n=4) in the presence of 100μM and 3mM EDC respectively. Furthermore, the blocking activity of EDC was accompanied by a concentration dependent reduction in the current amplitude of the ryanodine modified channel. In the presence of 3mM EDC the current amplitude was significantly ($p<0.05$) reduced from 23.0$\pm$0.23pA to 15.2$\pm$0.69pA (n=4). This reduced amplitude was maintained after washout but the reduction in *Po* was reversible. In the presence of 100μM EDC changes to both the current amplitude and *Po* were reversible. We conclude that the chemical modifier EDC enters the conduction pathway of the sheep cardiac SR Ca^{2+}-release channel causing block. Furthermore, at concentrations previously shown to chemically modify carboxyl groups of amino acid residues, EDC is causing an alteration of the ion handling characteristics of the channel. *Supported by the British Heart Foundation.*

Tu-Pos113

CONCENTRATION-DEPENDENT EFFECTS OF A REVERSIBLE RYANOID ON THE SR Ca^{2+}-RELEASE CHANNEL (RyR). ((**Alan J. Williams**, Luc Ruest*, William Welch† & John Sutko¶)) NHLI, Imperial College, London, SW3 6LY U.K.. *Dept. Chimie, Univ. Sherbrooke, Québec, Canada. †Dept. Biochem. and ¶Dept. Pharmacol., Univ. Nevada, Reno, NV.

We describe the consequences of the interaction of 21-amino-9α-hydroxy-ryanodine (21-amino-ryanodine) with the sheep cardiac muscle RyR. Purified RyRs were incorporated into planar phospholipid bilayers and single-channel current fluctuations were monitored under voltage-clamp conditions (holding potential +40 mV) with K^+ (600 mM) as the permeant ion. Channel open probability was increased by the addition of 100 μM EMD 41000. The binding of 21-amino-ryanodine to the cytosolic face of the channel modifies ion-handling, reducing single-channel conductance from 813±10 to 354±6 pS (SEM, n=14). Unlike ryanodine which binds irreversibly to RyR, 21-amino-ryanodine interacts reversibly with its receptor under steady-state conditions. Dwell times in the modified conductance state last seconds. Raising the concentration of 21-amino-ryanodine, in the range 50 to 900 nM, increases the probability of channel modification (P_{mod}). The relationship of P_{mod} to [21-amino-ryanodine] can be described by a simple single-site binding scheme with 50% saturation occurring at 105 nM. In the presence of 21-amino-ryanodine, the distributions of dwell times in both the unmodified and modified states can be described by single exponentials. In addition, the apparent rate constant of 21-amino-ryanodine association varies linearly with [21-amino-ryanodine] while the dissociation rate is independent of [21-amino-ryanodine]. These observations are consistent with a mechanism whereby modifications of RyR channel function induced by 21-amino-ryanodine result from the interaction of a single molecule of the ryanoid with each channel protein. *Supported by a grant from the BBSRC*

Tu-Pos114

A NEW SCORPION TOXIN STIMULATES RYANODINE RECEPTOR IN RABBIT SKELETAL MUSCLES THROUGH ITS INDIRECT PATHWAY. ((**S. KAWANO**[1], A. KUNIYASU[2], Y. HIRAYAMA[1], A. KURUMA[1]. YH. JI[3], K.XU[3], M. HIRAOKA[1], H. NAKAYAMA[2]))
[1]Dept. of Cardiovascular Diseases, Med. Res. Inst. Tokyo Medical and Dental Univ.,Tokyo, [2]Pharmaceutical Sciences, Kumamoto Univ., Kumamoto, Japan, [3]Shanghai Inst. of Physiology, Academia Sinica, Shanghai, People Republic of China.

Several scorpion toxins are known to modulate ryanodine receptor calcium release channels (RYR). In this study, we investigated a peptide toxin newly isolated and purified from a Chinese scorpion, Buthus martensi Karsch (Toxin), whether it affected RYR in the cardiac and skeletal sarcoplasmic reticulum (SR). Toxin activated [^{3}H] ryanodine binding to both cardiac and skeletal preparations, which showed the larger effects on skeletal preparations than on cardiac ones. In the skeletal triad preparations, Toxin increased both affinity and B_{max} of the [^{3}H]ryanodine binding. However, in purified RYR, Toxin-induced enhancements of [^{3}H] ryanodine binding were not observed. Single channel recordings of RYR also showed the similar results. Openings of RYR in skeletal triad preparations were increased by the application of Toxin (P_o=0.07 to 0.37 in the presence of ATP). However, in the purified RYRs, P_o were 0.33 and 0.33 before and after Toxin, respectively. From these results, we conclude that Toxin may affect on RYR indirectly, possibly by mediating other molecules closely associated with RYR, which may inhibit the activities of RYR.

Tu-Pos115

RYANODINE RECEPTORS FROM BRAIN MICROSOMES ARE INHIBITED BY THE ALKALOID TETRANDRINE. ((F S. Scornik, C. Sánchez Antelo, *B. Cassels and O. D. Uchitel)) Inst. de Biol. Celular y Neurocien. "Eduardo De Robertis". Fac. de Med., UBA, Argentina; *Dpto. de Quím. Fac. de Ciencias, U. de Chile, Chile.

Changes in the intracellular calcium play a key role in the physiology of the nervous system. Calcium release from intracellular stores underlies much of these changes. The ryanodine receptor (RyR) is central in regulation of the intracellular calcium concentration in other tissues. Its presence in the brain has been determined although its role is not well understood. Tetrandrine is a plant alkaloid known for its use in medicine. Most of its effects has been associated to blockade of membrane voltage activated calcium channels. However, some indirect evidence indicate that affect calcium release from internal stores. In this work we show that a channel obtained from total brain, incorporated into lipid bilayers that shows characteristics of a RyR is inhibited by the alkaloid tetrandrine. The channel studied is permeant for Ba^{2+}, Na^+ and K^+. The observed conductances were: 204 ± 7 pS in 500/50 mM $BaCl_2$, n = 9; 366.5 ± 6 pS in 250/50 NaCl, n = 13 and 622.5 ± 0.5 in 250/50 KCl, n = 2. When studied in bionic conditions the permeability for Na^+ and K^+ was the same. However when any of the monovalent ions where studied in bionic conditions with Ba^{2+}, the last one was more permeant. We also observed calcium, caffeine and ryanodine sensitivity. When 1 μM tetrandrine was added to the *cis* chamber, channel activity was lowered in a 50 to 90% of control channel activity (n=4). In this condition ryanodine still evoked a substate with a Po close to 1 (n=2). Preliminary results show that a higher dose (10 μM) of tetrandrine can inhibit the ryanodine induce substate. We show direct evidence of tetrandrine inhibition of the RyR. This is important as a pharmacological tool in the characterization of the channel and its implications as a therapeutic agent.

Tu-Pos116

RYANODINE & INOSITOL TRISPHOSPHATE RECEPTORS SHARE THE SAME CALCIUM POOL IN CEREBELLAR PURKINJE NEURONES. *((Kamran Khodakhah & Clay M Armstrong))* Department of Physiology, University of Pennsylvania, Philadelphia, PA 19104.

Activation of ryanodine receptors (Ry-R) by Ca^{2+}, and inositol trisphosphate receptors ($InsP_3$-R) by $InsP_3$ mediate release of Ca^{2+} from intracellular stores in many different cell types including cerebellar Purkinje neurones. While $InsP_3$ and ryanodine receptors are co-localized in Purkinje neurones, it is not established whether these receptors release calcium for a common Ca^{2+} pool, or modulate separate Ca^{2+} stores.
To investigate this question intracellular free calcium concentration ($[Ca^{2+}]_i$) was measured in whole-cell voltage-clamped Purkinje neurones fo!lowing flash photolytic release of $InsP_3$. Ryanodine, which clamps its channels in an open, subconducting state, was added to the intracellular patch pipette solution. The continuous efflux of Ca^{2+} from the modified Ry-R's depleted the ryanodine-sensitive Ca^{2+} pools without significantly altering $[Ca^{2+}]_i$. Under these conditions the ability of $InsP_3$ to mobilize Ca^{2+} was examined. If $InsP_3$ and ryanodine receptors modulate different calcium pools, depletion of ryanodine-sensitive pools by ryanodine should not alter $InsP_3$-evoked Ca^{2+}-release. Alternatively, if $InsP_3$ and ryanodine receptors share the same calcium pools, depletion of ryanodine-sensitive stores would also abolish $InsP_3$-evoked Ca^{2+}-release. In the presence of ryanodine $InsP_3$ failed to elevate $[Ca^{2+}]_i$ indicating that both $InsP_3$ and ryanodine receptors mobilize calcium from a common calcium pool.

Tu-Pos117

SELECTIVE EFFECTS OF RYANODINE AND INOSITOL TRISPHOSPHATE ON CALCIUM STORES IN ASCIDIAN OOCYTES.

((**Mireille Albrieux**, Michel Villaz))
Laboratoire de Biophysique Moléculaire et Cellulaire, DSV/DBMS, 17 rue des Martyrs, F-38054 Grenoble, France.

Using the whole cell patch-clamp technique, a low threshold activated calcium current is induced by depolarization in the oocyte of the ascidian *Phallusia mammillata*. We show here that either ryanodine or inositol trisphosphate at nanomolar concentrations induces a decrease in the intensity of this calcium current, when applied internally from the mouth of the patch pipette. This decrease in calcium current is prevented by intracellular application of the calcium chelator BAPTA, therefore imputable to calcium release. The inositol trisphosphate effect is slow (minutes) and elicited with no change in cell surface area. Ryanodine triggers a rapid decay (seconds) of calcium current intensity, going together with an increase in oocyte surface area, as measured by its capacitance. These ryanodine effects on unfertilized oocytes mimick two early postfertilization events: the loss of the low threshold calcium current and the membrane trafficking started at meiosis resumption. We discuss the methodological relevance of our patch-clamp approach, compared to fura-2 calcium imaging data, in connection with the possible biological role of a ryanodine receptor in early steps of development.

Tu-Pos118

cADPR ACTIVATES SINGLE CALCIUM CHANNELS PRESENT IN SEA URCHIN EGG MICROSOMES.

((C. Pérez, J.J. Marengo*, R. Bull, and C. Hidalgo)). Faculty of Medicine, University of Chile., and C.E.C.S., Santiago, Chile.

Cyclic ADP ribose (cADPR) has been proposed to act as an endogenous agonist of the ryanodine-receptors (RyRs) present in sea urchin eggs, where it triggers calcium release from IP_3-independent calcium stores. Yet the calcium channel properties of these RyRs, and the effects of cADPR on their activity are unknown. Microsomes were isolated from eggs of the sea urchin *Tetrapigus nyger*. Microsome fusion with planar lipid bilayers revealed calcium-selective channels that were activated by ryanodine (> 50 μM), inhibited by 20 μM Ruthenium Red, and unaffected by up to 5 μM IP_3. Cytosolic (*cis*) free calcium concentration ($[Ca^{2+}]$) in the μM range (1 to 30 μM) produced moderate channel activation, whereas channel inhibition was observed at *cis* $[Ca^{2+}]$ > 100 μM. *Cis* addition of cADPR (1-10 μM) at 10 μM *cis* $[Ca^{2+}]$ increased fractional open time (Po) in a concentration dependent manner. Channel activation by cADPR was abolished by Ruthenium Red, was independent of exogenous calmodulin, and was enhanced by μM *cis* $[Ca^{2+}]$ and by luminal $[Ca^{2+}]$. Increasing luminal $[Ca^{2+}]$ from 10 μM to 50 μM, at 10 μM *cis* $[Ca^{2+}]$, augmented Po from 0.01 to 0.15. These results suggest that these channels, that resemble the mammalian RyR-channels, underlie cADPR-induced calcium release in sea urchin eggs. Supported by Fondecyt 2950037 & 1940369. *Andes Foundation Doctoral Fellow.

Tu-Pos119

REGULATION OF THE INOSITOL 1,4,5-TRISPHOSPHATE RECEPTOR BY ARACHIDONIC ACID AND EICOSANOIDS. ((**F. Striggow** and B.E. Ehrlich)) University of Connecticut, Farmington, CT 06030-3505

Stimulation of many G-protein or tyrosine kinase-linked receptors leads to the activation of the inositol 1,4,5-trisphosphate receptor ($InsP_3R$) followed by the release of Ca^{2+} from intracellular stores into the cytosol. One of the many regulatory functions of Ca^{2+} is to mediate the translocation of cytosolic phospholipase A_2 ($cPLA_2$) to the membrane of the endoplasmic reticulum. There, $cPLA_2$ catalyzes the release of arachidonic acid, the precursor for the synthesis of the eicosanoids. We investigated the influence of arachidonic acid and some eicosanoids on the single channel properties of the $InsP_3R$ after incorporation of native cerebellar channels into lipid bilayers. We found that arachidonic acid inhibited the $InsP_3R$ completely (Ki = 27 nM). In contrast, arachidonic acid up to 100 µM had no influence on the activity of the ryanodine receptor/Ca^{2+} release channel. Furthermore, we did not observe effects of prostaglandin D_2, the major prostaglandin in brain, or prostaglandin H_2 on the single channel activity of either Ca^{2+} release channel. We suggest that arachidonic acid is an important component of the regulation of intracellular Ca^{2+}. Specifically, arachidonic acid-induced inhibition of $InsP_3R$ activity may provide feedback inhibition of the Ca^{2+}-dependent translocation of $cPLA_2$ to the endoplasmic reticulum.

Supported by NIH grants GM51480 and HL33026. FS was a fellow of the German Academic Exchange Service (DAAD).

Tu-Pos120

RELATIONSHIP BETWEEN THE BELL-SHAPED CALCIUM DEPENDENCE OF $InsP_3$-GATED CHANNEL ACTIVITY AND CALCIUM-DEPENDENT INHIBITION OF $InsP_3$ BINDING. ((**E.J. Kaftan**, B.E. Ehrlich, and J. Watras)) University of Connecticut, Farmington, CT

The calcium (Ca)-dependent regulation of the inositol 1,4,5-trisphosphate ($InsP_3$)-gated channel in response to $InsP_3$ production can be described by a bell-shaped Ca-dependence. We found that the Ca concentration at which peak activity occurs and the width of the bell-shaped curve increased as the $InsP_3$ concentration increased from 0.02 to 2 µM. When analyses of $InsP_3$ binding were done in media identical to that used for channel activity measurements, increasing the Ca concentration from 1 nM to 10 µM Ca resulted in a 3-fold increase in the apparent dissociation constant for $InsP_3$ binding. Complete inhibition of $InsP_3$ binding was not observed, even at 100 µM Ca whereas, $InsP_3$-gated Ca channel activity could be completely inhibited by 5 µM Ca. The inability to explain the descending phase of the bell-shaped curve by simple Ca-dependent inhibition of $InsP_3$ binding led to the development of a multistate model that could describe the Ca-dependent changes in both binding and channel activity. The observed shift in the bell-shaped Ca dependence curve extends the range of cytosolic Ca concentrations that can be achieved in response to $InsP_3$ production, especially during periods of prolonged stimulation of the phosphoinositide cascade.

Supported by NIH grants GM51480 and HL33026.

Tu-Pos121

MOLECULAR CLONING AND CHARACTERISATION OF A NOVEL HOMOLOGUE OF P64 FROM MAMMALIAN BRAIN.

((**Rory R. Duncan** and Richard H. Ashley)) Department of Biochemistry, University of Edinburgh, Edinburgh, EH8 9XD, UK.

We have previously reported the isolation of partial cDNA species from rat brain encoding two novel homologues of the bovine kidney Cl^- channel component, p64. Here we report the isolation of cDNA encoding a full-length protein for one of these homologues, p64H1. The cDNA and its gene product have been partially characterised. The tissue distribution of p64H1 has been studied using Northern and Western blot analysis. The membrane topology of p64H1 has been determined using a combination of *in vitro* translation in the presence or absence of microsomal membranes and protease protection assays. These studies show that p64H1 has a single, α-helical transmembrane domain, with the carboxy-terminus of the protein residing in the cytoplasm. We suggest that p64 is a member of an extensive gene family encoding proteins associated with Cl^- transport. Thorough characterisation of these proteins may lead to the elucidation of their function *in vivo*.

(Supported by the MRC).

Tu-Pos122

CELLULAR AND INTRACELLULAR LOCALISATION OF A RAT BRAIN HOMOLOGUE OF P64.

((**Richard H. Ashley**, Paul K. Westwood and Rory R. Duncan)) Department of Biochemistry, University of Edinburgh, Edinburgh, EH8 9XD, UK.

P64 is a component of an intracellular anion channel, isolated from bovine kidney microsomes. We have reported elsewhere the isolation and characterisation of two novel homologues of p64 in rat brain which we believe constitute members of a new gene family. Here we determine the cellular location in rat brain of one of these homologues, p64H1 using *in situ* mRNA hybridisation and immunohistochemical analyses. In addition, the *intracellular* location of p64H1 has been studied in cultured HEK293 cells expressing recombinant protein, using indirect immunofluorescence labelling. These studies together show p64H1 is expressed in neurons, notably in the hippocampus and in the Purkinje and granule cell layers of the cerebellum. Within the cell, p64H1 is highly localised to the membranes of the endoplasmic reticulum.

(Supported by the Wellcome Trust and the MRC).

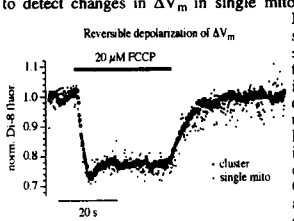
CARDIAC E-C COUPLING AND Ca REGULATION

Tu-Pos123

CONFOCAL MICROSCOPIC RECORDING OF MEMBRANE POTENTIAL IN SINGLE ISOLATED CARDIAC MITOCHONDRIA

((J. Hüser, T.R. Shannon, C.E. Rechenmacher, D.M. Bers & L.A. Blatter)) Dept. of Physiology, Loyola University Medical Center, Maywood, IL 60153

The electrochemical potential difference across the inner mitochondrial membrane is composed of the electrical potential difference (ΔV_m) and a pH gradient. ΔV_m in isolated mitochondria has been measured to study mitochondrial functions from substrate oxidation to ATP synthesis. ΔV_m-dependent fluorescent probes (redistribution dyes) have been used widely to detect changes in ΔV_m in suspensions of isolated mitochondria. However, the relatively slow response time of these indicators to changes in ΔV_m, the slow mixing time in cuvette experiments, and the heterogeneity within mitochondrial populations impede detailed analysis of the kinetics of ΔV_m changes. Using fast voltage-sensitive fluorescent membrane probes (Di-8-ANEPPS and RH-421) and laser scanning confocal microscopy we were able to detect changes in ΔV_m in single mitochondria isolated from rat heart (see fig.).

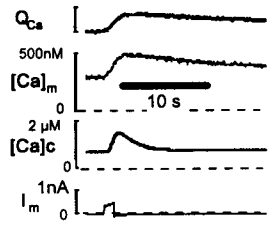


Mitochondria were superfused with buffer solution allowing solution changes with $t_{1/2} \leq 200$ ms. Using this technique, we found that ΔV_m was unaffected by rapid changes in extra-mitochondrial [Ca^{2+}] (1-100 µM) consistent with the slow kinetics of net ^{45}Ca uptake measured under similar conditions. However, in the presence of the Ca ionophore A23187, similar additions of Ca caused a decrease in ΔV_m. Thus, the rate of Ca uptake via the mitochondrial uniporter alone seems to be too low to affect ΔV_m during a single cardiac cycle.

Tu-Pos124

SIMULTANEOUS MEASUREMENTS OF CYTOSOLIC AND MITOCHONDRIAL Ca SIGNALS IN SINGLE VENTRICLE MYOCYTES.. ((Z. Zhou and D. M. Bers)) Department of Physiology, Loyola University Chicago, Maywood, IL.

We developed a method to measure the mitochondrial (Mito) Ca uptake (MCa-U) and intra-Mito free [Ca] ($[Ca]_m$) in single cells in real time. Cat and ferret myocytes were loaded by indo-1/AM and whole-cell clamped by a patch-pipette. Ca influx was evoked by depolarization to +10 mV (via Ca channel) or +110 mV (via Na/Ca exchanger). Cytosolic indo was either removed by dialysis via patch pipet (very slow, ≥40 min) or quenched by exposure to Mn (<10 min). Both methods indicated 75% of indo was in Mito. Using the selective MCa-U blocker RU-360 in the pipet prevented changes in $[Ca]_m$ such that $[Ca]_c$ could be measured by cytosolic indo after subtracting the Mito indo signals. $[Ca]_c$ could also be calculated directly from the cell length measurements (Bassani *et al., BJ* 68:1453-60, 1995) although the $[Ca]_c$ signal preceded the contraction signal by ~200 ms. In Mn quenched cells, $[Ca]_c$ was measured by cell contraction. The heavy Mito indo loading also allows calculation of the pure Ca influx into Mito (as Q_{Ca}, see Fig) since most of the Mito Ca influx is initally bound to indo (Zhou & Neher, *Pflug Arch*, 425:511, 1993). I_m shows Na/Ca exchange current. Decay of $[Ca]_m$ after Ca influx depends on both basal $[Ca]_c$ and $[Ca]_m$. Mito indo can also be saturated after excessive Mito Ca influx.

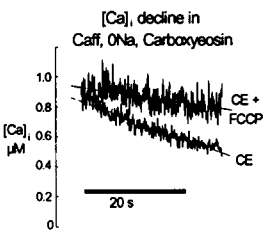


Tu-Pos125

A METHOD TO ESTIMATE MITOCHONDRIAL Ca INFLUX IN INTACT RABBIT CARDIAC MYOCYTES.

((J.W.M. Bassani, R.A. Bassani, & D.M. Bers)). Faculdade de Engenharia Elétrica e de Computação and Centro de Engenharia Biomédica, Universidade de Campinas, SP, Brasil and Dept. Physiology, Loyola University - Chicago, IL, USA.

We estimate mitochondrial (Mito) Ca uptake during the $[Ca]_i$ decline in intact rabbit ventricular myocytes, using the sum of Ca fluxes and change in Ca bound to cytosolic ligands: $d[Ca]_T/dt = J_{SR} + J_{NaCaX} + J_{SLCa\text{-}pump} + J_{Mito} + J_{Leak} + d(\Sigma Ca\text{-bound})/dt$. During $[Ca]_i$ decline we inhibited net Ca transport by 1) SR Ca-pump (J_{SR}) by caffeine, 2) sarcolemmal Ca-ATPase ($J_{SLCa\text{-}pump}$) by carboxyeosin and 3) sarcolemmal Na/Ca exchange (J_{NaCaX}) by removal of Na_o and Ca_o. Ca transients were evoked by rapid and sustained application of caffeine (10 mM) after 5 min of 0.5 Hz stimulation. Curve CE in the Fig shows $[Ca]_i$ decline due to J_{Mito} plus any leak (J_{Leak}). Inhibition of Mito with FCCP (CE +FCCP curve) shows $[Ca]_i$ decline due to J_{Leak}, such that the difference is the FCCP-sensitive J_{Mito} component. These curves were linear whether plotted as free $[Ca]_i$ (Fig) or total Ca. That is, Ca flux ($d[Ca]_T/dt$) was constant for 20-30 sec. Thus, the Ca flux due to net Mito Ca uptake may attain a value close to 1 μmol•l cytosol^{-1}•s^{-1} at $[Ca]_i$ ~0.5-1 μM. Since the slope of the curves does gradually decline as $[Ca]_i$ falls this J_{Mito} is an upper limit with respect to twitches where mean $[Ca]_i$ is lower. *Support: FAPESP (95/0355-3), SP-Brasil & NIH (HL-30077)*



Tu-Pos126

SARCOPLASMIC RETICULUM Ca RELEASE ACTIVATED BY MEMBRANE DEPOLARISATION, IN THE ABSENCE OF Ca^{2+} ENTRY, IN HEART CELLS FROM RABBIT, RAT AND GUINEA-PIG ((**Allan J. Levi**, Ion A. Hobai, Geoff Dalton, F. Chris Howarth, Vijay Pabbathi, Jules C. Hancox and Gregory R. Ferrier)) Department of Physiology, University of Bristol, Bristol BS8 1TD, U.K.

In guinea-pig heart cells dialysed internally with 50 μM cyclic AMP, membrane depolarisation by itself can activate SR Ca release, in the absence of Ca^{2+} entry (Levi & Ferrier, 1996; abstract this meeting). This work had two objectives - to detect whether this hitherto undiscovered "voltage-activated calcium release" (VACR) mechanism is present in other species, and to study its dependence on [cyclic AMP]. We performed experiments on rabbit, rat and guinea-pig cells at 37°C. Myocytes were dialysed with a Na-free pipette solution (to inhibit Ca release triggered by reverse Na/Ca exchange); Ca_i transients measured with Fura-2, or with a mixture of Fluo-3/Snarf; Na channels inhibited with 300 μM lidocaine. After a train of conditioning pulses to maintain Ca load, cells were held at -60mV for 4 seconds, and then a +20mV test depolarisation was applied. Rapid application of 5mM Ni two seconds before the test depolarisation inhibited the L-type Ca channel ($I_{Ca,L}$) and Na/Ca exchange completely (no inward current). With no cyclic AMP in the pipette, a switch to 5mM Ni abolished the test pulse Ca_i transient (consistent with $I_{Ca,L}$ being the main SR trigger under these conditions). However, in all three species, with 100 μM cyclic AMP in the pipette, large rapid (t_{pk} 70msec) Ca_i transients remained during 5mM Ni (rabbit 89.6+/-7.1%; rat 64.3+/-6.2%; guinea-pig 78.1+/-3.8%; of control Ca_i transient remained during 5mM Ni). Even with 5 μM cyclic AMP in the pipette, a substantial Ca_i transient could be observed during Ni application (e.g. rat 63.6+/-16.2%; rabbit 45.3+/-4.8%). We conclude the VACR mechanism is present in all three species, and the extent to which it triggers Ca release is modulated by internal [cyclic AMP].

Tu-Pos127

DEPRESSION OF CONTRACTIONS ACTIVATED BY THE CARDIAC VOLTAGE SENSITIVE RELEASE MECHANISM IN MYOCYTES FROM MYOPATHIC HAMSTERS. ((**S.E. Howlett**, G.R. Ferrier and C. Mapplebeck)) Dalhousie University, Halifax, Nova Scotia, Canada (Spon: J. C. Hancox).

Changes in properties of the voltage sensitive release mechanism (VSRM) for Ca^{2+} release from sarcoplasmic reticulum might contribute to contractile dysfunction in cardiomyopathic (CM) hamster heart. We compared activation-inactivation properties of the VSRM and density of ryanodine receptors in hearts from young (80-100 days) normal and CM hamsters. Myocytes were evaluated at 37°C with high resistance microelectrodes to minimize cell dialysis. Currents were measured with switch clamp and contractions measured with a video edge detector. I_{Na} was blocked with lidocaine (200μM) and I_{TO} was blocked with 4-aminopyridine (2mM). Contraction-voltage (CV) relations determined from a potential (V_{PC}) of -65 mV were sigmoidal, which is characteristic of the VSRM. However, in CM cells the threshold for activation of contraction was shifted by +15 mV and contraction amplitudes were depressed compared with normal ($p<0.05$). VSRM contractions exhibited steady-state inactivation with similar half-inactivation voltages and slopes in both groups. When the VSRM was inactivated by a V_{PC} of -40 mV, contractions activated by $I_{Ca\text{-}L}$ exhibited similar bell-shaped CV relations in normal and CM cells. Magnitude of $I_{Ca\text{-}L}$ also was similar in normal and CM cells. However, density of [^{3}H]-ryanodine receptors was reduced in homogenates from CM hearts compared to normal ($p<0.05$). Thus, selective depression of VSRM contractions, perhaps through a reduction in ryanodine receptor density, might contribute to development of contractile dysfunction in this model of CM and congestive heart failure.

Tu-Pos128

TETRACAINE BLOCKS A VOLTAGE-SENSITIVE RELEASE MECHANISM FOR CONTRACTION IN VENTRICULAR MYOCYTES. ((G.R. Ferrier and **C.A. Mason**)) Dalhousie University, Halifax, Canada. (Spon. R. Bose)

Tetracaine (TRC), which blocks I_{Na}, also inhibits opening of isolated sarcoplasmic reticulum Ca^{2+} release channels. Recently we described a voltage-sensitive release mechanism (VSRM) for SR Ca^{2+} which initiates contraction in cardiac myocytes. The present study investigates whether TRC inhibits contractions initiated by the VSRM or L-type Ca^{2+} current ($I_{Ca\text{-}L}$) in guinea pig myocytes. Experiments utilized single electrode voltage clamp to measure currents, and a video edge detector to measure contraction. Experiments were conducted in the presence of I_{Na} blockade with 200 μM lidocaine to eliminate effects of TRC mediated by block of I_{Na}. VSRM and $I_{Ca\text{-}L}$ contractions were separated by activation and inactivation properties. VSRM contractions were abolished by 300μM TRC with little effect on $I_{Ca\text{-}L}$ contraction or current. Control contraction-voltage (CV) relations were sigmoidal. When the VSRM was blocked with TRC, CV curves became bell-shaped, and proportional to $I_{Ca\text{-}L}$ (n = 13). Also, staircase phenomena were inhibited. Contractions remaining in the presence of TRC were abolished by either 0.1 mM ryanodine or 2.5 μM nifedipine. A higher concentration of TRC (1 mM) abolished $I_{Ca\text{-}L}$ and $I_{Ca\text{-}L}$ contractions as well as the VSRM. Thus at low concentrations TRC is a specific blocker of SR Ca^{2+} release by the VSRM, without inhibiting Ca^{2+}-induced Ca^{2+} release in response to $I_{Ca\text{-}L}$. Inhibition of the VSRM is independent of block of I_{Na} and occurs without depletion of SR Ca^{2+}.

Tu-Pos129

A ROLE FOR cAMP IN ACTIVATION OF THE VOLTAGE-SENSITIVE RELEASE MECHANISM FOR CARDIAC CONTRACTION. ((J.Q. Zhu, S.E. Howlett and **G.R. Ferrier**)) Dalhousie University, Halifax, Canada.

Previously we showed, with high resistance microelectrodes, that a voltage-sensitive release mechanism (VSRM) can initiate cardiac contraction by release of Ca^{2+} from the sarcoplasmic reticulum (SR) in the absence of L-type Ca^{2+} current ($I_{Ca\text{-}L}$) or I_{Na}. Studies with patch pipettes have failed to demonstrate the VSRM, possibly because of washout of an essential intermediate. We examined this possibility in guinea pig ventricular myocytes at 37°C. Contractions were measured with a video edge detector; currents were measured with whole cell patch clamp. Pipettes (1-3 MΩ contained a K^+-based solution with 8 or 0 mM NaCl, 4 mM MgATP and 0-50 μM 8-bromo-cAMP. I_{Na} was blocked with 200 μM lidocaine. VSRM and $I_{Ca\text{-}L}$ contractions were separated by activation and inactivation properties. With 0 μM cAMP, $I_{Ca\text{-}L}$ contractions were always present whereas VSRM contractions were transiently observed in only 10% of cells (n=31). Increasing concentrations of cAMP greatly increased the incidence, magnitude and persistence of VSRM contractions, increased $I_{Ca\text{-}L}$, but only modestly increased amplitude of $I_{Ca\text{-}L}$ contractions. In the absence of cAMP, contraction-voltage (CV) relations were bell-shaped and proportional to $I_{Ca\text{-}L}$. In contrast, with 50 μM cAMP in the pipette, the characteristic sigmoidal CV relation of the VSRM was demonstrable. These studies demonstrate that washout of essential intermediates such as cAMP in patch clamp experiments may alter cardiac excitation-contraction coupling profoundly.

Tu-Pos130

Ca RELEASE ACTIVATED BY MEMBRANE DEPOLARIZATION IN THE ABSENCE OF Ca ENTRY IN MAMMALIAN HEART. ((**Allan J. Levi** and Gregory R. Ferrier)) Department of Physiology, University of Bristol, U.K. and Department of Pharmacology, Dalhousie University, Canada.

The objective of this study was to determine whether Ca transients ($[Ca]_i$) measured with Fura-2 AM (340/380 nm excitation) can be initiated by a voltage-sensitive release mechanism for Ca^{2+} from sarcoplasmic reticulum, in the absence of Ca^{2+} influx. Currents were measured with whole cell voltage clamp at 35°C and utilized pipettes (1-3 MΩ containing 0 mM Na^+ to inhibit reverse Na-Ca exchange, and 50 μM cAMP to maintain phosphorylation. Na current was blocked with 250 μM lidocaine. Test steps were made from -60 mV, following conditioning pulses to maintain SR Ca. A test step to +20 mV elicited a large $[Ca]_i$. Rapid application of 100 μM Cd 1s before test steps blocked inward Ca current, but 81% of $[Ca]_i$ remained (n=11). Similarly 78% of $[Ca]_i$ remained with rapid switches to 5 mM Ni to inhibit Na-Ca exchange plus Ca currents. However, rapid switches to 0 mM Ca plus Ni abolished $[Ca]_i$, indicating an extracellular Ca requirement. Depletion of SR Ca with 10 mM caffeine also inhibited Ni-insensitive $[Ca]_i$. Termination of Ni-insensitive $[Ca]_i$ was coupled to repolarization of test steps with different durations. Steps to different potentials with rapid application of Cd revealed an activation curve for $[Ca]_i$ with V_{half} = -37.5 and k = 6.2 mV. SR release exhibited steady-state inactivation with V_{half} = -49.2 mV and k = 6.3 mV. This study demonstrates release of cardiac SR Ca directly controlled by sarcolemmal potential.

Tu-Pos131

EFFECTS OF HYPERTROPHY ON RELAXATION MECHANISMS AND NA/CA EXCHANGER mRNA EXPRESSION IN ISOLATED RABBIT VENTRICULAR MYOCYTES.

((**R.U. Naqvi**, *K.R Boheler and K.T. MacLeod)) Cardiac Medicine and *Cardiothoracic Surgery, Imperial College at NHLI, London SW3 6LY UK

Cardiac myocyte hypertrophy was produced by constriction of the ascending aorta in rabbits. Animals were kept for nine months following the procedure, allowing the development of a progressive pressure-overload on the heart. Upon sacrifice, left ventricular myocytes from 3 control (C) and 3 hypertrophy (H) hearts were isolated by enzymatic dissociation. A portion of the cells were loaded with the fluorescent calcium indicator indo-1(5 μM) and field-stimulated (0.5, 0.2 Hz; 2mM Ca, 22°C). Twitch time-course was prolonged in H compared with C animals. Time-to-peak contraction in C = 0.62±0.02s (n=22) vs H = 0.75±0.03s (n=28), $p<0.001$. The associated indo-1 Ca signal was also slowed; C = 0.35±0.006s (n=22) vs H = 0.38±0.01s (n=26), $p<0.01$. Time-to-90% relaxation (R90) of cell length was also prolonged (C = 0.69±0.05s vs H = 0.91±0.04s, $p<0.001$), together with the indo-1 Ca signal (C = 0.6±0.02s vs H = 0.75±0.03s, $p<0.0001$). Total RNA was extracted from the remaining cells. Na/Ca exchanger mRNA level relative to GAPDH, as determined by RNase protection assay, was increased in H (C = 0.12±0.03 vs H = 0.16±0.04). Effects of hypertrophy can therefore be seen at the level of gene expression in addition to changes in cell function.

Tu-Pos132

ANGIOTENSIN CONVERTING ENZYME INHIBITION INCREASES CaATPASE EXPRESSION AND IMPROVES MYOCYTE FUNCTION FOLLOWING CARDIAC HYPERTROPHY.

((*S.Y.Boateng, R.U.Naqvi, K.T.MacLeod and ***K.R.Boheler**)) Cardiac Medicine and *Cardiothoracic Surgery, Imperial College at NHLI, London, SW3 6LY, UK.

The effects of sub-antihypertensive doses of ramipril on CaATPase and phospholamban (PLB) expression following cardiac hypertrophy in rats were studied. Hypertrophy was induced by constriction of the abdominal aorta. Five weeks post surgery, rats received a daily sub-antihypertensive dose (50µg/kg/day) of ramipril or vehicle (polyethylene glycol) for 4 weeks. PLB mRNA and protein expression were unchanged in cardiac hypertrophy (H). Following ramipril treatment CaATPase mRNA increased by 35% ($p<0.05$ n=6) in H. Control animals (C) on ramipril treatment showed a 64% increase in the CaATPase mRNA levels compared with C rats on vehicle alone ($p<0.01$ n=5). PLB mRNA increased by 45% ($p<0.05$) in H and by 50% in C following ramipril treatment. PLB protein levels were not altered following ramipril treatment. CaATPase protein levels were normalized following ramipril treatment in the H group. In preliminary experiments using isolated myocytes we measured myocyte contractility in this model. Following cardiac hypertrophy, myocytes are slower to contract compared with control animals. Time to peak (TTP) was 0.27s±0.015 in C (n=4) and 0.36s±0.017 in H (n=6) $p<0.01$. Following ramipril, the TTP was 0.31s (n=2) in H and 0.24s (n=2) in C. Time to 50% relaxation (R50) was 0.15s±0.008 in Cand 0.24s0±0.018 in H, $p<0.01$. Following ramipril, the R50 was 0.22s (n=2) in H and 0.11s (n=2) in C. Thus ramipril can increase gene expression and may improve myocyte function following cardiac hypertrophy in rat.

Tu-Pos133

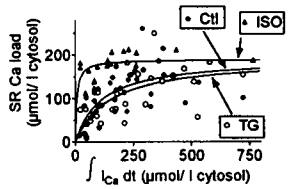
Ca^{2+}-SIGNALING IN TRANSGENIC MICE OVEREXPRESSING MUTANT CARDIAC Na^+-Ca^{2+} EXCHANGER (Δ680-685). ((**Y.J. Suzuki**, K.D. Philipson* and M. Morad)) Dept. of Pharmacology, Georgetown Univ., Washington, DC 20007 and *Cardiovascular Res. Lab., UCLA, Los Angeles, CA 90024.

We have previously examined transgenic mice overexpressing canine cardiac Na^+-Ca^{2+} exchanger (NCX) in whole cell-clamped myocytes dialyzed with fura-2, and found 3-fold overexpression of caffeine-induced Na^+-Ca^{2+} exchange current ($I_{Na/Ca}$). The present study examines the characteristics of $I_{Na/Ca}$ in ventricular myocytes from transgenic mice expressing a mutant NCX lacking six amino acids (NCXΔ680-685). Deletion of this segment in the main intracellular loop removes Ca^{2+} regulation, and the exchanger is no longer inhibited at low Ca^{2+}. Sarcolemmal vesicles from NCXΔ680-685 transgenic heart had a 1.2-fold increase in NCX activity. Freshly isolated ventricular myocytes from transgenic mice were whole-cell clamped and dialyzed with Cs^+-rich internal solution containing 0.2mM cAMP, 0.1mM fura-2 and 10mM NaCl. We found that caffeine-induced $I_{Na/Ca}$ in these myocytes was 2.4±0.2 pA/pF (n=19) vs 1.6 pA/pF in non-transgenic littermates. This value is, however, significantly smaller than that obtained in transgenic myocytes overexpressing the wild-type exchanger (4.9 pA/pF; Adachi-Akahane et al., *Biophys. J.* 70:A270, 1996). Despite smaller caffeine-induced $I_{Na/Ca}$ in these myocytes, depolarization from -40 to +100 mV in 10 mV steps resulted in a sigmoidal increase in $[Ca^{2+}]_i$ in the presence of nifedipine. The rates of rise of $[Ca^{2+}]_i$ in response to depolarization were slower under such conditions than those induced by I_{Ca} in the absence of nifedipine. The rise in magnitude of $[Ca^{2+}]_i$, on the other hand, was about the same at positive potentials as that triggered by I_{Ca}, suggesting that $I_{Na/Ca}$-induced Ca^{2+} release from the SR occurred with a lower efficiency than that induced by I_{Ca}. These results suggest that deletion of amino acids 680-685 from NCX enhances the Ca^{2+}-influx mode of the exchanger resulting in $I_{Na/Ca}$-induced Ca^{2+} release from the SR. (Supported by NIH HL16152 & HL48509)

Tu-Pos134

MODULATION OF SR Ca LOADING BY THAPSIGARGIN AND ISOPROTERENOL IN INTACT FERRET VENTRICULAR MYOCYTES. ((**K. S. Ginsburg**, C. R. Weber, and D. M. Bers)) Dept Physiology, Loyola Univ Chicago, Maywood, IL 60153

Previously we reported that SR Ca content reaches a limit (near 190 µmol/l cytosol) in response to stimulation designed to maximize Ca loading and isoproterenol (ISO) increased the maximal SR Ca load by ≤25%. Here we measured SR Ca content in myocytes in which the SR had been initially emptied with caffeine. Cells were repeatedly depolarized (0mV, 200msec) to admit Ca (via I_{Ca}). Cumulative Ca entry was measured by the integral of I_{Ca} ($\int I_{Ca}dt$) and $[Ca]_i$ was measured by Indo-1(AM) fluorescence. Na-free solutions were used to block Na/Ca exchange. SR Ca content was assessed by application of caffeine and Na (for 10 sec) and integration of the Na/Ca exchange current ($I_{Na/Ca}$) at −70 mV. Again, SR Ca content appeared to reach a similar limit without much rise in $[Ca]_i$. The decline of twitch $[Ca]_i$ attributed to the SR Ca-ATPase was slowed 2-3 fold by brief exposure to thapsigargin (TG) and sped up 2-3 fold by ISO (1 μM). The maximum SR Ca load was not altered by either TG or ISO, but the half-max SR Ca load was reached at lower $\int I_{Ca}$ for ISO and higher for TG. The lack of effect on maximum SR Ca load over a 5-fold range in SR Ca-pump activity suggests that backflux or thermodynamic gradient is important in limiting the maximal SR Ca load.



Tu-Pos135

MEASUREMENTS OF CALCIUM ENTRY AND SR CALCIUM CONTENT IN GUINEA-PIG AND RAT VENTRICULAR MYOCYTES

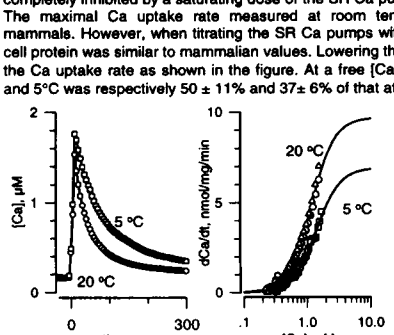
((**C.M.N. Terracciano** and K.T. MacLeod)) Cardiac Medicine, Imperial College at NHLI, London UK

In cardiac myocytes several mechanisms may contribute to the increase of cytoplasmic Ca during the action potential (AP). The amount of Ca entry through the sarcolemmal (SL) Ca channels was investigated using the AP clamp technique. Cells were stimulated (0.5 and 0.2 Hz) and voltage-clamped using switch clamp with high resistance microelectrodes. 10µM niflumic acid was added to the superfusate to inhibit Ca-activated Cl current. A typical AP was recorded for each myocyte and used as the voltage command. To calculate the amount of Ca influx via Ca channels, 2mM Cd was rapidly applied before an AP and the remaining current subtracted from the total current obtained during the previous cardiac cycle. Such application of Cd completely abolishes contractions and Ca transients. The amount of SR Ca content was calculated from the caffeine-induced transient inward current. We assume that (1) at steady-state, Ca influx via Ca channels is extruded via Na/Ca exchange; (2) both processes produce inward currents and Cd-sensitive current recorded during the cardiac cycle is the sum of these currents; (3) two thirds of the total current recorded is ascribed to Ca entry through SL Ca channels. In guinea-pig cardiac myocytes stimulated at 0.5 Hz and 0.2 Hz, Ca entry through SL Ca channels during a cardiac cycle was approximately 30% and 50 % of the SR Ca content respectively. In rat myocytes Ca entry via SL Ca channels at 0.5 Hz was approximately 3.5 % of the SR Ca content. In the presence of 500 nM thapsigargin Ca entry via SL Ca channels in guinea-pig cardiac cells was 39 % greater than control suggesting a larger contribution of this mechanism to the Ca transient when the SR is depleted of Ca.

Tu-Pos136

TEMPERATURE AND THAPSIGARGIN SENSITIVITY OF SR Ca TRANSPORT IN TROUT VENTRICULAR MYOCYTES. ((**L. HOVE-MADSEN**, A. LLACH and L. TORT)) Dept. Biologia Celular, Universidad Autonoma de Barcelona, 08193 Cerdanyola, Spain.

The role of SR Ca transport in cardiac myocytes from teleosts is largely unknown. We therefore measured SR Ca transport in permeabilized trout ventricular myocytes using Ca selective electrodes and indo-1 as described previously (*Circ. Res.* 73: 820-828, 1993). Ca uptake measured in the presence of ruthenium red and oxalate was completely inhibited by a saturating dose of the SR Ca pump inhibitor thapsigargin (TG). The maximal Ca uptake rate measured at room temperature was lower than in mammals. However, when titrating the SR Ca pumps with TG the $K_{0.5}$ of 200 pmol/mg cell protein was similar to mammalian values. Lowering the temperature to 5°C, reduced the Ca uptake rate as shown in the figure. At a free [Ca] of 1 µM the Ca uptake at 10 and 5°C was respectively 50 ± 11% and 37± 6% of that at room temperature. The lower Ca uptake rate was not due to an increase in the passive Ca leak from the SR since the leak induced by a saturating dose of TG was unaffected by a temperature change. This suggest that, during relaxation, the trout SR is capable of removing a significant part of the total cytosolic Ca transient. Furthermore, it can accumulate Ca at low temperatures that induce a net SR Ca release in mammalian myocytes.



Tu-Pos137

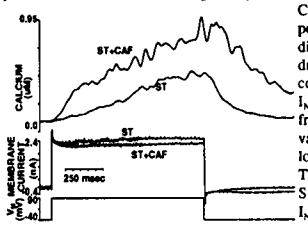
EFFECT OF 2,5-DI-TERT-BUTYLHYDROQUINONE (TBQ) ON RAT VENTRICULAR MYOCYTES. ((L.J. Cook, G.W. Bate, S.C. O'Neill and D.A. Eisner)) Veterinary Preclinical Sciences, The University of Liverpool, PO Box 147, Liverpool, L69 3BX, UK

Efficient systems to elevate intracellular Ca^{2+} and then extrude Ca^{2+} ensure continued cardiac muscle cell contraction. Disruption of this equilibrium leads to either a net gain or loss of Ca^{2+} by the cell. One important ion transport mechanism in this system is the SR Ca^{2+}-ATPase which sequesters Ca^{2+} into the SR. Previous studies have investigated the contribution of the Ca^{2+}-ATPase during a Ca^{2+} transient revealing its role in the removal of $[Ca^{2+}]_i$ during relaxation. In the present study we have used the reversible Ca^{2+}-ATPase inhibitor TBQ to study the Ca^{2+}-ATPase in single rat ventricular myocytes. In support of earlier work investigating the Ca^{2+}-ATPase (Negretti *et al*, 1993, J. Physiol. 468:35-52) TBQ caused a progressive decrease in both the magnitude and rate of decay of the Ca^{2+} transient, and this effect was maximal using 50 μM. In further experiments rat ventricular myocytes were voltage-clamped and loaded with fluo-3 allowing measurements of membrane current, and both cell averaged $[Ca^{2+}]_i$ and local changes in Ca^{2+}. Under resting conditions (-80 mV) application of TBQ (50 μM) caused a transient increase in $[Ca^{2+}]_i$ which was accompanied by an inward current. Subsequent application of caffeine (10 mM-to empty the SR), whilst still in the presence of TBQ, caused a further transient increase in $[Ca^{2+}]_i$ and an inward current, suggesting that this concentration of TBQ fails to completely block the SR Ca^{2+}-ATPase. In comparison to the effects of caffeine the response to TBQ was delayed in onset whilst the rate of recovery of both the $[Ca^{2+}]_i$ transient and inward current were greater. Furthermore, the elevation in $[Ca^{2+}]_i$ by TBQ tended to be due to initiation of a Ca^{2+} wave. This may suggest that TBQ is initiating calcium-induced release of SR calcium. Assuming TBQ removes one source of Ca^{2+} extrusion from the myoplasm (the SR) there are two possible mechanisms which may account for these effects: (1) Ca^{2+} 'leak' from the SR can now trigger CICR, as less can be sequestered by the SR (2) any spontaneous Ca^{2+} release (e.g. a spark) can now initiate CICR, again, as the removal of this local Ca^{2+} from the myoplasm is reduced.

Tu-Pos138

SARCOPLASMIC RETICULUM DIRECTLY BUFFERS CALCIUM INFLUX VIA THE SARCOLEMMAL SODIUM-CALCIUM EXCHANGER AND RAPIDLY MODULATES ITS IMPACT ON CALCIUM RELEASE IN CARDIAC MYOCYTES.
((A.M. Janczewski, H.A Spurgeon, E.G. Lakatta)) LCS, GRC, NIA, NIH, Baltimore, MD 21224. (Spon. by R. G. Hansford)

We developed a protocol permitting simultaneous and quantitative assessment of Ca^{2+} influx via the sarcolemmal Na^+-Ca^{2+} exchange current (I_{NaCa}), and of the sarcoplasmic reticulum (SR) Ca^{2+} loading and release, during a single beat. Cytosolic $[Ca^{2+}]$ ($(Ca^{2+})_i$) transients (CAT) and membrane current were measured in whole-cell-clamped guinea pig ventricular myocytes dialyzed with indo-1 and 10 mM Na^+ at 24°C. Ca^{2+} influx via I_{NaCa} during pulses from -40 to +90 mV, was graded by the pulse duration (100-1000 ms). Rapid, brief exposures to caffeine (CAF) were used to deplete the SR of Ca^{2+} prior to stimulation (ST) and/or to prevent SR Ca^{2+} accumulation during the voltage pulse (ST+CAF) following SR Ca^{2+} depletion. The configuration of the CAT induced by ST+CAF reflects the total, net Ca^{2+} influx via I_{NaCa}, while the CAT induced by ST reflects a portion of Ca^{2+} influx accessing the cytosol directly or via SR release (Fig.). The difference in CATs induced by ST+CAF and ST represents a portion of Ca^{2+} influx via I_{NaCa} which had been directly sequestered by the SR but not released during the depolarization. Thus under these conditions, we find that ≈80% of Ca^{2+} influx via I_{NaCa} is directly buffered by the SR, and that fractional release of accumulated Ca^{2+} is greatly varied (from ≈20 to 50%) by changes in SR Ca^{2+} loading that occur during the same depolarization. The results are consistent with a critical role of the SR in regulation of the impact of Ca^{2+} influx via I_{NaCa} on cardiac E-C coupling.



Tu-Pos139

DUAL EFFECTS OF TETRACAINE ON SPONTANEOUS CALCIUM RELEASE IN VENTRICULAR MYOCYTES ((Sandor Györke, Valeriy Lukyanenko and Inna Györke)) Texas Tech University HSC, Lubbock, TX 79430.

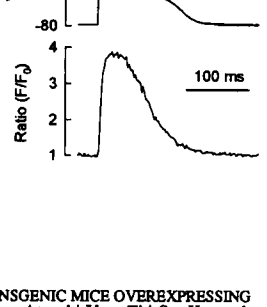
To define the mechanisms of initiation of spontaneous Ca release from the sarcoplasmic reticulum (SR) we investigated the effects of the Ca release blocker, tetracaine (T), on spontaneous local Ca release events (sparks) and propagating Ca waves in Ca-overloaded cardiac myocytes. The effects of T on Ca signals in intact cells were correlated with the effects of the drug on single cardiac SR Ca release channels (CRC) incorporated into lipid bilayers. In rat ventricular myocytes exposed to 10 mM Ca, T at low concentrations (0.25-1.25 mM) exerted clear biphasic effects on spontaneous Ca release. Initial inhibition of Ca sparks and propagating Ca waves was followed by a gradual increase in release activity. In the initial inhibitory phase (1-2 min after addition of T), the frequency of sparks was diminished, and up to a 2-3 - fold decrease in the magnitude of sparks was detected. In the delayed potentiatory phase (> 5 min after addition of T), the frequency, amplitude and duration of sparks all were considerably increased above the control levels. The increase in release activity culminated in a spectra of large scale responses, ranging from large amplitude propagating waves to non-propagating multifocal releases that occurred simultaneously in large areas of the cell. At high concentrations (>1.25 mM), T abolished all forms of spontaneous release without any apparent signs of secondary increase in Ca signals. As indexed by changes in the magnitude of caffeine-induced Ca transients, exposure of the cells to T resulted in a gradual increase in the SR Ca load. In single cardiac CRCs incorporated into lipid bilayers, T reduced the open probability (P_o) in a concentration-dependent manner, without any sign of secondary time-dependent alterations of channel activity during recording periods of 10-15 min. Elevating Ca from pCa 4.8 to pCa 2 on the luminal side of the channel caused an increase in channel activity under control conditions as well as in the presence of various concentrations of T. We conclude that the primary effect of T on SR CRCs is inhibition of channel activity both *in vitro* and *in situ*. The ability of T to reduce spark magnitude indicates that sparks are not due to activation of single channels or non-reducible clusters of channels, implying a multichannel origin of sparks. The paradoxical late potentiation of release by submaximal blocking concentrations of T could be accounted for by a gradual increase in SR Ca load and subsequent activation of the CRCs by Ca inside the SR. These experiments point to activation of release channels by increased luminal Ca as the mechanism responsible for spontaneous Ca release from the SR. Supported by NIH (HL 52620).

Tu-Pos140

REGULATION OF THE $[Ca^{2+}]_i$ DURING ACTION POTENTIAL IN ADULT MOUSE VENTRICULAR MYOCYTES ((T. Shioya & W.J. Lederer)) Departments of Physiology and Molecular Biology and Biophysics, University of Maryland School of Medicine and Medical Biotechnology Center, Baltimore MD, USA

Transgenic mouse models provide powerful tools to investigate cellular and molecular physiology of the heart. However, using transgenic mouse cardiac myocytes in the study of heart cell physiology has not been very successful because of difficulties in preparing single heart cells from adult mice. Here, we describe a new method to make single cells from adult mouse hearts, which gives a consistent yield. Using this preparation, we examined the electrical properties of single mouse ventricular myocytes using the whole-cell clamp technique and the $[Ca^{2+}]_i$ dynamics in these cells using a confocal laser scanning microscope and the fluorescent Ca^{2+} indicator fluo-3. We have identified distinctive electrical features of adult mouse ventricular myocytes at 37 °C; Importantly, the action potential shape was different from that reported in other small rodents.

The action potentials had 1) a negative plateau that centers on potentials around -40 mV, 2) a clear initial notch that precedes the plateau and 3) a duration of 148 ± 37 ms (mean ± SD) to 90% repolarization. The time-course of $[Ca^{2+}]_i$ transient had a close relation to that of the action potential. We provide evidence that the Na^+/Ca^{2+} exchanger plays an important role in coupling between the $[Ca^{2+}]_i$ transient and the action potential during the plateau. We will discuss novel features of mouse E-C coupling that involves the Na^+/Ca^{2+} exchanger. (Supported by the NIH and the Maryland Affilliate of the American Heart Association).



Tu-Pos141

Ca^{2+}-ACTIVATED Cl^- CURRENT CAN BE TRIGGERED BY Na^+ CURRENT-INDUCED SR Ca^{2+} RELEASE IN RABBIT VENTRICULAR MYOCYTES ((H. Sun, S. Nattel, and N. Leblanc)) Montréal Heart Institute, 5000 Bélanger St., Montréal (Québec) H1T 1C8, Canada.

The Ca^{2+}-activated Cl^- current ($I_{Cl,Ca}$) contributes to the repolarization of the cardiac action potential under physiological conditions. $I_{Cl,Ca}$ is known to be primarily activated by Ca^{2+} release from the sarcoplasmic reticulum (SR). L-type Ca^{2+} current ($I_{Ca,L}$) represents the major trigger for Ca^{2+} release in the heart. Recent evidence, however, suggests that Ca^{2+} entry via reverse-mode Na^+/Ca^{2+} exchange promoted by voltage and/or Na^+ current (I_{Na}) may also play a role. The purpose of this study was to test the hypothesis that $I_{Cl,Ca}$ can be induced by I_{Na} in the absence of $I_{Ca,L}$. Macroscopic currents and Ca^{2+} transients were measured using the whole-cell patch clamp technique in rabbit ventricular myocytes loaded with Indo-1. 10 μM nicardipine abolished $I_{Ca,L}$ at a holding potential of -75 mV as tested in Na^+-free external solution. In the absence of $I_{Ca,L}$, a 4-aminopyridine-resistant transient outward current was recorded in 44 cells accompanying a phasic Ca^{2+} transient. Both the current and Ca^{2+} transient were abolished by 10 μM ryanodine (n = 3), 10 mM caffeine (n = 6), 30 μM tetrodotoxin (n = 9), or removal of extracellular Ca^{2+} ions (n = 5). 100 μM niflumic acid inhibited the current without affecting the Ca^{2+} signal (n = 6). Double-pulse experiments showed that the transient outward current was mainly carried by Cl^- (n = 4). These properties are consistent with those of $I_{Cl,Ca}$ previously described in mammalian cardiac myocytes. We conclude that (1) $I_{Cl,Ca}$ can be recorded in the absence of $I_{Ca,L}$, (2) I_{Na}-induced SR Ca^{2+} release mechanism is also present in the rabbit heart and may play a physiological role in activating the Ca^{2+}-sensitive membrane Cl^- conductance. Supported by MRC,HSFQ and F.R.S.Q.

Tu-Pos142

EXCITATION CONTRACTION COUPLING IN TRANSGENIC MICE OVEREXPRESSING Na-Ca EXCHANGER (NCX1). ((Masafumi Kasama, Atsushi Yao, Zhi Su, Kenneth Philipson, William Barry and **John Bridge**)) CVRTI, University of Utah, Salt Lake City, UT 84112 (Spon. by Kenneth Spitzer)

We compared triggered contractions in myocytes from transgenic mice overexpressing NCX1 with wild type mice. We first voltage clamped cells from wild type and transgenic animals at 23°C with single micro-electrodes containing: 10 mM Na, and 100 nM Ca buffered with 14 mM EGTA. Li replaced K and the pH was buffered to 7.1 with Hepes. Cells were superfused with a modified Hepes buffered Tyrode solution containing 1.0 mM Ca. We activated reverse Na-Ca exchange by rapidly removing extracellular Na. The Ni sensitive exchange current was approximately three times greater in the transgenics. We then measured shortening voltage and Ca current (I_{Ca}) voltage relationships in both transgenic and wild type myocytes under voltage clamp with pipettes containing 10 mM Na and 134 mM K buffered to pH 7.1 with Hepes. In the wild type both I_{Ca} vs voltage and shortening vs voltage were bell shaped. In the transgenics I_{Ca} vs voltage was bell shaped but shortening vs voltage was sigmoid so that shortening did not decline significantly with voltage at positive potentials. This suggests that some other process bedsides I_{Ca} determines the relationship between shortening and voltage in transgenics. Wild type cells were field stimulated and Ca transients were measured in Fluo-3 AM loaded cells. Steady state application of 5μM nifedipine to block I_{Ca} resulted in abolition of 90% of the Ca transient in wild type cells but only reduced the Ca transients by approximately 70% in transgenics. We conclude that in wild type cells from mouse triggered SR Ca release by Na-Ca exchange is negligible but becomes significant in transgenics.

Tu-Pos143

SODIUM AND CALCIUM SIGNALS RECORDED WITH TWO-PHOTON EXCITATION CONFOCAL MICROSCOPY

((E. Niggli* and L. A. Blatter#)) Depts. Physiology, *University of Bern, Bern, Switzerland, #Loyola University, Chicago.

In cardiac muscle, I_{Ca} and I_{Na} contribute to subcellular Ca signalling and ec-coupling. The evidence linking I_{Na} to changes of intracellular [Ca] via the Na-Ca exchange is still rather indirect. We used two-photon excitation of the fluorescent ion indicators SBFI and Indo-1 to record changes in $[Na]_i$ and $[Ca]_i$. Fluorescence was imaged with a confocal microscope. Cultured neonatal adult cardiac myocytes were loaded with the appropriate ion indicator by exposure to the AM-ester. Rapid superfusion of the cells with depolarizing or caffeine solutions induced homogeneous changes of the Indo-1 ratio. Maintaining the depolarization resulted in spontaneous repetitive Ca signals (Ca waves and Ca "sparks"). Caffeine superfusion was accompanied by a partial quench of the Indo-1 fluorescence. Removal of extracellular Na led to a rapid ($t_{1/2}$ < 5 s) fall in $[Na]_i$, as recorded from SBFI loaded cells. Surprisingly, a significant subcellular Na-gradient extending from the surface to the cell interior and nucleus was present for up to 3 s. These results show that intracellular [Na] and [Ca] can be measured and imaged with two-photon excitation of ion indicators requiring UV-illumination. More importantly, the transient subcellular gradients of $[Na]_i$ indicate the possibility of local Na accumulation in the cytosol and presumably also in the subsarcolemmal space. This finding is consistent with the hypothesis that Na influx during I_{Na} subsequently could activate Na-Ca exchange in the Ca influx mode. (Supported by SNF and Schweppe Foundation Chicago).

Tu-Pos144

Dual Interactions of Extracellular Protons with Na-Ca Exchanger.

((M. Egger and E.Niggli)) Dept. Physiology, University of Bern, Switzerland.

The electrogenic Na-Ca exchange (stoichiometry 3 Na^+: 1Ca^{2+}) can be modulated by intracellular constituents, e.g. ATP and protons (pH_i). In this study we addressed the question how extracellular protons (pH_o) interact with the Na-Ca exchange under fixed physiological pH_i at 7.2. In order to investigate the extracellular pH effects (range: pH_o 4.0 up to pH_o 10), the Na-Ca exchange current (I_{NaCa}) was recorded from adult guinea-pig cardiac ventricular myocytes under voltage clamp conditions in the whole-cell configuration in the presence of 20 µM ryanodine. Rapid intracellular Ca^{2+} concentration jumps were generated by flash-photolysis of caged Ca^{2+} (DM-nitrophen) to activate I_{NaCa}. Under control conditions (pH_o 7.2) the Ca^{2+} concentration jump induced I_{NaCa} up to 265 pA at a fixed membrane potential of -40 mV. Fast ($t_{1/2}$<500 ms) changes of the extracellular pH were performed using a gravity driven superfusion system. The maximum I_{NaCa} was found at pH_o ≈7.5. Reducing the pH_o to 4.0 was accompanied by a decrease of I_{NaCa} to about 10%. However, I_{NaCa} was also inhibited in the same order of magnitude by increasing pH_o to 10. Block of I_{NaCa} induced by alkalinization and acidification was completely reversible. These findings indicate that at least two mechanisms exist for an interaction between extracellular protons and the Na-Ca exchanger. Although the peak I_{NaCa} was reduced at high pH_o the time-constant of current decay remained nearly unchanged and the integrated charge moved during I_{NaCa} decreased. This observation suggests that the rate of Ca^{2+} transport may be less affected than the charge moved per Ca^{2+}, a notion that would also be consistent with a change in the stoichiometry of the Na-Ca exchange cycle at elevated pH_o. (Supported by SNF).

Tu-Pos145

Ni^{2+} Uptake Mediated by the Human Cardiac Na-Ca Exchanger.

((M. Egger, A. Ruknudin[a], E. Niggli, D.H. Schulze[a] and W.J. Lederer[a])) [a]Dept. Physiology and [a]Dept. Microbiology, University of Maryland, Baltimore, USA; Dept. Physiology, University of Bern, Switzerland. (Spon. by J. Engel)

Extracellular $[Ni^{2+}]$ ($[Ni^{2+}]_o$) can block the electrical activity of the Na-Ca exchanger in cardiac myocytes by an unknown mechanism. In order to investigate functional aspects of $[Ni^{2+}]_o$ the cDNA of the human cardiac Na-Ca exchanger was subcloned into a baculovirus expression vector which was used to infect an insect cell line derived from ovarian cells of *Spodoptera frugiperda* (Sf9 cells). Na^+- dependent Ca^{2+}- transport of the expressed protein was assessed with a confocal microscope using the fluorescent Ca^{2+} indicator Fluo-3. Transmembrane Ca^{2+}- movements via the Na-Ca exchanger were only observed in transfected Sf9 cells. In the presence of 145 mM $[Na^+]_o$ the application of 10 mM $[Ni^{2+}]_o$ induced a remarkable decrease of the Fluo-3 (AM or salt) signal in transfected cells. In-vitro measurements of Fluo-3 showed that $[Ni^{2+}]$ quenches the fluorescence signal by about 64%. Therefore, the observed reduction of Fluo-3 fluorescence in Sf9 cells may result from Ni^{2+} uptake via Na-Ca exchange. Furthermore, with $^{45}Ca^{2+}$- flux studies we found that $^{45}Ca^{2+}$- influx and $^{45}Ca^{2+}$- efflux were inhibited by $[Ni^{2+}]_o$ (3 mM, $k_{1/2}$=0.66 mM) in transfected cells. Surprisingly, in 145 mM $[Na^+]_o$ significant $^{63}Ni^{2+}$- uptake was observed amounting to about 45 pmol mg^{-1} (12 min, 27°C) that was blocked with DCB (100 µM) to about 60%. In contrast, in the absence of $[Na^+]_o$ the $^{63}Ni^{2+}$- influx via Na-Ca exchange was ≈ 15 pmol mg^{-1} but insensitive to DCB. The $^{63}Ni^{2+}$- influx was dependent on the $[Ca^{2+}]_o$ and $[Ni^{2+}]_o$. These results indicate that Na^+-dependent and Na^+-independent electroneutral transport stoichiometries may be responsible for $[Ni^{2+}]_o$ uptake via the Na-Ca exchanger (e.g. 1 Ca^{2+}:1 Ni^{2+} and/or 2 Na^+:1 Ni^{2+} exchange). (Supported by NIH, SNF)

Tu-Pos146

ADRENERGIC MODULATION OF SODIUM-CALCIUM EXCHANGE IN SLOW SKELETAL MUSCLE FIBERS OF THE FROG. ((X. Trujillo, F. Andrade, C. Vasquez and M. Huerta)) Centro Universitario de Investigaciones Biomédicas. Universidad de Colima. Apdo. Postal 11; 28000 Colima, Col. México.

Skeletal muscle possess two different fibers: twitch and tonic or slow fibers. Slow fibers, in contrast to twitch fibers, can maintain tension during prolonged depolarization. The effects of adrenaline on sodium-calcium exchange were investigated in the slow muscle fibers. The sodium-calcium exchange was studied by contractile response in a solution where the extracellular sodium was withdrawn. Isometric tension was recorded from slow bundles of cruralis muscle from Rana pipiens. Normal solution was (mM): NaCl, 117.5; KCl, 2.5; CaCl2, 1.8. pH was adjusted to 7.4 with imidazol-chloride. Na-free solutions were prepared by replacing NaCl with an osmotically equivalent amount of TEA-Cl. Na-free solutions contained d-tubocurarine (50 µM) to prevent a possible acetylcholine-like effect. Experiments were done at room temperature (20-22 °C). Application of adrenaline (5 to 10 µM) to the bath abolished the tension evoked by sodium withdrawal in seven bundles of slow fibers. This effect was reversible. The present results suggest that adrenaline modulates tension evoked by sodium withdrawal. It could reflect a modulation of sodium-calcium exchange in these slow fibers.

Tu-Pos147

ANGIOTENSIN II EXERTS BIDIRECTIONAL EFFECTS ON MYOCARDIAL CONTRACTILITY.

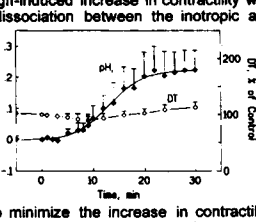
((**K. Harada**, O. Kohmoto, T. Takahashi)) Internal Medicine II, University of Tokyo, Tokyo 113, Japan

By using whole-cell patch-clamp technique, we studied the direct effects of angiotensin II (Ang II) on the L-type calcium channel current (I_{Ca}) and the cell shortening (CS) in isolated guinea pig ventricular myocytes. Ang II (100 nM) exerted an AT_1 receptor-mediated augmentation in the CS (119.6±6.3 % n=11) with a significant attenuation of run-down of the I_{Ca} (99.6±5.3 % vs. 88.9±2.1 % at 5 minutes) (5 mM HEPES in the pipette). This was inhibited by TA606A (1 µM), AT_1 receptor antagonist; 5-(N,N-Dimethyl)amiloride (100 µM); calphostin C (100 nM); or rottlerin (10 µM). The effect on the I_{Ca} was not inhibited by increases in $[Ca^{2+}]_i$ buffering capacity, which was consistent with PKCδ activation. In contrast, with 30 mM HEPES in the pipette, Ang II induced an AT_1 receptor-mediated inhibition in the CS (67.1±11.8 %, n=6) and the I_{Ca} (66.7±9.6 %, n=6). Both values were significantly less than those with 5 mM HEPES in the pipette. This effect was inhibited by TA606A, but not by rottlerin (up to 50 µM). Moreover, suppression of the I_{Ca} was blocked by 10 mM EGTA in the pipette. These results suggest the co-existence of AT_1 receptor-mediated positive inotropism via PKCδ -Na^+/H^+ exchanger activation ($[Ca^{2+}]_i$- independent pathway) and AT_1 receptor-mediated, $[Ca^{2+}]_i$ - dependent negative inotropism (probably through PLA_2 activation).

Tu-Pos148

ANGIOTENSIN II-INDUCED INCREASE IN INTRACELLULAR pH IS NOT THE PREDOMINANT MECHANISM OF THE POSITIVE INOTROPIC EFFECT OF THE DRUG. ((**A. Mattiazzi**, G. Pérez, B. Alvarez, I Ennis, M. Vila Petroff, M.C. Camilión de Hurtado and H.E. Cingolani)). Centro de Investigaciones Cardiovasculares, Facultad de Medicina, La Plata, Argentina.

We examined the mechanism of the positive inotropic effect of Angiotensin II (AngII) in 12 cat papillary muscles. The muscles were loaded with BCECF-AM for simultaneous measurement of intracellular pH (pH_i) and developed tension (DT). In agreement with our previous findings (Biophys J. 70:A54, 1996), in HEPES buffer AngII induced a significant increase in DT (224 ± 65%), before any detectable increase in pH_i. The increase in pH_i occurred when the AngII-induced increase in contractility was already fading. Compelling evidence of the dissociation between the inotropic and alkalinizing effects of AngII is shown in the Figure. In these experiments extracellular Na^+ was substituted for Li^+ to preclude the function of Na^+/Ca^{2+} exchanger without affecting the Na^+/H^+ antiport (Li^+ can be driven by the Na^+/H^+ antiport but it cannot be transported by the Na^+/Ca^{2+} exchanger). Replacement of Na^+ by Li^+ virtually suppressed the positive inotropic effect of AngII without affecting the pH_i increase (n=5). Similar results were obtained in 3 experiments in which Ca^{2+} was decreased to minimize the increase in contractility produced by Na^+ removal. However, in these muscles, the positive inotropic response to an increase in extracellular Ca^{2+} was present. Conclusions: The increase in pH_i was not the predominant mechanism of AngII-induced positive inotropism, as previously suggested (J Physiol 480:203-215, 1994). Other mechanisms, like AngII-induced activation of Na^+/Ca^{2+} exchanger (J Mol Cell Cardiol 28:11-17, 1996) may play a significant role in this effect.



Tu-Pos149

CALORIMETRY MEASUREMENTS IN THE ISCHEMIC HEART. ((**J.E. Ponce-Hornos**, A. Mazzadi, P. Bonazzola and A.C. Taquini)) Inst. de Invest. Cardiol. Fac. de Medicina and Cat. de Biofísica Fac. de Odontología UBA. M.T. de Alvear 2270 (1122) Bs. As. Argentina (Spon. S. Alonso)

A calorimetric method has been used to measure myocardial heat production simultaneously with mechanical parameters during 45 min global ischemia. The muscles (Langendorff perfused, rat hearts) were electrically paced at 1.5 Hz and left ventricular isovolumic pressure development (PD) and heat production were simultaneously measured. The experiments were performed under 25, 30 and 35 °C. The time course of the power released (H) during the ischemic intervention showed an initial and fast decrease of about 12 min duration, followed by a slower decrease that lasts even after PD turns non-detectable. Under 30 and 35 °C (but not under 25 °C) this slow phase is accelerated after about 28 and 18 min of ischemia respectively. For all three temperatures the time course of the PD/H ratio (used as an index of muscle economy) decreased under ischemia following a two exponential type function for about 100 s. After this initial period the economy decreased at a higher rate. Muscle reperfusion for 45 min showed a recovery of PD of 94.4%, 53.4% and 9.5% for 25, 30 and 35 °C conditions respectively, suggesting an association between the capability of recovering the mechanical function with the decrease in H observed at high temperature. This decreasing in H could be an indication of permanent damage.

Supported by grant OD 022 UBA

Tu-Pos150

DEUTERIUM OXIDE (D_2O) REDUCES L-TYPE CA^{2+} CURRENT (I_{Ca}) IN SINGLE RAT VENTRICULAR MYOCYTES

((K. Hongo, F.Brette* & **E.White**)) Department of Physiology University of Leeds, Leeds LS2 9NQ. U.K.*Laboratoire Physiologie des Cellules Cardiaques et Vasculaires, CNRS EP41, Faculte des Sciences 37200 Tours, France.

D_2O reduces the contraction of feline ventricular myocytes, possibly by causing proliferation of microtubules, which impedes sarcomere motion (Tsutsui et al., 1994, *Science* 260, 682-686). However in rat ventricular myocytes the negative inotropic effect of D_2O was not reduced by prior incubation with colchicine, an agent known to disrupt microtubules (Brette et al., 1996 *J. Physiol.* 491.P, 157P). Another possible mechanism for the negative inotropic effect of D_2O is a reduction of Ca^{2+} influx via I_{Ca}. To test this possibility cells were whole cell patch clamped and I_{Ca} evoked by step depolarizations from -40 mV to 0 mV for 200 ms at 0.33 Hz. Exposure to a bicarbonate-buffered Tyrode solution in which 50% of the H_2O was replaced by D_2O (50% D_2O) resulted in a decrease in the amplitude of I_{Ca} in all cells tested (from 0.72 ± 0.09 nA to 0.52 ± 0.09 nA, mean ± s.e.m. n = 6, $p < 0.05$ paired t-test). The effects on I_{Ca} were reversible upon washing. In 3 cells voltage-clamped using the perforated patch technique (with amphotericin B as the pore forming agent), exposure to 50% D_2O reduced I_{Ca} by 34 ± 4%. Contraction was simultaneously recorded in these cells and decreased by 46 ± 3%. Our observations may indicate that a reduction in I_{Ca} contributes to the negative inotropic effect of D_2O.

Supported by the British Heart Foundation.

Tu-Pos151

GLUTATHIONE IS A CO-FACTOR FOR H_2O_2-MEDIATED STIMULATION OF Ca^{2+}-INDUCED Ca^{2+} RELEASE IN CARDIAC MYOCYTES.

((**Y.J. Suzuki**, L. Cleemann and M. Morad)) Department of Pharmacology, Georgetown University Medical Center, Washington, DC 20007. (Spon. by L. Packer)

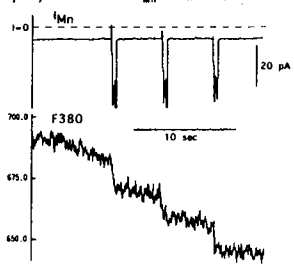
Reactive oxygen species are known to cause attenuation of cardiac muscle contraction in ischemia-reperfusion injury. This attenuation is usually preceded by transient augmentation of twitch amplitude as well as $[Ca^{2+}]_i$. The present study examines the role of endogenous antioxidant, glutathione in the mechanism of hydrogen peroxide (H_2O_2)-mediated augmentation of Ca^{2+} release from the sarcoplasmic reticulum (SR). Whole cell-clamped single rat ventricular myocytes were dialyzed with the Cs^+-rich internal solution containing 0.2mM cAMP, 0.2mM fura-2 and 2mM glutathione (reduced form). After equilibration of the dialysis solution with the cytosolic compartment, I_{Ca}-induced Ca^{2+} release from the SR was monitored. Superfusion with H_2O_2 (0.1-1mM) for 20 sec resulted in inhibition of I_{Ca} but enhancement of Ca^{2+}-transients, leading to transient (3-4 min) increase in efficacy of I_{Ca}-induced Ca^{2+} release ($\Delta[Ca^{2+}]_i/I_{Ca}$) in 71% of myocytes (n=7). H_2O_2 (0.1mM) caused 50% increase (from 20 to 30 nM pF pA^{-1}) while 1mM caused 3-fold increase (from 13 to 39 nM pF pA^{-1}) in the efficacy of I_{Ca}-induced Ca^{2+} release. When glutathione was excluded from the patch solution, the stimulation of I_{Ca}-induced Ca^{2+} release occurred only in a minority (20%, n=10) of the myocytes. H_2O_2-exposure did not increase the basal $[Ca^{2+}]_i$, suggesting that the mode of action is not to inhibit the SR Ca^{2+}-ATPase or to induce passive leak. H_2O_2-mediated stimulation of I_{Ca}-induced Ca^{2+} release was also observed in myocytes dialyzed with dithiothreitol (0.5 mM) which has more negative redox potential compared to glutathione. Therefore, reduced thiols (i.e. glutathione) play a physiological role in H_2O_2-mediated stimulation of I_{Ca}-induced Ca^{2+} release. We propose that the mechanism of glutathione action to support H_2O_2-mediated stimulation of I_{Ca}-induced Ca^{2+} release involves the formation of thiol-disulfide interchange within the ryanodine receptor or associated molecule. (Supported by NIH HL16152 and GUMC Research Starter Grant)

Tu-Pos152

L-TYPE MANGANESE CURRENT AND CURRENT-DEPENDENT QUENCHING OF FURA-2 SIGNALS IN RABBIT VENTRICULAR CELLS.

((**H. Masumiya**, M. Tateyama and R. Ochi)) Department of Physiology, Juntendo University School of Medicine, Hongo, Tokyo 113, Japan

We recorded Mn^{2+} currents (I_{Mn}) from whole-cell clamped rabbit ventricular myocytes and fluorescence signals from those loaded with fura-2. Test solutions contained Mn^{2+} and NMDG replacing Na^+ and Ca^{2+}. Depolarization pulses induced I_{Mn} in the presence of 5 and 20 mM Mn^{2+}. Nitrendipine (1μM) suppressed I_{Mn} but BAY-K 8644 (1μM) increased I_{Mn} only in the presence of 5 mM Mn^{2+}. Forskolin (10 μM) increased I_{Mn} to 211.6 + 17.5%. These modulations by drugs indicate that Mn^{2+} ions permeate through L-type Ca^{2+} channels. The figure shows that I_{Mn} elicited by step pulses to 10 mV with a duration of 400 ms in the presence of 20 mM Mn^{2+} produced a rapid quenching of fura-2 fluorescence. Mn^{2+} current could be a useful tool to study the mechanism of depolarization-induced Ca^{2+}, increase.



Tu-Pos153

INHIBITION OF GLYCOLYSIS BY IODOACETIC ACID (IAA) ALTERS Ca^{2+} RELEASE AND INHIBITS Ca^{2+} REMOVAL IN RAT VENTRICULAR MYOCYTES.

((James S.K. Sham, Li-Hua Deng, and Lewis C. Becker)) Department of Medicine, The Johns Hopkins Medical Institutions, Baltimore, MD 21224.

In cardiac myocytes, glycolytic ATP is known to fuel and modulate sacrolemmal ion transporters, such as Na^+,K^+-ATPase, Ca^{2+}-ATPase, and ATP-sensitive K^+ channels. A recent study showed that the entire chain of glycolytic enzymes from aldolase onward is present in vesicles of sarcoplasmic reticulum (SR) of cardiac and skeletal muscle, and glycolytic ATP is preferentially utilized by SR Ca^{2+}-ATPase (Xu et al. Circ. Res. 77:88-97, 1995). Therefore, we examined whether inhibition of glycolysis alters Ca^{2+} uptake and release in intact rat ventricular myocytes, using whole-cell voltage-clamp and Indo-1 fluorescence techniques. Myocytes were superfused with modified Tyrode's solution containing 10 μM tetrodotoxin to block Na^+ channels, and dialysed with a Cs-based internal solution containing 5 mM pyruvate, 1 mM KADP, 1 mM $MgCl_2$, and 1 mM KH_2PO_4 to provide substrates for oxidative phosphorylation, and 200 μM cAMP to optimize SR Ca^{2+} uptake. I_{Ca} and Ca^{2+} transients were activated by depolarizing pulses from -60 mV to a test potential of 0 mV. Inhibition of glyceraldehyde-3-phosphate dehydrogenase with low concentration (0.1 mM) of IAA caused a gradual reduction in peak I_{Ca}. Systolic $[Ca^{2+}]$ increased from 0.76±0.08 μM to 1.57±0.42 in the first 2-3 min of IAA exposure, followed by an abrupt reduction, whereas diastolic $[Ca^{2+}]$ increased progressively and was associated with cell contracture. IAA also caused gradual but marked reduction in the rate of Ca^{2+} removal, determined by fitting the declining phase of Ca^{2+} transients with a single exponential. The time-constant was significantly prolonged from 126±16 ms under control condition to 248±17 ms ($P<0.05$) in the presence of IAA. The changes in both I_{Ca} and Ca^{2+} transients happened more rapidly with 1 mM IAA. These results suggest that inhibition of glycolysis by IAA has multiple effects on I_{Ca}, Ca^{2+} release and uptake. Since SR Ca-ATPase is responsible for >80% of total Ca^{2+} removal in rat ventricular myocytes, the profound inhibition of Ca^{2+} removal by IAA is, therefore, consistent with the idea that glycolytic ATP may play an important role in fueling Ca^{2+} sequestration by SR Ca^{2+}-ATPase.

Tu-Pos154

CYTOSKELETON INVOLVEMENT IN EXCITATION-CONTRACTION COUPLING IN RAT VENTRICULAR CARDIOMYOCYTES.

((**V.A. Maltsev** and A.I. Undrovinas)) Henry Ford Heart and Vascular Institute, Detroit, MI 48202.

We investigated effects of cytochalasin D (CytD), a disrupter of F-actin on the key processes consecutively involved in excitation-contraction coupling, namely: action potentials (APs), L-type Ca current (I_{CaL}), Ca transients, and cell contractions. Parameters were measured in rat ventricular cardiomyocytes at 37°C by perforated and whole-cell patch-clamp, fluo-3 Ca indicator, and an edge movement detector, respectively. Cells were preincubated in CytD for at least 4 h. The resting potential, overshoot and duration of APs, as well as maximum density of I_{CaL} changed insignificantly (20 uM of CytD; n=10 for each parameter). CytD slowed decay and rising phase of Ca transient, but the amplitude of Ca transient and resting $[Ca]_i$ did not change (Table). However, contractions in the CytD treated cells were completely abolished thus indicating an uncoupling of excitation and contraction. The contraction block occurred within 5 min. after CytD was added and the effect was partially reversed on washout. A half contraction block occurred at 0.8 uM with relaxation phase being significantly slowed as characterized by the time constant of exponential fit to relaxation (τ, see Table). We conclude that sarcoplasmic reticulum function and myofibrilar performance are related to F-actin-based cytoskeleton.

Table. Parameters are shown as mean±SEM (*, †, ¶, $p < 0.01$)

Cells	Relaxation		Resting	Parameters of Ca transient			
	τ (ms)	n	$[Ca]_i$ (nM)	τ_{decay} (ms)	τ_{rise} (ms)	amplitude (nM)	n
Control	41±3*	22	91±11	28.1±1.3†	3.6±0.2¶	424±38	28
CytD	96±7*	10	89±7	47.3±2.8†	5.1±0.6¶	442±51	20

Tu-Pos155

THE SLOWING OF THE RATE OF DECREASE OF THE INTRACELLULAR FURA-2 RATIO (R) SIGNAL DURING TWITCH RELAXATION IN RAT TRABECULAE. ((M.F. Patterson, Y. Jiang, and F.J. Julian)) Department of Anesthesia, Brigham & Women's Hospital, Boston, MA 02115.

We have previously reported (Julian et al., Biophys. J. A294, 1992), during relaxation in single frog skeletal muscle fibers loaded with fluo-3, the presence of a transient rise in the fluorescence signal coincident with the onset of the rapid phase of relaxation. It was shown that motion artifact was highly unlikely to cause this because fiber motion was not observed during relaxation by direct visual observation through a high power light microscope. We now report similar findings in cardiac muscle. Very small trabeculae (≈3mm long, ≈150μm wide and ≈50μm thick) were isolated from the right ventricle of rats. In a key step, the ends of a trabecula were glued to wires using cyanoacrylate tissue adhesive. We have previously shown (Julian et al., Circ. Res. 49, 1300, 1981) that this method of attachment significantly decreases end compliance as demonstrated by flash photography and use of markers and spot-follower apparatus. Following gluing, the trabeculae were loaded iontophoretically through micropipettes with fura-2 salt to a level about 3x the autofluorescence as previously described (Backx & ter Keurs, AJP 264, H1098, 1993). The trabeculae were then immersed in an oxygen-rich, artificial bathing medium and stimulated electrically to produce twitch contractions at 0.2Hz at 25°C. Prior to loading with fura-2 salt, non-dye associated fluorescence (autofluorescence) was measured using photon counting and scanning from wavelengths 300-450nm (emission at 510nm), together with analog measurements at 345nm and 380nm (excitation wavelengths for fura-2). Throughout the experiments, the isometric force response was recorded as well as the fura-2 emission fluorescence at the excitation wavelengths. Fluorescence records were corrected by subtracting autofluorescence, and then R was formed by dividing the 345nm signal by the 380nm fluorescence signal. In response to electrical stimulation of the preparation, the R signal rose rapidly to reach a peak well before the twitch force. Thereafter, the R signal decayed smoothly to reach low levels as the twitch force decreased in the so-called linear phase of relaxation. At the onset of the much faster phase of force relaxation, the R signal characteristically changed from a steady decay to a transient slower or rising phase before resuming the steady decay to reach baseline levels as shown previously (Patterson and Julian, Biophys. J. A54, 1996). From above, the transient slowing of R should signal a rise in myoplasmic [Ca^{2+}] independent of motion artifact. Nevertheless, the absence of significant muscle motion during this phase of R was confirmed in the following way. Flash photographs (duration ≈1ms) of trabeculae were taken at various times during the twitch contraction and during rest, and revealed good striation patterns caused by the regular distribution of sarcomeres (Julian and Sollins, Circ. Res. 37, 299, 1975). The striation pattern during the course of the transient rise in R was stable and regular thus ruling out motion artifact as a significant complicating factor. In summary, we show here that during the transition from slow to rapid phase of force decay in a cardiac muscle twitch there is a transient rise in a signal reporting intracellular [Ca^{2+}], as in skeletal muscle. The rise of R is most likely due, also as in skeletal muscle, to a burst of Ca^{2+} released from myofibrillar Ca-binding sites consequent to the onset of synchronized cross-bridge cycling, which enhances release of Ca^{2+}, during non-uniform relaxation. Supported by NIH GM-48078.

Tu-Pos156

NEGATIVE INOTROPIC EFFECTS ON TWITCH PARAMETERS FROM RAT TRABECULAE CAUSED BY EITHER HALOTHANE OR LOW EXTRACELLULAR [Ca^{2+}] ARE MIRRORED IN THE FURA-2 RATIO (R) SIGNAL. ((Y. JIANG, M. F. PATTERSON AND F. J. JULIAN)) Department of Anesthesia, Brigham & Women's Hospital, Boston, MA 02115.

The mechanism(s) of action of various vapor anesthetics to cause significant side effects such as depression of cardiac muscle contraction remains an important subject. We have previously reported here (Julian et al., Biophys. J. V57, 167a, 1990) in cardiac muscle that halothane has a comparable negative inotropic effect on lightly loaded speed of shortening and peak force to that produced by lowering extracellular [Ca^{2+}]. It is the aim of the work reported here to discover whether, by use of fluorescent dye indicators signaling intracellular [Ca^{2+}], changes in the intracellular Ca^{2+} transient (ICT) following stimulation correlate with changes in the twitch force observed after treatment with the above two negative inotropic interventions. Very small trabeculae (≈3mm long, ≈150μm wide and ≈50μm thick) were isolated from the right ventricle of rats. In a key step, the ends of a trabecula were glued to wires using cyanoacrylate tissue adhesive (Julian et al., Circ. Res., V49, p1300, 1981). Following gluing, the trabeculae were loaded iontophoretically through micropipettes with fura-2 salt to confine the dye to the myoplasmic compartment to a level about 3x the autofluorescence as previously described (Backx and ter Keurs, Am. J. Physiol.,V264, H1098, 1993). The trabeculae were then immersed in an oxygen-rich, artificial bathing medium ([Ca^{2+}]=2mM, 25°C) and stimulated electrically to produce twitch contractions at 0.1Hz. Prior to loading with fura-2 salt, autofluorescence was measured using photon counting and scanning from wavelengths 300-450nm (emission at 510nm), together with analog measurements at 345nm and 380nm (excitation wavelengths for fura-2). Throughout the experiments, the isometric force response was recorded as well as the fura-2 emission fluorescence. Fluorescence records were corrected by subtracting autofluorescence, and then R was formed by dividing the 345nm signal by the 380nm fluorescence signal. This made it possible to display the time-course of both the twitch force response and the synchronized R signal. In response to electrical stimulation in control contractions ([Ca^{2+}]$_o$= 2mM), the R signal rose rapidly to reach a peak well before the twitch force. Thereafter, for the most part, the R signal decayed to reach baseline levels after the twitch force. Concentrations were chosen for halothane (≈0.5mM) or low extracellular [Ca^{2+}] (0.5mM) that depressed peak twitch force by about 75%. Importantly, corresponding similar effects were also observed in the ICT produced by each treatment in that the amplitude of the ICT in each case was depressed. It is reassuring that depressant effects on force produced by two different negative inotropic agents can be correlated with changes in the ICT. Otherwise, it would be necessary to postulate a very loose coupling between the ICT and force generation. Supported by NIH GM-48078.

Tu-Pos157

THE EFFECTS OF LY 368052 ON CONTRACTION IN ISOLATED CARDIAC MYOCYTES. ((E. McCall & M.I. Steinberg)), Cardiovascular Research, Lilly Research Laboratories, Indianapolis, IN 46285.

The positive inotropic effect of BDF 9148 has been well-documented in a variety of cardiac tissues (Ravens *et al.*, 1995. *Cardiovasc. Drug Rev.* 13: 260-274). LY 368052 is one of several inotropes which are structurally related to 9148. The aim of this study was to determine the inotropic activity of LY 368052 and to try to identify the mechanisms underlying this effect. All experiments were carried out using freshly isolated, adult rat ventricular myocytes, with cell contractility measured in response to regular field stimulation (steady-state, SS) at 0.1-1 Hz and on rapid caffeine application (caffeine contractures, CafC). Exposure to LY 368052 led to a concentration-dependent positive inotropic effect (peak SS contraction), with an EC_{50} value of 0.5 x 10^{-9} M at 0.1 Hz. The effect was also frequency-dependent, with greater inotropy shown at lower stimulation frequencies, e.g., exposure to 0.5 nM LY 368052 increased SS contraction over two-fold at 0.1 Hz whilst there was no effect at 1 Hz. The inotropic effect of LY 368052 was not accompanied by an increase in SS SR Ca content, e.g., CafC amplitudes at both 0.1 and 0.3 Hz were not different in the presence and absence of 0.5 nM LY 368052. The lack of effect on CafC amplitudes suggests that LY 368052 does not alter myofilament Ca sensitivity. The inotropic effect of LY 368052 may be mediated by an increase in the amount of Ca released from the SR during E-C coupling or increased Ca influx into the cell, which could activate the myofilaments directly.

Tu-Pos158

THE EFFECTS OF 4-AMINOPYRIDINE ON CONTRACTION AND SR Ca^{2+} CONTENT IN MAMMALIAN VENTRICULAR MUSCLE
((Simon M. Harrison, Zhao-Kang Yang, Nick Janvier and Mark R. Boyett)) Department of Physiology, University of Leeds, Leeds LS2 9NQ, U.K.

Action potentials and contractions were measured in canine ventricular myocytes. 5 mM 4-aminopyridine (4-AP) doubled the size and prolonged the timecourse of contractions. To exclude 4-AP dependent effects on action potential configuration due to blockade of I_{to}, similar experiments were carried out under voltage clamp control. Under these conditions 4-AP had qualitatively similar effects. In skeletal muscle, 4-AP blocks SR K^+ channels involved in counter ion movements during SR Ca^{2+} fluxes and this increases the Ca^{2+} content of the SR (e.g. Fink and Veigel, 1996, *Acta Physiol. Scand.* 156, 387-396). If 4-AP had similar effects in cardiac muscle this could contribute to the positive inotropic effect. To study this, saponin-skinned preparations from rat ventricle were maximally contracted (pCa ~4.0) for 3 min to load the SR with Ca^{2+}. The pCa of the bathing solution was then reduced to >9.0 to induce relaxation and the Ca^{2+} content of the SR determined by exposure to 10-20 mM caffeine. Caffeine-induced contractures were greater when preparations were Ca^{2+} loaded in the presence of 4-AP. Furthermore, when 4-AP was present only during the caffeine-induced release of Ca^{2+} from the SR, contracture magnitude was also enhanced. Similar results were observed with Cs^+, another K^+ channel blocker. These results show that 4-AP and Cs^+ can increase the Ca^{2+} content of the SR which could contribute to the positive inotropic effect of 4-AP. The effects of 4-AP and Cs^+ may result from blockade of SR K^+ channels which play a prominent role in the counter-ion charge balance across the SR membrane during Ca^{2+} loading and Ca^{2+} release.
Supported by The BHF, The Royal Society and The Wellcome Trust

SARCOPLASMIC RETICULUM

Tu-Pos159

OLIGOMERIC STRUCTURE AND DYNAMICS OF SPIN-LABELED PHOSPHOLAMBAN AND ITS MUTANTS IN SDS AND MEMBRANES.
((Christine B. Karim, John D. Stamm, Joseph M. Autry*, David D. Thomas, and Larry R. Jones*)) Department of Biochemistry, University of Minnesota Medical School, Minneapolis, MN 55455, and *Krannert Institute for Cardiology, Indiana University School of Medicine, Indianapolis, IN 46202.

The structure and molecular dynamics of recombinant phospholamban (PLB), and its mutants (L37A and C41L) were probed using electron paramagnetic resonance (EPR) of a thiol-reactive spin label (MTSSL), targeting cysteines in the hydrophobic C-terminal domain. On SDS-PAGE, PLB is pentameric, while L37A and C41L are monomeric and tetrameric, respectively. WT-PLB and L37A maintain their native oligomeric state upon spin labeling, but C41L displays a decrease in the level of oligomerization on SDS-PAGE (from tetramer to monomer) with increasing levels of bound spin label. These relative SDS-PAGE mobilities were confirmed by EPR of these proteins in SDS. After insertion of each protein into lipid (DOPC) bilayers, the EPR spectra indicate that the spin labels are more immobilized, indicating that the PLB subunits are more associated in WT than in L37A. The observed spin label motions provide direct insight into structural models for PLB in the membrane. Both PLB and the trypsin-digested transmembrane domain of PLB (PLB-TM) are pentameric on gels, but EPR shows that the local environment of the labeled cysteines is changed by removing the cytoplasmic domain of PLB. This suggests that subunit packing in the transmembrane domain of PLB depends on the cytoplasmic domain.

Tu-Pos160

PHOSPHOLAMBAN INTERACTION WITH CARDIAC SARCOPLASMIC RETICULUM Ca^{2+}ATPase: RECRUITMENT HYPOTHESIS. ((Jeffrey Smith, Edward McKenna, Richard Wiedmann, and Robert G. Johnson, Jr.)) Merck Research Laboratories, Department of Pharmacology, West Point, PA 19486

Cantilina et al., 1993 (J.Biol.Chem.268:17018) argue that the shift in apparent calcium sensitivity of cardiac SR Ca^{2+}ATPase observed following PLB phosphorylation or treatment with anti-PLB mAb is solely a kinetic effect on a Ca^{2+} dependent slow isomerization which is rate limiting for enzyme turnover. Another interpretation by Voss et al., 1994 (Biophys.J. 67:190) suggests that PLB regulation of the cardiac Ca^{2+}ATPase is a function of critical changes in protein dynamics and protein-protein interactions. According to their hypothesis, Ca^{2+}ATPase activity is dependent on its oligomeric state. Large aggregates have reduced rotational motion and activity relative to smaller more active oligomers. PLB decreases Ca^{2+}ATPase activity by reducing rotational motion and stabilizing large oligomeric Ca^{2+}ATPase complexes. Phosphorylation of PLB relieves this stabilization and allows the dissociation of these large aggregates into smaller active units. Similarly, anti-PLB mAb 1D11 or saturating micromolar Ca^{2+} produce the same effect. We would like to clarify their hypothesis to state that the PLB-aggregated Ca^{2+}ATPase molecules are enzymatically inactive. Disruption of PLB-regulation increases the availability of active Ca^{2+}ATPase units, i.e. recruitment of more pumps from a PLB-aggregated reserve. Our explanation is that unphosphorylated PLB aggregates and inactivates a population of Ca^{2+}ATPase molecules. PLB-phosphorylation or anti-PLB mAb treatment releases the inactive pool of Ca^{2+}ATPase causing a increase in the number of active pump units resulting in an apparent increase in the Ca^{2+} sensitivity of ATP hydrolysis and Ca^{2+} uptake. Data describing the stimulation of ATP hydrolysis, Ca^{2+} uptake and formation of phosphoenzyme intermediate by Ca^{2+}, anti-PLB mAb and quercetin will be presented in support of this hypothesis.

Tu-Pos161

SYNCHRONOUS ASSAY OF ATPASE ACTIVITY AND CALCIUM TRANSPORT IN ISOLATED SARCOPLASMIC RETICULUM MEMBRANES USING A NOVEL FLUOROMETRIC METHOD. ((C. Palahniuk and **J.S.C. Gilchrist**)) Dept. Oral Biology, University of Manitoba, Winnipeg, Manitoba, Canada. R3E 0W2

We have developed a fluorometric method for the synchronous assay of ATPase activity and Ca^{2+} transport by skeletal muscle sarcoplasmic reticulum (SR) vesicles derived from the terminal cisternae. Extraluminal Ca^{2+} transients were monitored by the fluorescence of the calcium color dye, Calcium Green-2 (E_x=506 nm/E_m=531 nm). SR Ca^{2+}-ATPase activity was synchronously followed through fluorescence measurements of NADH oxidation (E_x=377 nm/E_m=452 nm) in a coupled pyruvate kinase/lactate dehydrogenase enzyme pathway for ATP regeneration. All fluorescence measurements were performed using a Quantamaster-1 (model QM-1) fluorometer from Photon Technologies International. The method reveals that the apparent coupling of SERCA pump-mediated Ca^{2+}-ATPase activity to Ca^{2+} transport in these vesicles is governed by the opening and closing of the ryanodine receptor/Ca^{2+} release channel. When the Ca^{2+} release channel was closed a basal level of ATPase activity (~0.25 $\mu Mol.mg^{-1}.min^{-1}$) was observed. In contrast, activation of the Ca^{2+} release channel resulted in a >7 fold activation of ATPase activity. The responsiveness of the SERCA pump to ryanodine receptor states may reflect a mechanism for regulating Ca^{2+} fluxes within the non-junctional membrane regions of the terminal cisternae (Supported by the Medical Research Council of Canada)

Tu-Pos162

cAMP DEPENDENT PHOSPHOLAMBAN PHOSPHORYLATION IN DOG SOLUBILIZED SARCOPLASMIC RETICULUM VESICLES.
((Isabelle Berrebi-Bertrand, Marie-Paule Laville, Jean-Claude Camelin)) SmithKline Beecham, Laboratoires Pharmaceutiques, Saint Grégoire, France.

When used at low concentration Zwittergent 3-14 (Z) is one of the most powerful non anionic and pH insensitive detergents for the solubilization of native membrane proteins but nothing is established regarding the protein function in such solubilized membranes. The aim of the present work was to characterize further the Phospholamban (PLB) phosphorylation state using dog cardiac Sarcoplasmic Reticulum (SR) vesicles solubilized with Z. After Z treatment, PLB in the SR should be more accessible for regulatory proteins.We investigated the ability of the cAMP dependent protein kinase (PKA) to enhance the phosphorylation state of PLB on Ser 16. The method used was SDS PAGE electrophoresis followed by immunoblots using monoclonal antibodies raised against PLB. In control SR vesicles, pentameric PLB was mainly dephosphorylated (deP), but was 100% phosphorylated (P) with 2000 U PKA and 50% (P) by 200 units PKA (ratio PLB (P)/PLB (deP)=1). After solubilization of the SR vesicles with (Z) from 0.1 to 0.5 % this ratio was maintained. From 1% to 1.5% (Z) this ratio is enhanced implying that PLB is more (P) and less (deP). In fact, after solubilization with 1% (Z) 200 U PKA enabled complete PLB phosphorylation. The same result was obtained (100% (P)) with 5 U PKA whereas in control SR vesicles, PLB was mainly (deP) at this PKA concentration. Using more than 1.5% (Z) was deleterious for PLB and the ratio decreases suggesting PLB denaturation. In conclusion, our findings show that in (Z) 1% PLB is not denaturated and its enhanced accessibility allows its full phosphorylation with at least 400 fold less PKA versus non solubilized vesicles.These results are of great interest in the study of the structure-function relationship between PLB and SR proteins.

Tu-Pos163

TEMPERATURE DEPENDENCE OF Ca-ATPase ACTIVITY, Ca^{2+} UPTAKE, AND OLIGOMERIC STATE OF Ca-ATPase OF CARDIAC SARCOPLASMIC RETICULUM (SR). ((Howard Kutchai, Joseph J. Feher[1], and David D. Thomas*)) Dept. of Molecular Physiology. & Biological Physics, University of Virginia, Charlottesville, VA 22908; [1]Dept. of Physiology, Medical College of Virginia, Richmond, VA 23298; *Dept. of Biochemistry, University of Minnesota, Minneapolis, MN 55455

At temperatures from 5 to 40°C the apparent activation energies of Ca-ATPase activity and Ca^{2+} uptake of cardiac SR are essentially equal and they increase monotonically with decreasing temperature. By contrast, Arrhenius plots of Ca-ATPase activity of skeletal SR are fit by two straight lines that intersect at about 18°C. Ca-ATPase of cardiac SR was covalently labeled with erythrosin isothiocyanate or with erythrosin iodoacetamide and the time-resolved decay of the phosphorescence anisotropy was used to infer the distribution of the Ca-ATPase among its oligomeric forms. From 5°C to 30°C, there were only slight changes in the distribution among the oligomeric species. At 35 and 40° there was a dramatic increase in the proportion of Ca-ATPase oligomers that are too large to rotate on the time scale of the measurements (about 1 ms). The temperature dependences of Ca-ATPase activity, Ca^{2+} uptake, and the distribution of Ca-ATPase oligomeric states are all significantly different from those in SR from skeletal muscle. A striking difference between cardiac and skeletal SR is the apparent formation of large oligomers of Ca-ATPase at 35 and 40°C in cardiac, but not in skeletal SR.

Tu-Pos164

PHOSPHOLAMBAN PHOSPHORYLATION ALTERS BOTH THE MAXIMUM VELOCITY AND CALCIUM SENSITIVITY OF THE SR Ca^{2+}-ATPASE. ((M.E. Kargacin, Z. Ali, and **G.J. Kargacin**)) Dept. of Physiology and Biophysics, University of Calgary. Calgary, Alberta T2N 4N1

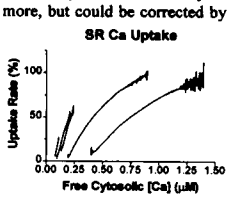
The protein phospholamban (PLB) interacts with the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase in cardiac muscle. When not phosphorylated PLB inhibits the pump. When PLB is phosphorylated the inhibition is removed. Experiments in which $^{45}Ca^{2+}$ was used to measure SR Ca^{2+} uptake showed a decrease in $Ca_{50\%}$ (Ca^{2+} concentration at half maximal pump velocity) for the pump upon phosphorylation of PLB; this results was mimicked by an antibody to PLB (A1). In our experiments, in which fura-2 was used to measure net Ca^{2+} transport by cardiac SR vesicles, phosphorylation of PLB by the catalytic subunit of protein kinase A resulted in a large increase in the V_{max} of the pump (up to 160% of control) as well as a decrease in the $Ca_{50\%}$. A1 also increased the V_{max} (average increase was 140% of control) and decreased $Ca_{50\%}$. Results of our experiments indicate that PLB phosphorylation has a significant effect on the V_{max} of SR Ca^{2+} transport. Our experiments also indicate that when PLB is phosphorylated by protein kinases the effect on Ca^{2+} uptake is different than when A1 binds to PLB.

This work was supported by the Heart and Stroke Foundation of Alberta, MRC Canada, and the Ruth Rannie Memorial Endowment.

Tu-Pos165

SR Ca UPTAKE RATE IN PERMEABILIZED VENTRICULAR MYOCYTES IS LIMITED BY REVERSE MODE OF THE SR Ca PUMP. ((T.R. Shannon, K.S. Ginsburg, & D.M. Bers)) Department of Physiology, Loyola University Chicago, Maywood, IL 60153

Cardiac SR Ca uptake is often described by the classical Hill equation. However, this equation describes transport only in the forward direction, assuming no transport in the reverse mode (i.e. from SR to cytosol). We hypothesize that this equation is not adequate to describe SR Ca uptake under physiological conditions where free [Ca] in the SR is expected to rise during relaxation. This hypothesis was tested in digitonin permeabilized myocytes in a cuvette. [ATP] was maintained by a regeneration system (CP-CPK) and SR Ca uptake was measured by the disappearance of Ca (using indo-1). No precipitating anions were present. Sequential addition of Ca to the cuvette produced progressively slower Ca uptake into the SR as the intra SR Ca content progressively increased (see Fig). At early Ca pulses, SR uptake could be reasonably well described by a simple Hill equation. Subsequent Ca pulses deviated more, but could be corrected by accounting for reverse Ca fluxes through the SR Ca pump. Voltage clamp experiments (with Na-free solutions to block Na/Ca exchange and indo-1 to measure $[Ca]_i$ and depolarizing pulses to add Ca via I_{Ca}) were also done to show this effect in intact myocytes. We conclude that SR Ca accumulation may prevent the Hill equation from adequately describing SR Ca uptake under physiological conditions.



Tu-Pos166

EFFECTS OF THYROXINE ON CARDIAC FUNCTION IN PHOSPHOLAMBAN-DEFICIENT MOUSE HEARTS. ((E.Kiss, **A.G.Brittsan**, I.Edes, W.Luo, I.L.Grupp, G.Grupp, and E.G.Kranias)) Univ. Cincinnati, Cin., OH 45267. (Spon. by L.Lane)

Alterations in cardiac contractility induced by thyroxine have been suggested to reflect alterations in the expression levels of several proteins, including the SR Ca^{2+}-ATPase (SERCA) and its regulator phospholamban (PLB). To determine the relative contribution of these proteins in the altered contractility of hypothyroid and hyperthyroid hearts, PLB and SERCA protein levels and cardiac function were evaluated in wild-type (WT) and PLB-deficient (PLB-KO) mice. Quantitative immunoblot analysis of SERCA revealed similar increases in hyperthyroid and similar decreases in hypothyroid WT and PLB-KO hearts compared to their respective euthyroid groups. However, the levels of PLB were decreased in hyperthyroid and increased in hypothyroid WT hearts. To examine whether the observed changes in SR Ca^{2+} handling proteins were reflected in altered cardiac function, contractile parameters were assessed in isolated work performing hearts. In WT hearts, hypothyroidism was associated with a significant depression of the speed to relaxation, while hyperthyroidism enhanced both the contraction and relaxation rates. In the PLB-KO hearts, hyperthyroidism was not associated with any further enhancement of the contractile paramters, while hypothyroidism resulted in a significant depression of the rates of both contraction and relaxation. However, the contractile parameters in the hypothyroid PLB-KO hearts were still higher than those in the euthyroid WT hearts. These findings indicate that alterations in thyroxine levels: a) have an inverse effect on PLB and SERCA expression levels, thereby affecting contractility by altering the PLB/SERCA ratio; and b) induce similar changes in the SERCA levels in WT and PLB-KO hearts. Furthermore, increases in thyroxine levels do not further stimulate the hyperdynamic cardiac function of PLB-KO mice, while hypothyroidism is associated with depressed cardiac contractile parameters in the PLB-KO mice.

Tu-Pos167

ACIDOSIS ENHANCES ISOPROTERENOL-INDUCED PHOSPHOLAMBAN PHOSPHORYLATION IN THE INTACT HEART BY INCREASING PHOSPHORYLATION OF Thr^{17} RESIDUE. ((**C. Mundiña-Weilenmann**, L. Vittone, M. Ortale, H.E. Cingolani, A. Mattiazzi)). Centro de Investigaciones Cardiovasculares. Facultad de Medicina. La Plata, Argentina. (Spon. by R. A. Venosa).

Previous experiments showed that acidosis enhances isoproterenol-induced phospholamban (PHL) phosphorylation (Am J Physiol 270:C107-114, 1996). The availability of phosphorylation site-specific antibodies to PHL prompted us to reexamine the issue in isolated Langendorff perfused rat hearts to gain further insight into the mechanism involved in this effect. At normal pH_o (7.40), PHL phosphorylation -measured in ^{32}P labelled hearts- and phosphorylation of Thr^{17} and Ser^{16} residues, attained a "plateau" at 30 nM isoproterenol. Acidosis (pH_o 6.70) produced a further increase in ^{32}P incorporation into PHL from 132 ± 26 (30 nM Isoproterenol, pH_o 7.40) to 243 ± 38 pmol ^{32}P/mg membrane protein (30 nM Isoproterenol, pH_o 6.70) (n=5). This increase was exclusively due to an increase in phosphorylation of Thr^{17} (n=5) and was associated with a significant enhancement of isoproterenol-induced relaxant effect. Since acidosis increases $[Ca]_i$ (J Physiol 384:431-449, 1987), we explored the possibility that the enhancement of Thr^{17} phosphorylation could be due to an activation of Ca-calmodulin-dependent protein kinase (CaMKII). Increasing $[Ca]_o$ at pH_o 7.40 in the presence of 30 nM isoproterenol produced an increase in Thr^{17} phosphorylation (n=3), suggesting that CaMKII can be further activated in the presence of isoproterenol. In addition, acidosis produced a significant inhibition of sarcoplasmic reticulum associated phosphatase activity which can contribute to phosphorylation of PHL. The experiments indicate that acidosis enhancement of isoproterenol-induced PHL phosphorylation is exclusively due to Thr^{17} phosphorylation and may occur by simultaneous activation of CaMKII and inhibition of PHL phosphatase.

Tu-Pos168

AN AUTOINHIBITORY PEPTIDE FROM THE ERYTHROCYTE Ca-ATPASE AGGREGATES AND INHIBITS BOTH MUSCLE Ca-ATPASE ISOFORMS. (Yongli Shi, Laxma G. Reddy, Adelaida G. Filoteo,* John T. Penniston,* and David D. Thomas) Dept. Biochem., Univ. of Minnesota; *Dept. Bioch/Mol.Biol, Mayo Clinic.

We have studied the effects of C28R2, a basic peptide derived from the autoinhibitory domain of the plasma membrane Ca-ATPase, on enzyme activity, oligomeric state, and conformational equilibrium of the Ca-ATPase from skeletal and cardiac sarcoplasmic reticulum (SR). Time-resolved phosphorescence anisotropy (TPA) was used to detect Ca-ATPase oligomerization in SR. C28R2 inhibits and aggregates the Ca-ATPase of both skeletal and cardiac SR. Inhibition and aggregation are Ca-dependent in skeletal SR, but not in cardiac SR. At low Ca, the inhibition of Ca-ATPase by C28R2 is greater in skeletal SR than in cardiac SR, but an opposite effect was observed at high Ca. In cardiac SR at low Ca, phospholamban (PLB) antibody (functionally equivalent to PLB phosphorylation), increased the inhibitory effect of C28R2, diminishing the difference between skeletal and cardiac SR. Fluorescence of FITC-labeled SR suggests that C28R2 stabilizes the E2 conformation of the Ca-ATPase in cardiac SR, whereas in skeletal SR it stabilizes E1. We conclude that C28R2 affects Ca-ATPase activity, conformation, and self-association, but these effects are different in cardiac and skeletal SR. The differences are due, at least in part, to the presence of PLB in cardiac SR.

Tu-Pos169

PHOSPHORYLATION OF PHOSPHOLAMBAN DECREASES THE SELF-ASSOCIATION OF THE CARDIAC MUSCLE CALCIUM PUMP (Min Zhao, Laxma G. Reddy and David D. Thomas) Dept. of Biochemistry, University of Minnesota, Minneapolis, MN 55455.

We have used time-resolved phosphorescence anisotropy (TPA) to detect the microsecond rotational dynamics, and thereby the protein-protein interactions, of cardiac Ca-ATPase as a function of phospholamban (PLB) phosphorylation. The Ca-ATPase was labeled specifically in cardiac sarcoplasmic reticulum vesicles with erythrosin 5'-iodoacetamide (ErIA), which did not affect its calcium-dependent ATPase activity. The up-regulation of the ErIA-labeled Ca-ATPase by PLB phosphorylation was normal. Phosphorylation of PLB increased the rotational mobility of the Ca-ATPase in the 50-300μs time range. The time-resolution of TPA prevents the ambiguity of EPR and other steady-state techniques, and shows clearly that phosphorylation decreases protein association, without a change in the protein's internal structure. Simulations of TPA decays indicate that 40% ± 5% of PLB is disaggregated by PLB phosphorylation. This is consistent with the Western blot detection of PLB phosphorylation by gel shift, which indicates that the percentage of phosphorylated PLB is 42% ± 5% under TPA conditions. We conclude that enzyme self-association is involved in the mechanism and regulation of the Ca-ATPase in cardiac SR.

Tu-Pos170

NUCLEOTIDE ANTAGONISTS REVERSE PHOSPHOLAMBAN REGULATION OF CARDIAC SARCOPLASMIC RETICULUM Ca^{2+}ATPase. ((Edward McKenna, Kathleen E. Coll, and Robert G. Johnson, Jr.)) Merck Research Laboratories, Department of Pharmacology, West Point, PA 19486

In cardiac sarcoplasmic reticulum (SR), low micromolar concentrations of ellagic acid and tannin stimulate calcium-activated ATP hydrolysis and $^{45}Ca^{2+}$ uptake at submicromolar free Ca^{2+}. Two types of concentration dependence curves were found. Tannin, like quercetin [McKenna et al., 1996 JBC 271:24517], exhibited a biphasic curve with nearly complete inhibition at concentrations above 10 μM. Ellagic acid exhibited a sigmoidal curve with a plateau over a wide range of concentrations. The maximal stimulation rivaled the effects of anti-phospholamban mAb 1D11 and produced the characteristic increase in calcium sensitivity without altering the V_{max} as observed with mAb 1D11 or phospholamban phosphorylation. Phospholamban peptides, PLB 1-25 and PLB 1-30, could not prevent stimulation by either compound. Neither compound stimulated Ca^{2+}ATPase activity in skeletal muscle SR or cardiac muscle SR at micromolar free Ca^{2+}, although the inhibitory effects of tannin remained. Both compounds are well-known inhibitors of ATP-dependent enzymes and altered the MgATP dependency of Ca^{2+}ATPase. These compounds increased the apparent $K_{m(MgATP)}$ in a concentration dependent manner. In addition, they dissociate PLB from the Ca^{2+}ATPase as evidenced by an increase in the $V_{max(MgATP)}$ like that measured in the presence of mAb 1D11. Dixon plots revealed different mechanisms of action. Tannin, like quercetin, produced a linear Dixon plot consistent with a simple competitive inhibition with nucleotide. In contrast, a curvilinear plot was observed with ellagic acid suggesting a complex partial inhibition. It is known that phospholamban does not alter nucleotide binding affinity, but nucleotide binding may effect phospholamban binding. This work suggests that the nucleotide and phospholamban binding sites are functionally related.

Tu-Pos171

SELF-ASSOCIATION OF THE SKELETAL MUSCLE RYANODINE RECEPTOR/CALCIUM RELEASE CHANNEL

((**C.C Yin***, P.N.T. Unwin§ and F.A. Lai*)) *MRC National Institute for Medical Research, Mill Hill, London NW7 1AA, UK. §MRC Laboratory of Molecular Biology, Cambridge CB2 2QH, UK.

Ryanodine receptors (RYRs)/calcium release channels form extensive ordered arrays *in situ* in the sarcoplasmic reticulum (SR) membranes of skeletal muscle [Ferguson et al. *J.Cell Biol.*99:1735;1984]. It is not known whether the association is due to receptor-receptor interaction between RYRs or is mediated through other proteins such as dihydropyridine receptor, triadin and calsequestrin, which have been shown to interact with RYR. To address this issue, RYR was purified from skeletal SR and the conditions under which ordered arrays could form were examined. Purified RYR in high ionic strength buffer containing detergent was dialysed against the same buffer without detergent. No association between RYRs was observed. When a low ionic strength buffer that mimics the physiological conditions was used, RYRs spontaneously associated into two-dimensional ordered arrays. The arrays formed belong to the P4 layer group, with RYRs arranged in a corner-to-corner fashion with spacings almost identical to the arrays observed *in situ*. In the presence of Mg^{2+}, a completely different arrangement was observed. RYRs arranged in the arrays almost side-by-side. Interestingly, these arrangements were observed previously in muscle tissues *in situ* [Loesser et al. *J.Muscle Res.Cell Motil.*13:161;1992]. The data suggests that the formation of RYRs into ordered arrays is due to self-association between RYRs; no other proteins are required to mediate the association.

Tu-Pos172

SEQUENCES OF CARDIAC TRIADIN IN MOUSE DEDUCED FROM cDNA. ((**A.H. Caswell**, T. Miller, and N.R. Brandt)) Dept Mol and Cell Pharmacology, University of Miami School of Medicine, P.O.Box 016189, Miami FL 33101.

We have screened a mouse cardiac library for triadin using either the C terminal antibody mAbGE4.90 or using cDNA probes. Two presumably alternatively spliced isoforms of cardiac triadin have been detected which have identical N terminal regions coding for aa's 1 to 292 and are also identical to a skeletal muscle transcript. This region of the sequence is closely related to the human and rabbit skeletal and cardiac sequences. One mouse isoform corresponds to the reported rabbit CT1 having a homologous 3' open reading frame, but completely different aa's at the C terminus. The other mouse isoform has a broad but very incomplete homology to the C terminal half of the rabbit and human skeletal sequences, indicating a high degree of polymorphism among mammalian species. The putative α helical and three short trans-membrane sequences are well preserved in skeletal and cardiac sequences from mouse, rabbit and human. Some mouse clone cDNAs have revealed several extensive deletions in both the coding and 3' untranslated regions of triadin. The presence of the skeletal C terminal sequence in the heart has been confirmed in rabbit, mouse and rat using mAbGE4.90. These sequences indicate that the putative three cytoplasmic regions of triadin which are proposed to make contact with transverse tubular proteins are well conserved while the luminal segment can undergo extensive evolutionary modification. Supported by NIH AR43355.

Tu-Pos173

THE NEGATIVELY CHARGED D3 REGION OF SKELETAL MUSCLE RYANODINE RECEPTOR IS INVOLVED IN ION CONDUCTION AND Ca-DEPENDENT INACTIVATION OF THE Ca RELEASE CHANNEL.

((**Manjunatha B. Bhat**, Jiying Zhao, Salim Hayek, Eric C. Freeman, Hiroshi Takeshima*, and Jianjie Ma)) Dept. of Physiol. & Biophysics, Case Western Reserve University, and Dept of Pharmacology*, Univ. of Tokyo.

To understand the contribution of the foot structure to the function of the Ca release channel, we studied a RyR deletion mutant, ΔD3-RyR, in which a region that is rich in glutamate and aspartate residues (a.a. 1641-2437) was removed. The deleted portion includes one of the divergent regions (D3) between the skeletal and cardiac subtypes of RyR. The wild type RyR expressed in CHO cells forms functional Ca release channels in the lipid bilayer membrane with linear current-voltage relationship, similar to that of the native RyR channel in the skeletal SR membrane; whereas the channels formed by ΔD3-RyR exhibit significant inward-rectification, i.e. currents moving from cytoplasm into SR lumen are ~20% less than that in the opposite direction. While opening of both wild type and ΔD3-RyR channels requires the presence of micromolar [Ca] in the cytoplasmic solution, the Ca-dependent inactivation was significantly different, as inhibition of the ΔD3-RyR channel requires ~ten-fold more Ca than that of the wild type RyR channel. Our data provide direct evidence that the negatively charged domain in the foot region of the skeletal muscle RyR participates in both ion-conduction and Ca-dependent regulation of the Ca release channel. Supported by NIH, AHA and HHMI.

Tu-Pos174

THE 3D LOCATIONS OF CALMODULIN (CaM) AND FK506-BINDING PROTEIN (FKBP12) BINDING SITES ON THE RYANODINE RECEPTOR.

((**M. Samso**, M. Radermacher, R. Grassucci, J. Berkowitz, *H.-B. Xin, *S. Fleischer, and T. Wagenknecht)) Wadsworth Center, New York State Dept. of Health, Albany, NY 12203 and *Department of Molecular Biology, Vanderbilt University, Nashville TN 37235.

The calcium channel activity of the skeletal muscle ryanodine receptor (RyR1) is modulated by CaM and FKBP12. We have now determined the locations where these two ligands bind on RyR1 by cryoelectron microscopy and three-dimensional image reconstruction of complexes prepared by in vitro assembly. The CaM:RyR1 complexes were prepared under conditions (0.1mM Ca^{2+}) that favor four moles of bound CaM per mole of tetrameric receptor. Studies under conditions (<0.1 μM Ca^{2+}) favoring up to 16 moles of bound CaM have begun, and preliminary results indeed show differences from the behavior at higher [Ca^{2+}]. The 3D reconstructions show that both CaM (0.1 mM Ca^{2+}) and FKBP12 bind to four symmetrically related locations at the periphery of the cytoplasmic assembly of RyR1, ≥ 10 nm from the putative site of the transmembrane ion channel. The distance between neighboring FKBP12 and CaM is 7 nm, with CaMs occupying crevices on the SR-facing side and FKBP12s near the transverse-tubule facing side of RyR1. The results are consistent with an allosteric mechanism of modulation by these ligands.

Supported by NIH AR40615 (TW), HL32711 (SF), HL46681 (SF), Muscular Dystrophy Assoc. (SF), and Ministry of Education and Science (Spain) (MS).

Tu-Pos175

PROPERTIES OF HOMOTETRAMERIC RYR3 RYANODINE RECEPTOR IN MAMMALIAN DIAPHRAGM MUSCLE.

((**T. Murayama** and Y. Ogawa)) Dept. Pharmacol., Juntendo Univ. Sch. Med., Tokyo 113, Japan.

In addition to type 1 isoform of ryanodine receptor (Ryr1), which plays an important role in excitation-contraction coupling in skeletal muscles, type 3 isoform (Ryr3) has been recently detected in several mammalian skeletal muscles. Here we report the properties of Ryr3 in mammalian diaphragm muscle using an antibody specific for Ryr3. On Western blot analysis, a single band was detected with the antibody just below the location of Ryr1 in sarcoplasmic reticulum (SR) vesicles from rabbit diaphragm but not in those from back muscle. The antibody specifically precipitated a single polypeptide of a molecular mass slightly smaller than that of Ryr1 from solubilized diaphragm SR vesicles. Sedimentation pattern of Ryr3 through sucrose gradients and the absence of Ryr1 co-precipitated with the antibody revealed that Ryr3 forms a homotetramer. Ryr3 showed caffeine-sensitive [^{3}H]ryanodine binding with high affinity (K_D=1.6 nM). From the B_{max} of ryanodine binding, the amount of Ryr3 was estimated to be less than 1 % of Ryr1 in rabbit diaphragm. These results showed the occurrence of the functional homotetrameric Ryr3 in mammalian diaphragm muscle.

Tu-Pos176

CYCLOSPORIN A TREATMENT ALTERS THE CHARACTERISTICS OF CALCIUM RELEASE CHANNEL IN SARCOPLASMIC RETICULUM OF RAT HEART

((**K.S. Park**, T.K. Kim and D.H.Kim)) Dept. of Life Sci., Kwangju Inst. of Sci. & T echnol. (K-JIST) Kwangju, Korea.

Cyclosporin A (CsA), an important therapeutic agent for the prevention of a graft rejection in organ transplantation, displays a clinically important cardiotoxicity. To define the mechanism of CsA-induced cardiotoxicity, Sprague-Dawley (SD) rats were treated with CsA (15 mg/kg body wt./day) for 21 days, and characteristics of Ca^{2+} release channels (CRC) of sarcoplasmic reticulum (SR) in the CsA-treated rat hearts were examined by equilibrium [^{3}H]ryanodine binding. Cremophor-treated SD rats served for control. Our results show that upon the treatment of rats with CsA, the maximal ryanodine binding (B_{max}) to heart whole homogenates decreased significantly (0.52 ± 0.02 vs. 0.37 ± 0.04 pmoles/mg protein, p< 0.05), whereas the dissociation constant of ryanodine (K_d) increased significantly (2.47 ± 0.36 vs. 4.55 ± 0.56 nM, p< 0.05). However, the characteristics of ryanodine binding in slow- and fast-twitch skeletal muscles (type I:soleus; type IIA: the deep region of the vastus lateralis; type IIB: the superficial region of the vastus lateralis) were not changed by CsA treatment. These results suggest that the heart muscle specific quantitative and qualitative alterations of CRC may be associated with the CsA-induced cardiotoxicity.

Tu-Pos177

VISUALIZATION OF SPATIALLY DISTINCT AND FUNCTIONALLY DISCRETE Ca^{2+} STORAGE COMPARTMENTS IN THE SARCO/ENDOPLASMIC RETICULUM.

((**V.A. Golovina**, and M.P. Blaustein)) Dept. of Physiol., Univ. of Maryland Med. Sch., Baltimore, MD 21201.

The dynamics of Ca^{2+} storage and release in the sarco/endoplasmic reticulum (S/ER) of intact cultured rat astrocytes and arterial myocytes were studied by using high-resolution digital imaging of Furaptra and Fura-2FF fluorescence. The S/ER was identified by staining with the lipophilic fluorochrome $DiOC_6(3)$. In resting cells, the S/ER apparent free Ca^{2+} concentration ($[Ca^{2+}]_{s/er}$) was 104±4 μM (n=36 cells; with Furaptra) and 96±3 μM (n=8; with Fura-2FF). Cyclopiazonic acid (CPA, 10 μM), which inhibits S/ER Ca^{2+}-ATPase and depletes IP_3-sensitive stores, caused $[Ca^{2+}]_{s/er}$ to fall by 26±7 μM (n=14; with Furaptra) in most S/ER regions (termed "CPA-sensitive"), and to rise in the remainder of the S/ER. Similar results were obtained with Fura-2FF. Subsequent addition of caffeine (CAF, 10 mM), which releases Ca^{2+} from IP_3-insensitive stores, reduced $[Ca^{2+}]_{s/er}$ by 18±4 μM (n=12) in the latter ("CAF-sensitive") S/ER regions. The effects of CPA and CAF were reversible and repeatable. The CAF-releasable S/ER store could also be depleted by 1 μM ryanodine. Heterogeneity of the S/ER Ca^{2+} stores was further revealed in cells activated by physiological agonists. Serotonin (5-HT, 10 μM) caused $[Ca^{2+}]_{s/er}$ to decline by 23±5 μM (n=7) in about 54% of the total S/ER area in Furaptra-loaded arterial myocytes, and to rise in about 40% of the S/ER area. CPA reduced $[Ca^{2+}]_{s/er}$ and increased $[Ca^{2+}]_{s/er}$ in, respectively, the same regions that were reduced and increased by 5-HT. Conversely, CAF reduced $[Ca^{2+}]_{s/er}$ in those regions in which the levels were increased by 5-HT. In astrocytes, glutamate (100 μM) evoked oscillations in $[Ca^{2+}]_{s/er}$ (with swings as large as 40-60 μM): $[Ca^{2+}]_{s/er}$ transiently rose in some areas and fell in adjacent areas, and then reversed direction. These findings indicate that S/ER Ca^{2+} stores are organized into small, spatially distinct compartments that appear to function as discrete units.

Tu-Pos178

CALCIUM REGULATION OF MODAL GATING OF CARDIAC RELEASE CHANNELS.

((**R. Armisén**, P. Vélez*, M. Fill* and B.A. Suárez-Isla)) Dept. Fisiología y Biofísica, Fac. Medicina, Universidad de Chile, Santiago 7, Chile and *Dept. Physiology, Loyola University Chicago, Maywood, Ill. 60153.

Ca^{2+} dependence of bursting modal behavior of ryanodine-sensitive Ca^{2+} release channels (cRyR) from canine sarcoplasmic reticulum was characterized in steady-state recordings by analysis of burst sequences and of *gamma* distributions of average intraburst open (T_o) and closed times (T_c). As shown for skeletal and neuronal RyR isoforms (Armisen et al.,(1996) Am. J. Physiol. 271:C144-C153), trains of low P_o bursts (L-mode) were interspersed with trains of high P_o bursts (H-mode). The *gamma* distributions of T_o had two *gamma* components, suggesting the existence of two distinct burst types. In contrast, the *gamma* distributions of T_c had only one component in all cases. Time correlations between consecutive burst pairs defined in terms of T_o and then statistically tested by 2x2 matrix contingency analysis, indicated that the probability that the sequential burst patterns were generated by random occurrence was very low ($p < 0.001$; two-tailed Fisher's exact test). These data confirms that cRyR can switch between distinct modes of gating. It is proposed that the relaxation time constants of adaptation observed after activation of RyR by flash photolysis of caged Ca^{2+} ($\tau \approx 0.98$ s for cRyR and 4.18 s for skRyR) (Vélez et al.,(1995) Biophys. J., 68, A356) reflects the slow switching between H- and L-modes that is revealed under steady state conditions (steady state rates of 0.1 s^{-1} for cRyR and 0.2-0.5 s^{-1} for skRyR). A minimal branched model with 3 open and 3 closed states explains activation by Ca^{2+} as a concentration dependent change in the availability of each gating mode.

Supported by FONDECYT Grant 1950632 to B.A.S.I. and a graduate student fellowship to R.A. from Conicyt, Chile.

Tu-Pos179

DANTROLENE INHIBITION OF SKELETAL MUSCLE RYANODINE RECEPTOR SUGGESTS ACTION AT DISCRETE HIGH-AFFINITY DANTROLENE SITE.

((B.R. Fruen, J.R. Mickelson, and C.F. Louis)) Department of Veterinary PathoBiology, University of Minnesota, St. Paul, MN.

Dantrolene inhibits the progression of a Malignant Hyperthermia (MH) crisis by limiting the uncontrolled increase in intracellular Ca^{2+} that underlies this pharmacogenetic disorder of skeletal muscle. The molecular basis of dantrolene's action has remained unclear, however, due in part to an inability to demonstrate unambiguous inhibitory effects of clinical concentrations on Ca^{2+} channel function in isolated preparations. We now describe evidence of a high-affinity, monophasic inhibition by dantrolene of ryanodine receptor (RYR) Ca^{2+} channel function in isolated sarcoplasmic reticulum (SR) vesicles prepared from MH-susceptible and normal pig skeletal muscle. In media simulating resting myoplasm (3 mM MgAMPPCP, 1 μM calmodulin, 100 nM Ca^{2+}, 150 mM Kpropionate, 15 mM PIPES, pH 7.0), SR [^{3}H]ryanodine binding was inhibited > 50% in the presence of dantrolene (IC_{50} = ~200 nM for both MH and normal SR). SR [^{3}H]ryanodine binding was also inhibited by analogues of dantrolene (azumolene ≥ dantrolene > > aminodantrolene); however, in the presence of 2 μM dantrolene, azumolene (up to 30 μM) had no additional inhibitory effect, suggesting that these drugs act at a common, saturable site. We postulate that the observed inhibitory effects of dantrolene on RYR channels may explain the clinical actions of this drug in controlling Ca^{2+} release from intracellular stores. Whether this inhibition reflects a direct action of dantrolene on the RYR channel protein or rather results from dantrolene binding to an associated regulatory component remains in question.

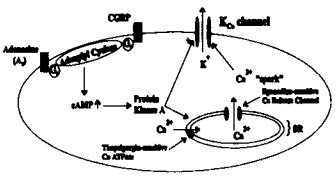
Supported by NIH grant GM-31382 and by AHA-MN Affiliate.

Tu-Pos180

cAMP/PKA ACTIVATES Ca^{2+} SPARKS AND K_{Ca} CHANNELS IN CEREBRAL ARTERY MYOCYTES

((V.A. Porter, A. Bonev, T. Kleppisch, J.Lederer & M.T Nelson))University of Vermont, MRF, Colchester, VT 05446 (Spon by M. Rubart)

We explored a mechanism by which cAMP could dilate arteries by examining the effects of adenosine, forskolin & cAMP on calcium release from the SR (Ca^{2+} sparks) and on K_{Ca} channels in smooth muscle cells isolated from small myogenic cerebral arteries. Forskolin, an activator of adenylyl cyclase, increased the frequency of the Ca^{2+} sparks (measured using the fluorescent Ca^{2+} indicator fluo-3)(control 2.4±0.38 sparks/cell; forskolin 10μM 5.38±0.45 sparks/cell or 2.2 fold) but not in the presence of H89, a blocker of protein kinase A. The amplitude, spread and time course of the sparks were not affected by forskolin. Currents through K_{Ca} channels ('STOCs') activated by Ca^{2+} sparks were measured in single cells using the perforated patch configuration. Forskolin increased STOC frequency (2.8±0.5 fold) and amplitude (1.2±0.07 fold). These effects were reversed by addition of H-89. STOC frequency and amplitude were also increased by adenosine (amp 1.3±0.13 fold; freq 2.6 ±0.7 fold) and db-cAMP (amp 1.12±0.09 fold; freq 2.0±0.2 fold). The effects of forskolin on the NP_O of single K_{Ca} channels were studied, using whole cell voltage clamp, in the presence of thapsigargin to prevent generation of sparks, and thus also STOCs. Forskolin increased the open probability 1.3 ±0.07 fold. These results suggest a novel mechanism for the regulation of cell constriction, through cAMP /PKA synergistic effects on Ca^{2+} spark frequency and K_{Ca} channel open probability.

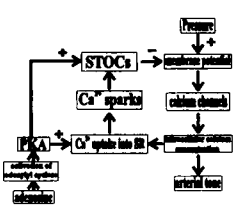


Tu-Pos181

DYNAMIC REGULATION OF CEREBRAL ARTERY DIAMETER BY cAMP/PKA INVOLVES ACTIVATION OF Ca^{2+} SPARKS AND K_{Ca} CHANNELS

((V.A.Porter, A.Stevenson, H. Knot & M.T.Nelson)) University of Vermont, MRF, Colchester, VT 05446

We tested the hypothesis that cAMP/PKA dilates arteries through increased Ca^{2+} spark frequency and open probability of K_{Ca} channels (see our other abstract & Nelson et al 1995, *Science 270, 5236, p633*). This hypothesis would predict that forskolin- (an activator of adenelyl cyclase) dilations would be inhibited by blockade of Ca^{2+} sparks or STOCs but diltiazem- (which blocks Ca^{2+} channels directly) dilations would not. In rat myogenic cerebral arteries pressurized to 60mmHg, the mean arterial diameter was 115±7μm. Forskolin, 100nM, produced a sub-maximal dilation to 140±16μm. Subsequent application of ryanodine, 10μM (blocks SR Ca^{2+} release channel) inhibited the forskolin dilation with a final diameter of 85±20μm. Application of ryanodine and the K_{Ca} channel blocker iberiotoxin (100nM) together did not cause a greater inhibition (83±20μm). In contrast, diltiazem (1μM) dilations to 153±13μm were not reversed by ryanodine, with the diameter remaining at 147±13μm. In the presence of 60mM K^+, which constricts to 59±6μm, forskolin failed to dilate the arteries (60±6μm), suggesting that forskolin acts through the opening of K^+ channels. Intracellular calcium was measured in intact pressurized arteries using the Ca^{2+} indicator fura-2. Forskolin dilations were accompanied by a fall in intracellular Ca^{2+} in a dose dependent manner. In conclusion, forskolin dilates arteries via an increase in the local release of intracellular Ca^{2+} (Ca^{2+} sparks). Ca^{2+} sparks cause hyperpolarization via the K_{Ca} channel, closing voltage-dependent Ca^{2+} channels, causing the observed reduction in global intracellular Ca^{2+} and vasodilation.



Tu-Pos182

ACTIVATORS OF PROTEIN KINASE C (PKC) DECREASE CALCIUM SPARK FREQUENCY IN CEREBRAL ARTERY MYOCYTES.

((1A.D. Bonev, 2M. Rubart, 1J.H. Jaggar and 1M.T. Nelson)) 1Department of Pharmacology, University of Vermont, Colchester, VT 05446 and 2Krannert Institute of Cardiology, Indiana University Medical School, Indianapolis, IN 46202.

Vasoconstrictors, which can activate PKC, depolarize and constrict arteries. We explored the possibility that PKC could act in part by inhibiting local Ca^{2+} release (Ca^{2+} sparks) through ryanodine-sensitive Ca^{2+} release channels in the sarcoplasmic reticulum of the smooth muscle cells (Nelson et al., *Science*, 270: 633, 1995), which in turn would decrease K_{Ca} channel activity and cause membrane depolarization. Calcium sparks were measured in freshly isolated smooth muscle cells from rat basilar artery using a laser scanning confocal microscope and the fluorescent calcium indicator fluo-3. Activators of PKC (either PMA (10 nM, n=30) or 1,2-dioctanoylglycerol (DOG, 1 μM; n=30)) reduced calcium spark frequencies 5.4- and 2.8-fold, respectively, whereas 4α-PMA (10 nM), a non active analogue of PMA, did not significantly alter calcium spark frequency. A decrease in the calcium spark frequency would be expected to lower the activity of K_{Ca} channels in the form of STOCs (Spontaneous, Transient Outward Currents). In voltage clamped cells (perforated patch configuration; holding potential, -30 mV), PMA (10 nM; n=6) was found to decrease the frequency of STOCs 15.5 - fold, whereas 4α-PMA (10 nM; n=4) had no significant effect. PMA (100 nM) did not alter either the peak amplitude or the time course of caffeine (10 mM)-induced $[Ca^{2+}]_i$ transients as measured with fura-2 (n=4). These data suggest a new mechanism for regulation of blood vessel function by PKC through reduction of Ca^{2+} spark frequency, possibly through an affect on ryanodine-sensitive Ca^{2+} release channel.

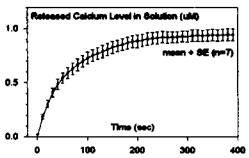
Supported by NIH and American Heart Association, Indiana Affiliate.

Tu-Pos183

TOTAL SR Ca^{2+} RELEASE ESTIMATED IN CULTURED SMOOTH MUSCLE CELLS USING QUANTITATIVE FLUORESCENCE MEASUREMENT.

((**Takao Sugiyama**)) Cardiovascular Division, Institute for Adult Diseases, Asahi Life Foundation, Shinjyuku, Tokyo 160 Japan

The total released Ca^{2+} from sarcoplasmic reticulum (SR) in saponin-permeabilized A7r5 cells were measured by photomultiplier using fura-2. Cells were cultured on the bottom surface of the well (W 9 x L 35 x D 0.1 mm) made on a small quartz slide which was covered by a quartz lid with an inlet and an outlet in order to create a perfusion volume. After the saponin-permeabilization, cells were perfused with the Ca^{2+} loading solution containing 200 nM Ca^{2+}, 1.5 mM Mg^{2+} and 3.15 mM ATP for 10 min. Ca^{2+} release was initiated by changing the solution to the release solution containing 1.5 mM Mg^{2+}, 0.423 μM (contaminated) Ca^{2+}, 0 mM ATP, and 3.16 μM free fura-2. During Ca^{2+} release, the flow of the release solution was stopped and the released Ca^{2+} was accumulated in the solution and measured by free fura-2. By using measured values for the average cell volume (9.3 pl/cell), percentage of saponin permeabilized cell number (80%), cultured cell density (1.9 x 10^4 cells/cm^2) and SR/cell volume ratio (5%, by Somlyo), the results indicate that 1.30 mmol/l SR Ca^{2+} was released in the initial 5 min. And moreover, when this release procedure was repeated 5 times without any Ca^{2+} loading to SR during Ca^{2+} release, total released Ca^{2+} was estimated about 5.11 mmol/l SR (n=1).



Tu-Pos184

PURIFICATION AND CHARACTERIZATION OF CANINE CARDIAC TRIADIN-1 OVEREXPRESSED IN INSECT CELLS.

((**Yvonne M. Kobayashi** and Larry R. Jones)) Krannert Institute of Cardiology, Dept. of Biochemistry & Molecular Biology, Indiana University School of Medicine, Indianapolis, IN 46202.

Triadin is an intrinsic junctional sarcoplasmic reticulum (JSR) protein postulated to play a role in excitation-contraction coupling. Molecular cloning and immunological studies have shown triadin to have three isoforms in rabbit heart of 35-, 40-, and 92-kDa. To further characterize the cardiac JSR protein complex, we have cloned the cDNA for the canine cardiac triadin-1 isoform. The protein contains 278 amino acids with a calculated molecular weight of 31,000. The deduced amino acid sequence shows that the canine protein has two 4-amino acid deletions in its lumenal domain compared to the rabbit isoform. The areas of deletion are within a "KEKE motif" proposed to compose part of the "polar-zipper" involved in protein-protein interactions. When recombinant canine cardiac triadin-1 was expressed and purified from Sf21 insect cells it exhibited an electrophoretic mobility indistinguishable from that of the 35-kDa protein in dog heart JSR vesicles. Recombinant triadin-1 also bound to canine cardiac calsequestrin in overlay assays. Polyclonal antibodies raised to recombinant canine cardiac triadin-1 reacted with two prominent bands of 35-kDa and 40-kDa on Western blots of canine cardiac JSR vesicles. We are currently raising antibodies to the unique C-terminus of canine cardiac triadin-1 and overexpressing the protein in transgenic animals to define its role in cardiac excitation-contraction coupling.

Tu-Pos185

LOW-RESOLUTION LOCALIZATION OF THE HIGH AFFINITY CALMODULIN BINDING SITE OF THE CARDIAC RYANODINE RECEPTOR (RYR) ((**Badr A. Al-Seikhan**, Gale M. Strasburg, Larry R. Jones)) Krannert Institute of Cardiology, Indiana University School of Medicine, Indianapolis, Indiana 46202. (Spon. L. Jones)

Calmodulin (CaM) is a known inhibitor of Ca release through the RyR. Previously, it was shown that the RyR is the major ^{125}I-azido-CaM binding protein in cardiac SR vesicles [Seiler *et al.* JBC (1984) 259:8550-8557]. To localize CaM binding site on the cardiac RyR, we raised a monoclonal antibody (mAb1E9) to amino acid residues 2666-2833 of the canine cardiac RyR which contains the CaM Kinase phosphorylation site (Ser 2809) and one predicted CaM binding site (residues 2775-2807). The RyR in canine cardiac junctional SR vesicles was photoaffinity labeled with ^{125}I-Bz-CaM and the vesicles were proteolyzed with calpain II, which selectively degrades the RyR into a series of large peptide fragments. SDS-PAGE and autoradiography demonstrated that two proteolytic fragments of 150 kD and 105 kD retained virtually all of the CaM label incorporated into the RyR. These two fragments were recognized by mAb1E9, and appear to be partial and limit peptides, respectively. However, neither of these two fragments reacted with a polyclonal antibody made to RyR residues 2805-2819. These results suggest that the high affinity CaM binding site resides somewhere between amino acid residues 1900-2804 of the cardiac RyR. Currently, we are attempting to determine the precise CaM binding site by peptide sequencing of labeled fragments.

Tu-Pos186

SPHINGOSYLPHOSPHORYLCHOLINE MODULATES Ca^{2+} RELEASE FROM CARDIAC SARCOPLASMIC RETICULUM. ((R. Betto[1], A. Teresi[1], G. Salviati[1], R.A. Sabbadini[2], K. Krown[2], C.C. Glembotski[2], L.A. Kindman[3], C. Dettbarn[4], Y. Péréon[4], K. Yasui[4], **P.T. Palade**[3])) [1]CNR-Muscle Biol. Unit, Padova, Italy, [2]Dept. Biol., San Diego State Univ., San Diego, CA 92182, [3]Dept. Med., Duke Univ. Med. Ctr., Durham, NC 27710, [4]Dept. Physiol., Univ. TX Med. Br., Galveston, TX 77555-0641. (Spon. by S.A. Lewis)

In this study we present evidence that sphingosylphosphorylcholine (SPC) stimulates Ca^{2+} release from the cardiac sarcoplasmic reticulum by activating the ryanodine receptor. Action of SPC on the ryanodine receptor is demonstrated by the observation that ryanodine, ruthenium red and sphingosine antagonized the SPC-induced Ca^{2+} release. In the absence of ATP, SPC also stimulated [^{3}H]-ryanodine binding to isolated cardiac sarcoplasmic reticulum vesicles. SPC shifts to the left the Ca^{2+} dependence of [^{3}H]ryanodine binding, but only at high pCa values. In intact cardiac myocytes, even in the absence of extracellular calcium, SPC causes a rise in diastolic Ca^{2+}, which is greatly reduced when the sarcoplasmic reticulum is depleted of Ca^{2+} by prior treatment with thapsigargin. The presence of a ruthenium red-insensitive SPC-induced Ca^{2+} release suggested the possible involvement of another SPC sensitive efflux mechanism in cardiac sarcoplasmic reticulum. Recently, a new intracellular SPC-gated calcium channel, distinct from other previously identified channels, has been discovered. The SPC-gated channel/modulator (SCaMPER) has been recently cloned and when expressed into oocytes it causes SPC-induced Ca release from internal stores (Mao et al., PNAS 93:1993-1996). Using ribonuclease protection assays, we have determined that its message is expressed in rat heart at levels comparable to that of the dihydropyridine receptor. In addition, SCaMPER has been detected at similar levels in other tissues (brain, skeletal muscle). We conclude that SPC releases calcium from cardiac sarcoplasmic reticulum membranes by activating the ryanodine receptor and possibly another intracellular Ca^{2+} release channel, which we have identified for the first time in cardiac tissue. Supported by Telethon Italy (469), CNR, MPI, NIH, AHA.

Tu-Pos187

SEPARATION OF ACTIVATOR FROM DEACTIVATOR EFFECTS OF RYANOIDS ON CALCIUM-RELEASE CHANNELS: EFFECTS IN RAT HEARTS. ((**H.R. Besch, Jr.**, K.R. Bidasee, J.T. Emmick, K. Gerzon, J.A. DiMicco, J. Pfaffenberger, P.V. Sulakhe*, T. Morris* and X. Vo*)). Dept. of Pharmacol. and Toxicol., Indiana Univ. Sch. of Med., Indianapolis, IN 46202 and *Dept. of Physiol., Univ. of Saskatchewan, Saskatoon, Canada.

The alkaloid ryanodine (Ry) activates (open) calcium-release channels (CRCs) at low concentrations (nM to low μM) and deactivates them at higher μM concentrations. Certain of our C_{10} ester derivatives of Ry, namely C_{10}-O_{eq} β-alanyl ryanodine (βARy) and C_{10}-O_{eq} guanidino propionyl ryanodine (GPRy) exhibit affinities greater than that of the parent molecule, but exclusively activate the CRCs when assessed using junctional sarcoplasmic reticulum membrane vesicles. In single channel experiments, these compounds cause CRC to enter into states of reduced conductance that have high open probabilities. However, our studies show that heart rate and blood pressure changes induced by βARy and GPRy in anesthetized rats differ little from those of parent Ry, with but one exception, the latter compounds were less toxic. To investigate this lack of difference, we used digitonin-permeabilized cardiac myocytes isolated from adult rat hearts. The SR of such cells was loaded with $^{45}Ca^{2+}$ (with or without PO_4 or oxalate) and then incubated with ryanodine or with βARy and GPRy. In these cells, Ry showed a threshold of 0.1 nM, maximal Ca^{2+}release at 0.3 μM, and complete abolition of Ca^{2+} release at 100 μM. βARy and GPRy had similar thresholds, but showed no ability to diminish Ca^{2+} loss, up to 100 μM. Caffeine's actions were exclu-sively activator over the range of 0.1 to 10 mM. Thus, ryanoids' effects on isolated CRCs mimic that in digitonin-permeabilized myocytes. These data also suggest that ryanoids effect on cardiovascular parameters accrue from the activator actions of these drugs.

Supported in part by the Showalter Trust

Tu-Pos188

AFFINITY OF RYANOIDS FOR CALCIUM-RELEASE CHANNEL DOES NOT PREDICT THEIR CHANNEL MODULATING CHARACTERISTICS. ((**K.R. Bidasee** and H.R. Besch, Jr.)) Dept. of Pharmacol. and Toxicol., Indiana Univ. Sch. of Med., Indianapolis, IN 46202-5120.

Ryanodine (Ry) is peculiar among channel-active ligands in that its concentration-effect curve has two distinct limbs, the first for opening and the second for closing calcium-release channel (CRCs). In prior efforts to separate these limbs, we focused on modifying the C_{10} equatorial hydroxyl on the C-ring. Most of these semi-synthetic ryanoids were differentially effective at opening (activating) CRCs. In the present study we investigate modifications on the A-ring (C_3) in combination with those on the C-ring previously described. Expansion of the pyrrole ring on the C_3 carbon of Ry to a pyridine ring as in ryanodyl-3-nicotinate (Rn) or hydrolysis of this ester to form ryanodol (Ryol) significantly reduces affinity for CRCs (IC_{50} of 700nM and 8000nM, respectively, compared to Ry's 6.2nM). These modifications also alter the ryanoids' channel modulating profile in a manner not parallel to their affinities (Ry exhibits an EC_{50act} = 4.25μM and $EC_{50deact}$ = 250μM, open CRCs to 81.5% of maximum, Ryol has EC_{50act} of 1000μM, $EC_{50deact}$ of 1.7mM, and open CRCs to 48% of maximum while Rn has EC_{50act} of 3.0μM, $EC_{50deact}$ of 2400 μM, open CRCs to maximum). The loss in affinity of Rn and Ryol for CRCs could be partially restored by esterifying their C_{10} secondary hydroxyls with guanidino propionyl (IC_{50} of 20nM for C_{10}-O_{eq} guanidino propionyl Rn and IC_{50} = 400nM for C_{10}-O_{eq} guanidino propionyl Ryol, respectively). Interestingly, this modification produced ryanoids that were more potent at activating CRCs than their parent. These data indicate that affinity differences among ryanoids fail to predict their corresponding channel modulating profiles.

Supported in part by the Showalter Trust

Tu-Pos189

STOCHASTIC MODEL OF THE RYANODINE RECEPTOR AND THE SPARK-TO-WAVE TRANSITION IN CARDIAC MYOCYTES. ((G. D. Smith, J. Keizer)) Institute of Theoretical Dynamics, Section of Neurobiology, Physiology, and Behavior, and Biophysics Graduate Group, U.C. Davis, Davis, CA 95616.

As part of a theoretical analysis of the spark-to-wave transition in Ca^{2+}-overloaded cardiac myocytes (Cheng et al., 1996), we present a stochastic model of an isolated ryanodine receptor (RyR) derived from a deterministic model used to analyze Ca^{2+} influx-dependent Ca^{2+} oscillations in the bull-frog sympathetic neuron (Keizer and Levine, 1996). This stochastic model inherits fast Ca^{2+}-activation and slower adaptation from the deterministic model and similarly reproduces peak and plateau open probability results (Györke and Fill, 1993 and 1994; Györke et al., 1994). Most importantly, the stochastic model accounts for the presence of elevated domain Ca^{2+} ($[Ca^{2+}]_d$) when the channel is open and Ca^{2+} is the current carrier. Assuming Ca^{2+} sparks are generated by an isolated RyR, we fit measurements of open and closed dwell times made both *in vitro* (~ 1 ms; Valdivia et al., 1995) in the absence of elevated $[Ca^{2+}]_d$ and *in vivo* (~ 15 ms; Cheng et al., 1993) in the presence of $[Ca^{2+}]_d$. We implement a numerical scheme which simulates the interaction between Ca^{2+} sparks by calculating buffered Ca^{2+} diffusion between spatially separated model RyRs responsive to the local Ca^{2+} concentration.

Tu-Pos190

THERMAL EFFECTS ON THE STRUCTURE AND FUNCTION OF SARCOPLASMIC RETICULUM ATPase. AN INFRARED STUDY. ((I. Echabe, **J.L.R. Arrondo** and F.M. Goñi)) Dept. Biochemistry, Univ. Basque Country, E-48080 Bilbao, Spain.

Infrared spectroscopy can be used in the study of the conformation of membrane proteins. The presence of α-helix is dominated by a band around 1650 cm^{-1} whereas ß-sheet or intermolecular extended chains give rise to two bands located at 1620-1630 and 1675-1690 cm^{-1}. One of the difficulties encountered in the application of infrared spectroscopy to the study of protein is the assignment of the bands to specific structural features. Because of the sensitivity of the infrared bands to changes in hydrogen bonding and dihedral angles geometry, band assignment can be made by attributing certain spectral intervals to givem structures. Recently, we have shown that some changes in the canonical wavenumber of infrared bands are due to specific confomational characteristics of the protein. Also, quantitation of structures by infrared spectroscopy has still some difficulties because it is not known undoubtedly if all the protein structures have the same molar absorptivity. The work presented here deals with the structure and assignment of sarcoplasmic reticulum ATPase. The use of temperature and limited proteolysis allows us to detect an α-helix structure coupled to ß-sheet, that gives rise to a band located at ~1640 cm^{-1} in H_2O. Thermal denaturation is not a two-state process, but presents an intermediate conformation before denaturation that is associated with oligomer-monomer transitions.

Tu-Pos191

NEGATIVELY CURVED CALCIUM WAVES IN CARDIAC MYOCYTES: VELOCITY CURVATURE RELATIONSHIP

((M.H.P. Wussling, K. Scheufler)).
Department of Physiology, Martin Luther University of Halle-Wittenberg, D-06097 Halle, Germany.

When spherical calcium waves with positive curvature collide with each other, new wavefronts which propagate perpendicularly to the original direction develop and drift apart with an abruptly enhanced velocity. Those wavefronts are considered to be negatively curved.
We have investigated development, collision, and annihilation of spontaneous calcium waves in freshly prepared rat cardiac myocytes loaded with 5 µM fluo-3 AM. For the measurement of spatio-temporal Ca^{2+}-patterns we used the confocal laser scanning microscope INSIGHT PLUS (Meridian, Okemos, MI).
Negatively curved waves propagate faster than those with positive curvature but the velocities of positively and negatively curved wavefronts, respectively, aim at the same value at zero curvature. It is suggested that the mechanism of CICR (calcium induced calcium release) underlying spontaneous Ca^{2+} waves in cardiomyocytes may be described in terms of the Belousov-Zhabotinskii reaction.

(Supported by Deutsche Forschungsgemeinschaft, Wu 194/3-2)

Tu-Pos192

DETERMINATION OF VELOCITY AND CURVATURE OF COLLISION - INDUCED CALCIUM WAVES

((V. Drygalla[1], S. Schmerling[1], and M. H. P. Wussling[2])).
[1]Institute of Numerical Mathematics, [2]Dept. of Physiology, Martin Luther University of Halle-Wittenberg, D-06097 Halle, Germany.

Colliding spherical calcium waves in cardiac myocytes develop new wavefronts analog to "cusp"- like structures as described in chemical systems of the Belousov-Zhabotinskii reaction. To determine negative curvature and propagation velocity, calcium wavefronts in the region of collision were acquired by confocal microscopy (using the fluorescent Ca^{2+} indicator fluo-3 AM) and approximated in pairs by parabolic functions. The procedure of approximation described in this study results (i) in the curvature K which is considered to be negative at the vertices of the parabolae, and (ii) in the normal velocity N of the apart drifting vertices.
The data {N,K} based on negatively curved calcium waves in rat cardiocytes was fitted using the velocity curvature relationship $N = c - D^*K$ (c = velocity of planar waves, D = diffusion coefficient) by orthogonal weighted regression, and resulted in $N = 85.7 - 120^*K$ with $R_{crit} = 1/K = 1.4$ µm at N = 0 and $D = 1.2 * 10^{-4}$ mm²/s.
(Supported by Deutsche Forschungsgemeinschaft WU 194/3-2)

MUSCLE REGULATORY PROTEINS II

Tu-Pos193

PHOSPHOLAMBAN GENE EXPRESSION IN CELLS AND TRANSGENIC MICE ((**K. Haghighi**, V.J. Kadambi, K.L. Koss, and E.G. Kranias)) Univ. of Cincinnati, Collage of Medicine, Cincinnati, OH (Spon. by E. Kranias, Ph.D.)

Phospholamban (PLB) is an integral sarcoplasmic reticulum (SR) phosphoprotein, that regulates the activity of the SR Ca^{2+}-ATPase. Alterations in the levels of PLB relative to the SR Ca^{2+}-ATPase are associated with alterations in cardiac mechanics. In order to identify regulatory factors that participate in modulating PLB gene expression, we initiated PLB promoter analysis both *in vitro* and *in vivo*. Seven PLBCAT fusion genes were generated containing 7, 5, 2, 0.6 ,0.2, 0.1 and 0.05 kb of the 5' upstream region from the transcriptional start site (tss), exon I, the entire intron, and 88 bp of exon II fused to the CAT reporter gene. In parallel, two additional constructs designated 5Δ2.8 and 2Δ2.8, which are identical to the 5 and 2 kb fusion genes except for the absence of a 2.8 kb intronic fragment, were generated. This 2.8 kb intronic fragment contains several repetitive elements which may regulate PLB gene expression. All fusion genes were transiently transfected into H9C2 and L6 rat myoblasts, and the results are summarized as % CAT activity for each construct relative to the pSVCAT (100%) in the Table below. To assess the activity of these fusion genes *in vivo*, five of the fusion genes (7, 5, 5Δ 2.8, 2 and 2Δ2.8 kb), were utilized to generate transgenic mice. Preliminary data indicate that the 7, 5, 5Δ2.8 and 2 kb constructs, were highly active, while the 2Δ 2.8 was silent *in vivo*. These results suggest that: a) the regulatory elements controlling PLB gene expression *in vitro*, are located within 600 bp upstream from the tss; and b) the 2.8 kb intronic fragment appears to contain important regulatory elements which are capable of modulating PLB gene expression *in vivo*.

PLBCAT	7	5	5Δ2.8	2	2Δ2.8	0.6	0.2	0.1	0.050
H9C2	78±1	25±2	88±1	63±2	82±2	65±1	34±1	7±2	8±1
L6	82±1	34±1	89±2	66±2	85±1	61±1	33±1	9±2	7±1

Tu-Pos194

PHOSPHOLAMBAN INHIBITORY EFFECTS UPON ITS REINTRODUCTION INTO THE KNOCKOUT BACKGROUND.

((**Yoji Sato**, Wusheng Luo, Vivek J. Kadambi and Evangelia G. Kranias)) Univ. of Cincinnati, Cincinnati, OH 45267. (Spon. by Guoxiang Chu)

Phospholamban-knockout (PLB-KO) mice exhibit significant enhancement in basal contractile parameters and attenuation of β adrenergic responses in either isolated cardiac myocytes or perfused beating hearts. To determine whether these phenotypic alterations can be reversed by PLB expression, we reintroduced PLB into the PLB-KO mouse using a cardiac specific α-myosin heavy chain promoter. We obtained two transgenic mouse lines, M_1 and M_2, with different expression levels of the reintroduced PLB (70 and 200 % of wild-type (100 %) mice, respectively). The hearts of the PLB-reintroduced mice were perfused by the Langendorff method in parallel with hearts from wild-type and PLB-KO mice. The left ventricular function was monitored through a fluid-filled catheter-transducer system with frequency-response being flat (± 10%) at least up to 47 Hz. The hearts from M_2 mice exhibited complete reversal of the enhanced basal contractile parameters and the attenuated isoproterenol response, observed in the PLB-KO hearts. However, the M_1 hearts exhibited partial reversal of the enhanced contractility in PLB-KO hearts (71 and 73 % in basal + and $-dP/dt_{max}$, respectively). These results suggest that reintroduced PLB in the PLB-KO background is capable of reversing the physiological alterations, observed upon ablation of PLB, and further confirm that the basal cardiac contractility and its responses to isoproterenol are dependent on the PLB levels expressed in the heart.

Tu-Pos195

POLYAMINE EFFECTS ON CALCIUM SENSITIVITY IN SKINNED CARDIAC MYOCYTES. ((**S.P. Harris**, K.T. Strang, and R.L. Moss)) Dept. of Physiology, University of Wisconsin, Madison, WI 53706.

Polyamines are naturally occurring polycationic substances present in most cells at high concentrations. Aside from their essential roles in cellular growth and differentiation, endogenous polyamines (e.g. spermine, spermidine, and putrescine) are thought to participate in intracellular calcium (Ca^{2+}) homeostasis, membrane excitability, and regulation of contractility in cardiac myocytes. Although many of these effects may be attributed to their interactions with membrane proteins (e.g. sarcoplasmic reticulum Ca^{2+}/ATPase), it is unclear whether polyamines exert direct effects on myofilament proteins as well. In the current study, we therefore utilized skinned cardiac myocytes to assess the effects of polyamines on myofilament Ca^{2+}-sensitivity. Single, skinned myocytes were obtained via enzymatic digestion of rat ventricles followed by cell permeabilization in Triton X-100. Isometric tension-pCa relationships were then measured in the same cell in the absence or presence of spermine (1 mM). In the presence of spermine the [Ca^{2+}] required for half-maximal tension was increased (right shifted tension-pCa curve) compared to control conditions, indicating a reduction in the Ca^{2+}-sensitivity of tension development. The slopes of the tension-pCa relationships were comparable, however, indicating that spermine did not affect the apparent molecular cooperativity of activation. Washout of spermine returned Ca^{2+}-sensitivity to control values. These results indicate that spermine exerts direct effects on the calcium sensitivity of myofilament proteins and suggest that endogenous polyamines may modulate cardiac contractility through direct interactions with contractile proteins. Supported by NIH HL47053.

Tu-Pos196

A NEW FACTOR IN THE CROSSTALK BETWEEN CARDIAC MYOCYTES AND VASCULAR ENDOTHELIAL CELLS. ((S. Winegrad*, D. Henrion**,G. McClellan*, J.L. Samuel#, L. Rappaport#)). Dept. of Physiology, School of Medicine, University of Penna., Phila. Pa.* & INSERM U127# & U141**, Hopital Lariboisiere, Paris.

Coronary vascular endothelial cells release substances that modify the contraction of the cardiac myocytes. The major positively inotropic substance is endothelin. This action has been studied in the isolated perfused working heart by collecting the coronary venous effluent, reoxygenating it and then assaying it for cardioregulatory activity by using it as the bathing medium for an isolated cardiac trabecula. The nature of its effect on contractility is closely related to the pO_2 in the coronary venous effluent before it was reoxygenated: the higher the pO_2, the greater the positive inotropy and the higher the concentration of endothelin in the perfusate. The relation between pO_2 and positive inotropy does not require the vascular endothelium in the perfused heart but does require the endothelial cells in the assay trabecula. These observations have led to the hypothesis that cardiac myocytes in response to local pO_2 secrete a factor that stimulates endothelial cells to secrete endothelin. This hypothesis has been tested with cultures of isolated adult rat cardiac myocytes by exposing the myocytes to different oxygen tensions and assaying the conditioned incubation media for a factor that stimulates the release of endothelin in relation to the pO_2 during the incubation. The results show that the cardiac myocytes are oxygen sensors and produce a substance that causes aortic rings to secrete endothelin and to generate greater force. This substance can act to coordinate the contractility of the cardiac myocytes and vascular smooth muscle.(Sup. by Fogarty Fellowship, NIH grant HL 16010, & INSERM)

Tu-Pos197

CGP-48506, A UNIQUE MYOFILAMENT CA-SENSITIZER, REVERSES ACIDOSIS-INDUCED DEPRESSION OF MYOCYTE CONTRACTION ((**H.Minami, B.M.Wolska,** M.O. Stojanovic and R.J.Solaro)) College of Medicine, Dept. of Physiology & Biophysics, University of Illinois, Chicago, IL 60612-7342

A reduced myofilament response to Ca^{2+}, involving both a decrease in myofilament Ca^{2+}-sensititivy and maximum activity, is a prominent mechanism for the decrease in cardiac contractility associated with acidosis. We tested whether CGP-48506, a unique agent that increases the myofilament response to Ca^{2+}, is able to reverse the depression of cardiac contraction associated with hypercapnic acidosis in isolated rat cardiomyocytes. Unlike other agents in this class, CGP-48506 has no detectable inhibition of phosphodiesterase type III. We (Wolska et al. Am. J. Physiol. 1996; 270: H24-H32) showed that CGP-48506, which is a benzodiazocine derivative, has direct effects on the actin-myosin reaction that result in an increase in myofilament Ca^{2+}-sensitivity with little change in maximum force. Compared to controls superfused at pH 7.4, cells at pH 6.8 demonstrated a significantly reduced extent of cell shortening (42.1±3.2% of control, mean±SE, n=27) with little increase in the peak of Ca^{2+}-transient. Moreover, cells in acidosis showed small but significant decreases in time to peak shortening to 50 and 75% relaxation. Superfusion of the cells with 3, 7 and 10μM CGP-48506 restored the inhibited contractility as a function of concentration, with no effect of Ca^{2+}-transient. Moreover, 10μM CGP-48506 completely reversed the depressed cardiac contraction with increase in time to peak shortening to 50 and 75% relaxation. Our results indicate that the depression of contractility associated with acidosis is due to a reduced myofilament response to Ca^{2+}, which can be overcome by agents working through a direct effect downstream from troponin C.

Tu-Pos198

α-ADRENERGIC RECEPTOR STIMULATION IMPROVES POST-ISCHEMIC MYOCARDIAL FUNCTION CONCOMITANT WITH A DECREASE IN MYOFIBRILLAR CA^{2+} ACTIVATED ACTOMYOSIN MGATPASE. ((**W.G. Pyle** & P.A. Hofmann))
Department of Physiology, University of Tennessee, Memphis, TN 38163.

A 25-35% decrease in ventricular myocyte maximum velocity of unloaded shortening is observed following stimulation with known cardioprotective agents such as α-adrenergic (Strang & Moss, *Circ Res* 77:114, 1995) and adenosine (Lester & Hofmann, *Am J Physiol* 271:In Press, 1996) receptor agonists. We hypothesize that protein kinase C-dependent phosphorylation of myofilament proteins, the slowing of actin-myosin cycling kinetics, and resulting reduction in myocyte ATP consumption contributes to the cardioprotective ability of these activators of PKC. To test this hypothesis isolated rat hearts perfused at a constant pressure were subjected to 30 min of global no-flow ischemia (37°C), reperfused, myofibrils isolated in the presence of a phosphatase inhibitor, and maximum activity of myofibrillar Ca^{2+}-dependent actomyosin MgATPase determined. Hearts were either untreated, or exposed to 10 μM phenylephrine (PHE; α-adrenergic receptor agonist) prior to ischemia. Left ventricular developed pressure (LVDP) following ischemia and 5 min of reperfusion were 70% of pre-ischemic LVDP in untreated and 90% of pre-ischemic LVDP in PHE treated hearts. PHE induced an approximate 40% decrease in maximum activity of myofibrillar Ca^{2+}-dependent actomyosin MgATPase. These results are consistent with the hypothesis that α-adrenergic agonists enhance post-ischemic function through a reduction in Ca^{2+} activated actin-myosin ATP consumption. We hypothesize that the decrease in ATP utilization by myofilaments is cardioprotective due to maintained function of ATP-dependent channels and pumps responsible for a low intracellular $[Ca^{2+}]$.

Tu-Pos199

CALCIUM BINDING TO THIN AND THICK FILAMENTS OF VERTEBRATE SKELETAL MUSCLE FIBERS STRETCHED TO BEYOND OVERLAP. ((J. G. Eichen and **M. E. Cantino**))
Dept. of Physiology and Neurobiology, U-131, Univ. Conn., Storrs, CT 06269.

Effects of crossbridge attachment on the binding of Ca to troponin C are thought to contribute to the cooperativity in the force-pCa relationship in striated muscles. In a previous paper (Biophys. J, 64:211 1993) we used electron probe x-ray microanalysis (EPXMA) to show that Ca levels were enhanced in the region of filament overlap in rabbit psoas muscles in rigor. This was interpreted as showing a direct effect of rigor crossbridge attachment on the binding of Ca to TnC. In the present study we investigated whether the increased Ca observed in the overlap region might result from increased Ca binding intrinsic to the ends of either thin or thick filaments. Freeze dried cryosections were prepared from rabbit psoas or frog semitendinosus fibers frozen in rigor as described previously. Data from digital EPXMA images of several sarcomeres were combined to yield an average Ca distribution profile along the A and I bands. Initial studies with rabbit psoas fibers showed little variation in Ca binding along either thick or thin filaments, however filament alignment in these overstretched sarcomeres was poor. Further studies with frog semitendinosus fibers, in which much better filament alignment could be maintained at long lengths, showed similar results. Uniform distributions were found for Ca levels expected to saturate TnC calcium binding sites, as well as for levels below saturation. These results support a direct effect of rigor crossbridge attachment on Ca binding to TnC.

Tu-Pos200

THE RELATIVE INDEPENDENCE OF REGULATED THIN FILAMENT SLIDING SPEED ON THE FRACTION OF ACTIVATED REGULATORY UNITS AT SATURATING Ca^{2+}. ((**C.A. Morris**, A. Bobkova, L. Tobacman and E. Homsher)) Dept. Physiology, UCLA, Los Angeles, CA, 90095 and Dept. Medicine, U. Iowa, Iowa City, IA 52242.

Regulation of thin filament sliding speed by Tn/Tm was investigated using a mutant TnC, CBMII, containing an inactivated regulatory site II. Mutant troponin (MutTn), reconstituted from native TnT, TnI and CBMII TnC, binds to thin filaments 2.2 times stronger than native cTn at pCa 5.0 and with cTm completely inhibits acto-S1 MgATPase (Huynh et al. Biophys. J. [1996], 70: 1447). The role of inactive Tn units on thin filament sliding speed in the motility assay was investigated by varying the ratio of MutTn/cTm: native cTn/cTm, maintaining a constant [Tn/Tm], added to rhodamine-phalloidin labeled F-actin. Thin filament sliding speed was measured in a motility chamber (pCa 5.0) in the presence of the 0.1 μM exogenous regulatory proteins at an ionic strength of 27 and 50mM at 25 C. Complete regulation (no movement) of the thin filaments was observed with both MutTn/cTm at pCa 5.0 and cTn/cTm at pCa 9.0 when 0.1μM exogenous Tn/Tm was added to the assay chamber. At 50mM ionic strength, the sliding speed of the thin filaments containing as much as 72% MutTn was >90% (96% of the filaments moving) of that of native Tn/Tm regulated controls. At 90% MutTn, the thin filament sliding speed dropped to 20% of control values (37% of the filaments moving). This data suggest that when the fraction of activated regulatory units is >0.25 the filament sliding speed is maximal at 50mM ionic strength. At 27mM ionic strength, 40% MutTn did not reduce sliding speed (95% moving) while 70% MutTn decreased sliding speed to 42% (75% of filaments moving) of control the value. These parallel results, with the decline in sliding speed occurring at >40% MutTn, suggest that weak binding by HMM to the thin filaments exerts a greater influence on sliding speed at lower ionic strengths. (Supported by NIH grants AR 30988 (EH) and HL 38834 (LST)).

Tu-Pos201

Non-Guanyl Cyclase-Mediated Anti-adrenergic Effects of NO_x on Protein Phosphorylation in Rat Ventricular Myocytes. ((**M.O. Stojanovic**, B.M. Wolska, G.M. Wahler, and R.J. Solaro)) Department of Physiology and Biophysics, University of Illinois, Chicago IL 60612

We have shown *(J.Mol.Cel.Cardiol. 28(6): A127, 1996)* that anti-adrenergic effects of nitric oxide (NO) in cardiomyocytes include a decrease in the phosphorylation of proteins that are substrates for protein kinase A. We examined whether these effects are guanyl cyclase/cGMP-mediated. ODQ (1H-[1,2,4]oxadiazolo[4,3-a]) was used as a selective inhibitor of soluble guanyl cyclase (sGC). Rat cardiomyocytes were preincubated with 10 μM ODQ and treated with: i) 0.5 μM Isoproterenol (ISO) + ODQ, or ii) ISO + ODQ + 200 μM SIN-1. We observed a significant decrease in phosphorylation of phospholamban (PLB) and troponin-I (TnI) in group ii (TnI=68±16%; PLB=41±26%, n=5) as compared to group i (TnI&PLB=100%). In another experiment, cells were treated with: iii) ISO + 50 U/ml Superoxide Dismutase & 300 U/ml Catalase (SOD/Cat), or iv) ISO + SOD/Cat + 200 μM SIN-1. The addition of SIN-1 to ISO+SOD/Cat did not result in a decrease in PLB and cTnI phosphorylation. These results indicate that anti-adrenergic effects of NO that produce the decrease in PLB and TnI phosphorylation: 1) are not mediated by sGC, and 2) may be induced by NO related congeners (NO_x) including peroxynitrite.

Tu-Pos202

COOPERATIVE ACTIVATION OF CONTRACTION OF CARDIAC MUSCLE BY BOUND CROSS-BRIDGES. ((**S.H. Buck***, P.J. Konyn*,R.L. Moss#))
Departments of *Pediatrics and #Physiology, University of Wisconsin, Madison WI 53706.

Activation of cardiac muscle appears to be a highly cooperative process. Previous work has demonstrated that rigor and cycling cross-bridges increase thin filament Ca^{2+} affinity suggesting that myosin head binding may participate in the process of activation. To assess the contribution of strong-binding myosin heads to the cooperative activation of contraction, we examined the effect of NEM-S1, a strong-binding non-force-generating myosin S1 analogue, on the tension-pCa relationship of single skinned ventricular myocytes from rat. Steady state force at sarcomere length 2.3 μm and 15°C was determined at maximal $[Ca^{2+}]$ (pCa 4.5) and submaximal $[Ca^{2+}]$ by slackening the myocyte after steady force was achieved. After the baseline tension-pCa relationship was determined, myocytes were incubated in 6 μM NEM-S1 in relaxing solution (pCa 9) and the tension-pCa relationship was determined again. NEM-S1 enhanced submaximal but not maximal tension (n=8). The greatest effect was observed at pCa > pCa_{50}, resulting in a decrease of the Hill coefficient from 3.7 to 1.6 ($p < .001$). The effect of NEM-S1 to increase tension, particularly at low $[Ca^{2+}]$, suggests that strong-binding myosin heads facilitate myocardial contraction by cooperatively activating adjacent regions of the thin filament regulatory strand. To investigate observed differences in activation of contraction by bound cross-bridges in skeletal and cardiac muscle, experiments are proposed utilizing transgenic mice in which skeletal thin filament regulatory proteins are expressed in heart.
Supported by NIH HL47053 and K08HL03134 and the AHA.

Tu-Pos203

IDENTIFICATION OF MYOCARDIAL MUSCLE PROTEINS SUSCEPTIBLE TO ISCHEMIA/REPERFUSION INJURY ((J.E. Van Eyk[1], F.M. Powers[3], W.R. Law[3], R.S. Hodges[2] and R.J. Solaro)) Dept. Physiol., Queens University, Kingston , Dept. Biochemistry, University of Alberta, Edmonton[2] and Dept. Physiol. & Biophysics, University of Illinois at Chicago, Chicago[3].

Degradation and loss of myocardial proteins may, in part, be responsible for the increased calcium sensitivity (0.26 pCa units) and decreased maximum force (68%) observed in skinned muscle bundles obtained from rat hearts subjected to 60 minutes ischemia and 45 minutes reperfusion. Heart tissue as well as effluent samples collected during reperfusion were analyzed using SDS PAGE, western blot and micro-sequencing techniques. Analysis of tissue showed significant loss of α-actinin, tropomyosin, troponin I (TnI), and the myosin light chains are associated with ischemia/reperfusion. Degradation of TnI was detected using an anti-TnI peptide monoclonal antibody (E2) which recognizes residues 137-148. Isolation of the TnI degradation product (MW=21,000) which co-migrate with myosin light chain 1 on a 12.5% SDS polyacrylamide gel will be discussed. There is also an increase in the quantity of mitochondrial proteins, H^+-transporting ATP synthatase OSC protein (MW=20,500) and ATP synthatase gamma chain (MW=30,200), associated with the ischemic/reperfused tissue. The cytosolic proteins, troisephosphate isomerase and glyceraldehyde phosphate dehydrogenase as well as, the myofibril proteins, α-actinin, tropomyosin, troponin T, myosin light chain 1 are present in reperfusion effluent. However, neither actin or troponin I were detected suggesting only certain myofilament proteins are suspectible to damage. Degradation and loss of these myofilament proteins could alter actomyosin regulation and/or myofilament spacing resulting, in the myocardial dysfunction associated with ischemia/reperfusion injury.

Tu-Pos204

CONFORMATION DEPENDENT PROTEOLYSIS IN LOBSTER SKELETAL MUSCLE MYOSIN FILAMENTS ((**L.D.Brown** and M.E. Cantino)) Dept. of Physiology and Neurobiology, U-131, Univ. Conn., Storrs, CT 06269.

Contraction of muscle is regulated by Ca-dependent processes on the actin or myosin filaments. The lobster superficial abdominal muscle (SFM) is regulated by both actin- and myosin-linked Ca-dependent processes (J. Gen. Physiol. 77:1 1975) however, the nature of the myosin-linked component has not been confirmed. We have used a modification of the proteolytic susceptibility procedure by Frado and Craig (J. Cell Biol. 109:529 1989)to investigate whether the myosin filaments from the SFM are regulated by a Ca-dependent myosin light chain kinase. Myosin filaments were prepared from the SFM by brief homogenization in a relaxing solution containing no calcium. Some filaments were treated with Ca alone or with Ca, calmodulin, and myosin light chain kinase. Each sample was subjected to proteolysis by papain, which attacks the myosin head/rod junction. Preliminary results suggest this region of myosin is more susceptible to proteolysis in the presence of Ca, calmodulin, and light chain kinase, than with Ca alone or under relaxing conditions, suggesting a light chain kinase dependent activation mechanism. We are currently investigating whether activation induced changes in the ordering of crossbridges can be detected by electron microscopy in isolated and synthetic filament preparations.

Tu-Pos205

ALTERATIONS IN CARDIAC GENE EXPRESSION DURING TRANSITION TO COMPENSATED HYPERTROPHY FOLLOWING MYOCARDIAL INFARCTION. ((M. Gidh-Jain, **B. Huang**, P. Jain and N. El-Sherif)) VAMC @ Brooklyn, NY 11209. (Spon. by N. El-Sherif).

To determine time dependent changes in gene expression in an animal model of left ventricular dysfunction, we measured mRNA levels of selective cardiac genes in the LV myocardium of rats, 3 days and 3 weeks after left coronary arterial ligation or sham operation. RNase protection assay was performed to assess the expression of *c-fos*, ANF, BNF, α 2/3 isoform of Na-K Atpase, cardiac α actin and α/β isoform of MHC. The table shows the changes in gene expression in the post-MI remodeled hypertrophied myocardium and compares these changes with those reported in pressure-overload hypertrophy models.

	Pressure overload hypertrophy		Post-MI hypertrophy	
	3 Days	3 Weeks	3 Days	3 Weeks
c-fos/ANF	↑	↔	↑	↑
BNF	↑	↑	↔	↑
α2 Na-K Atpase	↓	↓	↓	↔
α3 Na-K Atpase	↔	↑	↔	↑
α MHC	↓	↓	↓	↔
β MHC	↑	↑	↑	↔

These findings indicate that during left ventricular remodeling and hypertrophy following acute myocardial infarction, there is an upregulation of early response genes and fetal isogene expression. The pattern of activation, however, is distinct from that observed in other overload models, indicating the possible involvement of alternate signal transduction pathways.

Tu-Pos206

INCREASED TROPONIN-I PHOSPHORYLATION AND DECREASED Ca^{2+} SENSITIVITY IN CARDIAC MYOCYTES FROM THE SPONTANEOUSLY HYPERTENSIVE RAT. ((**Bradley K. McConnell**, Christine S. Moravec and Meredith Bond)) Dept. of Physiology & Biophysics, Case Western Reserve University & Dept. of Molecular Cardiology & Center for Anesthesiology Research, Cleveland Clinic Foundation.

We have shown that the decreased inotropic response to β-adrenergic stimulation in hypertrophied hearts from the spontaneously hypertensive rat (SHR) is associated with increased protein kinase A (PKA) dependent phosphorylation of troponin I (TnI) (McConnell et al., submitted). We therefore hypothesized that decreased sensitivity of the myofilaments to Ca^{2+} could contribute to this impaired response. Since phosphorylation of TnI by PKA decreases myofilament Ca^{2+} sensitivity, a higher free $[Ca^{2+}]$ would be required in the SHR to achieve the same degree of actomyosin ATPase activity or force development. We measured actomyosin ATPase activity as a function of increasing $[Ca^{2+}]$, in response to β-adrenergic stimulation in myofibrillar fractions isolated from the SHR with compensatory cardiac hypertrophy (26 weeks) and decompensatory hypertrophy (76 weeks) and in age-matched Wistar Kyoto (WKY) controls. In summary, our results indicate that increased PKA-dependent TnI phosphorylation in the 26 week SHR further decreases the Ca^{2+} sensitivity of troponin C and thus would decrease force development at any given intracellular free $[Ca^{2+}]$. In the 76 week SHR, the greater rightward shift of the Ca^{2+}-dependence of actomyosin ATPase activity is attributed primarily to increased myofilament Ca^{2+} sensitivity under baseline conditions.

strain of rat	EC_{50} *for* Ca^{2+} (control)	EC_{50} *for* Ca^{2+} (ISO)	ΔEC_{50}
26 WKY	5.90 ± 0.02 (1.27 μM)	5.72 ± 0.03* (2.11 μM)	0.18
26 SHR	5.90 ± 0.05 (1.27 μM)	5.55 ± 0.02*† (3.06 μM)	0.35
76 WKY	5.85 ± 0.03 (1.39 μM)	5.59 ± 0.05* (2.64 μM)	0.26
76 SHR	6.12 ± 0.04♦ (0.76 μM)	5.58 ± 0.04* (2.88 μM)	0.54

*p < 0.001 vs control; †p < 0.001 vs 26 wks WKY (ISO); ♦p < 0.001 vs 76 wks WKY (control)

Tu-Pos207

DISSOCIATION OF FORCE GENERATING MYOSIN CROSS-BRIDGES RESULTS IN AN INCREASE IN THE OFF RATE OF Ca^{2+} FROM TROPONIN IN RAT PAPILLARY MUSCLE. ((**Y. Wang**, G. Lui, K. Guth* and W.G.L. Kerrick)) Dept. Physiol. & Biophys., Univ. of Miami Sch. of Med., Miami, FL 33136 and *Scientific Instruments GmbH, Heidelberg, Germany

Rat papillary muscles were loaded with Fura-2 and subjected to quick stretches and releases, length vibrations, and isotonic shortening. The hypothesis tested is that dissociation of myosin cross-bridges during activation of contraction results in the release of Ca^{2+} from troponin. The Fura-2 (340/380) fluorescence ratio (a measure of intracellular Ca^{2+}) and force were measured simultaneously using the Guth Muscle Research System. Quick stretches and releases, length vibrations, and isotonic shortening suddenly applied during the falling phase of the action potential-initiated intracellular Ca^{2+} transient were all associated with a sudden increase in intracellular Ca^{2+} concentration. This mechanically induced increase in intracellular Ca^{2+} could only be elicited in the presence of force generating myosin cross-bridges. These results were interpreted as evidence for an increase in the off rate of Ca^{2+} from the thin filament when force generating myosin cross-bridges dissociate. Quick stretches and releases, and short periods of length vibration and isotonic shortening applied during an isometric twitch not only resulted in a release of Ca^{2+} from troponin, but significant deactivation of the muscle for the remainder of the isometric twitch. These data suggest that once the cross-bridges dissociate during a twitch, the affinity of troponin for Ca^{2+} is significantly decreased resulting in deactivation. Supported by AHA and NIH

Tu-Pos208

THE EFFECT OF pH ON THE RATE CONSTANT FOR THE DISSOCIATION OF FORCE GENERATING MYOSIN CROSS-BRIDGES IN RAT VENTRICULAR SKINNED FIBERS. ((**Y. Xu**, Y. Wang, D. Rowe and W.G.L. Kerrick)) Dept. Physiol. & Biophys., Univ. of Miami Sch. of Med., Miami, FL 33136 (Spon. by P.E. Hoar)

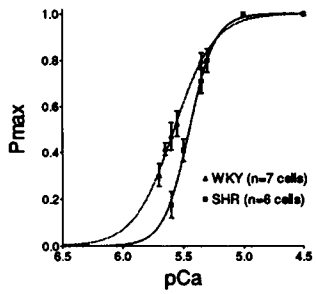
Force and ATPase activity were measured simultaneously in skinned rat ventricular fibers using the Guth Muscle Research System. Graded Ca^{2+}-activation was carried out at pH=6.5, 7.0, and 7.5. In contrast to previous studies where Ca^{2+} concentration was predicted by computer calculations, Ca^{2+} concentration in this study was accurately measured at the three different pH values using the fluorescent Ca^{2+} indicators, Calcium Green-2 and Oregon 5N. The Kds for these fluorescent Ca^{2+} indicators at different pH values were directly measured for the test solutions without the customary use of EGTA or BATA. The results show that 50% of maximal Ca^{2+}-activation of ATPase activity occurred at a lower concentration of Ca^{2+} than 50% of maximal force, and this difference in Ca^{2+} -sensitivity between ATPase and force increased with decreasing pH. At pH=6.5 Ca^{2+}-activation of ATPase reached maximum at approximately 70% of maximum force. Increasing Ca^{2+} concentration further caused force to increase more with a corresponding decrease in ATPase activity. According to Huxley's 1957 model and the assumption that ATP hydrolysis is proportional to cross-bridge turnover, the ratio of ATPase rate/force is proportional to the rate of dissociation of force generating myosin cross-bridges (g_{app}). Thus g_{app} decreases during Ca^{2+}-activation of force and is more pronounced at acid pH. Supported by the NIH and AHA.

Tu-Pos209

ISOLATED CARDIAC MYOCYTES FROM THE SPONTANEOUSLY HYPERTENSIVE RAT SHOW A DECREASED SENSITIVITY TO Ca^{2+}.

((R.E. Palmer and K.P. Roos)) Cardiovascular Research Lab., and Dept. of Physiology, UCLA Medical School, Los Angeles, CA 90095-1760.

Left ventricular myocytes isolated from the spontaneously hypertensive rat (SHR), and age-matched control Wistar-Kyoto (WKY) rat hearts, were attached using double-barreled micropipettes to a force transducer (Palmer et al., *Am.J. Physiol.*,270,C697, 1996). The cells were chemically skinned (Triton X-100 for 10 mins.) and rapid solution exchange allowed the examination of the force/pCa relationship at a sarcomere length of 2.10 μm. The hypertrophied cells exhibited a decreased sensitivity to Ca^{2+} (pCa_{50} = 5.45 ± 0.01), but had an increased Hill coefficient (n_H = 4.06 ± 0.2), as compared to the WKY (pCa_{50} = 5.58 ± 0.01; n_H = 2.62 ± 0.14). Absolute tension generation was increased in the SHR by 58.3 % compared to the WKY. The data indicates alterations in the contractile proteins in this model of hypertrophy.



Tu-Pos210

RAPID RELAXATION AND ACTIVATION OF MYOFIBRILS IN SKINNED VENTRICULAR TRABECULAE FROM RAT AND GUINEA-PIG HEARTS.

((Sue Palmer & Jonathan C. Kentish)) Dept. of Pharmacology, U.M.D.S., St. Thomas' Campus, London SE1 7EH, U.K.

The factors that determine the intrinsic rate of relaxation of cardiac myofibrils are unclear. To examine the relative roles of Ca^{2+} dissociation from TnC and of cross-bridge detachment in determining this rate, we compared relaxation rates in Triton-skinned trabeculae isolated from rat and guinea-pig hearts. Since rat has a higher myofibrillar ATPase activity, cross-bridge detachment in rat is likely to be faster than in guinea-pig. The pCa values for 50% activation were similar (rat: 5.60 ± 0.03, mean ± S.E., n=35; guinea-pig: 5.58 ± 0.04, n=8), suggesting that the rate of dissociation of Ca^{2+} from TnC (k_{off}) was the same in the two species. Rapid relaxation from Ca^{2+} activation was induced by flash photolysis of diazo-2 (0.25 mM) in a muscle bath at 22°C. In the guinea-pig, relaxation was fitted by a single exponential of rate constant 3.0 ± 0.3 s^{-1} (n=8). In the rat, the corresponding value was 18.9 ± 0.9 s^{-1} (n=11). This difference in rate makes it likely that relaxation rate is limited by cross-bridge detachment rather than by k_{off}. We also studied the rapid activation of skinned muscle using flash photolysis of NP-EGTA. Activation was much faster in the rat (14.4 ± 0.9 s^{-1}, n=20) than in the guinea-pig (2.6 ± 0.1 s^{-1}, n=8); activation rate is probably determined by the rate of cross-bridge attachment, since the rates were similar to the rates of force redevelopment after a release/re-stretch (k_{tr}) protocol at maximum Ca^{2+} activation. We conclude that cross-bridge kinetics largely determine the rates of myofibrillar activation and relaxation in skinned cardiac muscles.

Supported by the Royal Society and the British Heart Foundation.

Tu-Pos211

EFFECTS OF SKELETAL α-ACTIN ISOFORM EXPRESSION ON CONTRACTILE FUNCTION OF CARDIAC MYOCYTES ISOLATED FROM BALB/c MICE ((Joseph M. Metzger)) Dept. of Physiology, Univ. of Michigan, Ann Arbor, MI 48109.

During cardiac development there is a transition in α-actin isoform expression from co-expression of the skeletal and cardiac isoforms in early cardiac development, to the nearly exclusive expression of the cardiac isoform in the adult myocardium of rodents. The possible significance of this transition on Ca^{2+} activated contractile function in single cardiac myocytes has not been tested. Owing to a partial duplication in the cardiac α-actin promoter in the BALB/c mouse strain, the α-actin isoform expression pattern is about 50% skeletal and 50% cardiac in the myocardium of the adult animals. Ventricular myocytes were isolated from female adult BALB/c mice, and for controls from C57BL/6 mice. The tension-pCa relationships were not significantly different, in either the position or steepness of the relationship, between these two groups during activation under physiological conditions. Under acidic conditions it is known that maximum Ca^{2+} activated tension is depressed by a greater extent in adult cardiac myocytes compared with skeletal fibers. It is of great interest to define the myofilament isoforms that are responsible for this muscle lineage-dependent phenotype. Thus, we tested whether the cardiac α-actin isoform may confer, at least in part, the greater deficit in maximum tension under acidic conditions. Interestingly, maximum tension was depressed by a greater extent in ventricular myocytes from BALB/c mice [0.41 ± 0.01 Po (pH 6.20/pH 7.00), n = 17] compared with myocytes from C57BL/6 mice (0.48 ± 0.02 Po, n = 7). These findings are evidence that differential expression of α-actin isoforms do not account for the greater acidic pH-induced decrease in maximum force observed in cardiac compared with skeletal muscle fibers.

Tu-Pos212

ADENOVIRUS-MEDIATED TROPONIN T GENE TRANSFER AND EXPRESSION IN ADULT CARDIAC MYOCYTES. ((E.M. Rust, D.E. Michele, M.V. Westfall and J.M. Metzger)) Department of Physiology, University of Michigan Medical School, Ann Arbor, MI 48109-0622.

We report here a new approach to study cardiac contractile protein structure-function relationships which allows the replacement of an endogenous myofilament protein with a genetically engineered mutant without disturbing the appropriate isoform expression and stoichiometry of the intact myocyte. Adenovirus vectors are a highly effective means to introduce foreign genes into adult cardiac myocytes in primary culture. Previous experiments have shown that the stability of the cardiac myocyte contractile assembly is unaltered after adenovirus infection per se. Here we have examined isometric tension development during controlled Ca^{2+} activations to determine the effect of adenovirus infection on adult cardiac myocyte contractile function. Importantly, the shape and position of the tension-Ca^{2+} relationship were not altered in the control and adenovirus-infected myocytes over time in primary culture. In other experiments, recombinant adenovirus vectors have been constructed which contain the CMV promoter driving expression of several myofilament genes including the embryonic isoform of cardiac troponin T (AdCMVeTnT) and human α-tropomyosin (AdCMVSKαTM). Western blot analysis of samples collected from control and AdCMVeTnT-infected cardiac myocytes 5 days post-infection demonstrated expression of the embryonic cTnT isoform only in myocytes infected with AdCMVeTnT. Control non-infected myocytes at 5 days post-isolation expressed only the adult cTnT isoform and not the embryonic isoform. Taken together, these results indicate that adenovirus-mediated gene transfer provides a novel approach to directly study the structural and functional effects of expression of altered myofilament genes in the intact adult cardiac myocyte.

Tu-Pos213

PHOSPHORYLATION AND Ca^{2+} BINDING TO THE REGULATORY LIGHT CHAINS OF MYOSIN IN THE REGULATION OF SKELETAL MUSCLE CONTRACTION ((Danuta Szczesna, Jiaju Zhao, Georgianna Guzman, Gang Zhi[§], James Stull[§] and James D. Potter)) Dept. Mol. & Cell. Pharmacology, Univ. of Miami School of Medicine, Miami FL 33136; [§]Dept. Physiology, UT Southwestern Medical Center, Dallas TX 75235

We have previously shown (Szczesna *et al.*, Bioph. J., 70 A380, 1996) that phosphorylation of the regulatory light chains (RLCs) of rabbit skeletal myosin with Ca^{2+}/CaM activated MLCK increases 1) steady state force development in skinned rabbit skeletal muscle fibers and 2) the Ca^{2+} sensitivity of force development. In the present study we have tested the kinetics of force activation using flash photolysis of the caged Ca^{2+} chelator, DM-nitrophen. We found that the rate of force development increases 1.7±0.4 fold upon RLCs phosphorylation. The same was true when RLCs-depleted fibers were reconstituted with prephosphorylated P-RLCs. The endogenous level of RLCs phosphorylation in the fibers was quite variable (between 20-70%) and in some cases it was necessary to treat the fibers with Mn^{2+} activated phosphatase (PP1) to reduce the endogenous level of phosphorylation.

Recombinant chicken skeletal RLCs (WRLCs) gave the same steady state results as the rabbit RLCs described above. When the WRLCs Ca^{2+} binding site was inactivated (D47A), all of the above steady state effects of phosphorylation were lost. Thus, Ca^{2+} (or possibly Mg^{2+}) binding to RLCs plays a crucial role in the ability of RLCs phosphorylation to modulate force and Ca^{2+} sensitivity.

Tu-Pos214

CHARACTERIZATION OF Ca^{2+}-INDEPENDENT MYOSIN LIGHT CHAIN PHOSPHORYLATION IN CHICKEN GIZZARD MYOFIBRILS. ((J.E. Andrea and M.P. Walsh)) Smooth Muscle Research Group, University of Calgary, Calgary, Alberta T2N 4N1, Canada.

The principal mechanism of smooth muscle contraction involves Ca^{2+}/calmodulin (CaM)-dependent phosphorylation of the regulatory light chains (LC_{20}) of myosin at Ser19 by myosin light chain kinase (MLCK). Intact tissue studies have also implicated a role for Ca^{2+}-independent LC_{20} phosphorylation by an unidentified kinase. We have examined Ca^{2+}-dependent and -independent phosphorylation of LC_{20} in chicken gizzard myofibrils and compared it to phosphorylation by purified MLCK. In both preparations, LC_{20} is rapidly maximally phosphorylated in the presence of Ca^{2+}, CaM and okadaic acid. In the absence of Ca^{2+}, a slow increase in LC_{20} phosphorylation is observed. ITP and UTP can substitute for ATP in Ca^{2+}-dependent, but not Ca^{2+}-independent, LC_{20} phosphorylation. Ser19 is exclusively phosphorylated by purified MLCK in the presence or absence of Ca^{2+} and by the Ca^{2+}-dependent myofibrillar kinase activity. Ca^{2+}-independent LC_{20} phosphorylation in myofibrils occurs on both Ser and Thr residues. Only the Ca^{2+}-dependent LC_{20} phosphorylation by purified MLCK is effectively inhibited by MLCK pseudosubstrate peptides. Ser and Thr residues within peptide SM-1 are phosphorylated in the presence and absence of Ca^{2+} with myofibrils, but not with purified MLCK. The resting level of LC_{20} phosphorylation *in vivo* (0.1-0.2 mol P_i /mol LC_{20}) may thus be due to a Ca^{2+}-independent kinase distinct from MLCK. (Supported by MRC Canada.)

Tu-Pos215

CALDESMON INHIBITS THIN-FILAMENT ACTIVATION OF SMOOTH MUSCLE MYOSIN. **((J.R. Haeberle* and L.P. Adam**)) *Dept.Molecular Physiology and Biophysics, The University of Vermont, Burlington, VT, 05405 and **Boston Biomedical Research Institute, Boston, MA, 02114**

We previously reported that dephosphorylated smooth muscle myosin (dephos SMM) is activated by actin•Tm filaments that have been "turned-on" by high-affinity cross-bridge binding (rigor-dependent activation). In the present study, we have examined the capacity of mammalian smooth muscle caldesmon (CaD) to inhibit this activation. Isometric force and velocity were measured using a standard motility assay with actin•Tm filaments , moving over SMM coated coverslips. Changes in isometric force were measured by adding NEM-modified skeletal muscle myosin to the coverslip to impose a mechanical load on the filaments. An index of relative isometric force (Fnem) was determined as the minimum molar ratio of "NEM-modified myosin/SMM" at which filament motion was completely inhibited (i.e. isometric conditions). Fnem values were normalized to Fnem for thiophos SMM at 1 mM MgATP. Normalized Fnem for control dephosphorylated SMM was <0.005, confirming that there was little or no activation of dephos SMM under control conditions (1 mM MgATP). Reducing the MgATP concentration resulted in an increase in Fnem to a maximum of 0.52 for dephos SMM (0.1 mM MgATP) and 8.6 for thiophos SMM (0.2 mM MgATP). Half-maximal inhibition (I_{50}) of the rigor-activated force with either dephos or thiophos SMM occurred at 50 nM CaD. At higher CaD concentrations (50-500 nM), force production by dephos SMM was completely inhibited. For thiophos SMM, the rigor-dependent increase in force (0.2 mM MgATP) was completely inhibited by 50 nM CaD. In striking contrast, greater than 40 uM CaD was required to inhibit rigor-independent force (1 mM MgATP). These studies suggest that one regulatory function of caldesmon may be to inhibit thin-filament activated contraction of smooth muscle.

Tu-Pos216

CALDESMON'S EFFECTS ON ACTIN FILAMENT MOTILITY, *IN VITRO*, ARE REVERSED BY PHOSPHORYLATION WITH MAPK. ((L.P. Adam[a], P. Graceffa[a] and J.R. Haeberle[b])) [a]Boston Biomedical Res. Inst., Boston, MA, 02114 and the [b]Dept. of Mol. Physiol. and Biophys., The Univ. of Vt, Burlington, VT, 05405.

An *in vitro* motility assay was used to determine the effects of caldesmon phosphorylation by MAPK on the ability of caldesmon to inhibit isometric force produced by myosin on actin-tropomyosin filaments. We used F_{NEM} as an index of the relative isometric force generated by myosin; F_{NEM} is equal to the ratio of NEM-modified/unmodified myosin at which actin motility ceases. As the [ATP] in the motility assay was lowered from 1 to 0.2 mM, F_{NEM} increased approximately 8 fold. The increase in isometric force that resulted from lowering the [ATP] was inhibited by caldesmon ([50 nM]). However, when caldesmon was phosphorylated to a level of 1.45 mole phosphate/mole caldesmon by MAPK, the inhibition of F_{NEM} was not observed. Thus, phosphorylation of caldesmon by MAPK reverses caldesmon's ability to inhibit isometric force production in an *in vitro* motility assay. In addition to this effect, caldesmon-dependent tethering of actin-tropomyosin filaments to myosin in the *in vitro* motility assay was likewise reversed upon phosphorylation by MAPK. For comparative purposes, we measured the ATPase activity of phosphorylated myosin using MAPK-phosphorylated and unphosphorylated caldesmon, *in vitro*. Caldesmon inhibited actomyosin ATPase activity with 50% inhibition at an actin:caldesmon ratio of 25:1, and this was not altered by phosphorylation of caldesmon with MAPK. The phosphorylation of caldesmon, as occurs during smooth muscle stimulation, results in a significant alteration of caldesmon function as assessed using the *in vitro* motility assay that is not observed in solution ATPase assays.

Tu-Pos217

EXPRESSION OF CALDESMON DURING DIFFERENTIATION OF SMOOTH MUSCLE IN CHICKEN GIZZARD. ((Chandrakala Menon and Samuel Chacko)) University of Pennsylvania, Philadelphia, PA 19104.

Caldesmon is an actin/calmodulin-binding protein located in the thin filaments and microfilaments of smooth muscle and nonmuscle cells, respectively. Two isoforms of caldesmon, *h*- and *l*-types shown to exist in vertebrate smooth and nonmuscle cells, are produced by a single gene by alternative splicing of the caldesmon mRNA. Using a developing chicken gizzard, we studied the expression of *h*- and *l*-caldesmon during the differentiation of mesenchymal cells into smooth muscle cells. Proteins and total RNA from gizzards of 5, 7, 9, 13, 17 and 21-day embryos and 2-day post-hatch chicks were extracted and analyzed for caldesmon expression at both protein and mRNA levels. SDS PAGE and Western blot analysis was carried out using a polyclonal antibody against *h*-caldesmon. Total RNA was analyzed by Northern Blot analysis using a caldesmon cDNA probe, and *h*- and *l*-caldesmon cDNAs were identified due to the difference in their molecular sizes(4.8 and 4.1kb respectively). The mRNA was also analyzed by RT-PCR and Southern Blot analysis. Our results, show that the l- caldesmon was expressed at higher levels during the early stages and decreased gradually during growth. The h -caldesmon, however, is not expressed at day 5 and 7 and was turned-on by day 9. Hence, the expression of smooth muscle type caldesmon serves as a marker for differentiation of smooth muscle. Supported by DK 47514 and DK 39740 .

Tu-Pos218

COFACTORS OF MAPK AND PKC DURING Ca^{2+}-INDEPENDENT SMOOTH MUSCLE CONTRACTION. ((**C.B. Menice**[1,2], J.W. Hulvershorn[2], L.P. Adam[2], and K.G. Morgan[1,2])) [1]Harvard Medical School, Beth Israel Hospital, [2]Boston Biomedical Research Institute, Boston, MA, 02114 (Sponsored by J.P. Morgan)

A co-redistribution of MAP kinase (MAPK) and PKC-ε to the surface membrane has been reported to occur during phenylephrine(PE)-induced shortening of ferret aorta vascular smooth muscle cells (FA) in the absence of $[Ca^{2+}]_e$ (Khalil and Morgan, 1993). In the present study we searched for cofactors and missing links associated with Ca^{2+}-independent signaling involving PKC-ε and MAPK. The activation of MAPK during Ca^{2+}-independent PE-induced contraction was confirmed by the time dependent increase of a single 46kD band on an anti-phosphotyrosine Western blot. Western blots with specific antibodies for ERK1 p44, ERK2 p42, and JNK1 p46, indicated their presence in FA as well as other proteins at 38kD, 54kD, and 90kD that cross-react with a Pan ERK Ab. Since the ERK1 Ab which was utilized in the imaging studies recognizes a band at 46kD and the peptide sequence used to generate the ERK1 Ab has some homology to regions in JNK1, the 46kD tyrosine phosphorylated band could be either ERK1 or JNK1. Proteins at MW's corresponding to 36kD, 90kD, 110kD, 180kD, and 200kD were found to co-immunoprecipitate with a MAPK running at 46kD. The protein at 36kD was identified as calponin by immunoblotting a sample of the immunoprecipitate. An ERK1 p44 overlay assay on recombinant calponin further confirmed an interaction between ERK1 p44 and calponin even though ERK1 p44 did not phosphorylate calponin *in vitro*. Digital confocal studies confirmed that CaP undergoes a redistribution in FA cells during PE-stimulation. These results suggest that the mechanism of $[Ca^{2+}]_e$-independent contraction may involve an association between p46 MAPK and calponin. Support: NIH HL31704\ HL42293\HL07374

Tu-Pos219

α_1-ADRENOCEPTOR-MEDIATED PHOSPHORYLATION OF MYOSIN IN RAT TAIL ARTERIAL SMOOTH MUSCLE. **((M. Mita and M.P. Walsh)) Smooth Muscle Research Group, University of Calgary, Calgary, Alberta T2N 4N1, Canada.**

The mechanism of α_1-adrenoceptor-mediated contraction was investigated in helical strips of the rat tail artery. Muscle strips de-endothelialized contracted in response to the α_1-adrenergic agonist cirazoline with $EC_{50} \sim 0.2$ μM. The contractile response to a sub-maximal concentration of cirazoline (0.3 μM) was biphasic with a rapid phasic component peaking at ~ 30 s followed by sustained tonic contraction. Myosin light chain (LC_{20}) phosphorylation in response to cirazoline was also biphasic and closely matched the time course of contraction. Resting myosin phosphorylation levels were ~ 0.2 mol P_i /mol LC_{20} and reached a maximum of ~ 0.55 mol P_i /mol. Phosphopeptide mapping and phosphoamino acid analysis revealed that LC_{20} phosphorylation occurred exclusively at serine[19]. The sustained phase of contraction was eliminated by removal of extracellular Ca^{2+} and the phasic response was eliminated by depletion of endogenous Ca^{2+} stores. Both phases of the contractile response were restored by re-addition of Ca^{2+} to the bathing medium. LC_{20} phosphorylation and both phases of the contractile response to cirazoline were inhibited by the myosin light chain kinase inhibitor ML-9 (30 μM). Resting LC_{20} phosphorylation, however, was unaffected by ML-9. Finally, both phasic and tonic responses to cirazoline were partially inhibited by chloroethylclonidine (50 μM) indicating the involvement of both α_{1A} and α_{1B} adrenoceptors in these contractile responses. (Support: Heart and Stroke Foundation of Alberta.)

Tu-Pos220

INHIBITION OF Ca^{2+}-DEPENDENT, BUT NOT Ca^{2+}-INDEPENDENT, PHORBOL ESTER-INDUCED VASCULAR SMOOTH MUSCLE CONTRACTION BY A PKC-β C2 DOMAIN PEPTIDE ((**R. Laporte**[1], C.L. Sougnez[1], M.P. Walsh[3], K.G. Morgan[1,2])) [1]Boston Biomedical Research Institute, Boston, MA 02114; [2]Dept. of Medicine, Harvard Medical School, Boston, MA 02215; [3]Dept. of Medical Biochemistry, University of Calgary, Canada, T2N 4N1.

The phorbol ester 12-deoxyphorbol 13-isobutyrate 20-acetate (DPBA) induces a strictly Ca^{2+}-dependent contraction in the ferret portal vein (FPV) but a Ca^{2+}-independent contraction in the ferret aorta (FA). To assess the involvement of Ca^{2+}-dependent and -independent PKC isoforms in these contractions, we used a peptide (PKC-β C2-2) corresponding to a portion of the PKC-β C2 domain (M186-L198). This peptide is reported to inhibit translocation of the Ca^{2+}-dependent isoform PKC-β in cultured neonatal rat cardiomyocytes and to inhibit *Xenopus* oocyte maturation (Ron et al., JBC, 1995). The FPV and FA were β-escin-permeabilized in an identical manner. The control pCa-force curve for the FPV was consistently right-shifted compared to that for the FA and, in both cases, no contractions were induced by pCa ≤ 7. DPBA significantly increased force generation induced by pCa > 7. Additionally, DPBA induced a contractile response from pCa 9 to pCa 7 in the FA. Exposure to the PKC-β C2-2 peptide completely reversed the effect of DPBA in the FPV, while DPBA's effect was resistant to the peptide in the FA. PKC-β was not immunologically detectable in the FPV; α was the sole Ca^{2+}-dependent PKC isoform detectable. PKC-ε and possibly PKC-μ were the only phorbol ester-binding Ca^{2+}-independent isoforms immunologically detectable in the FA. We propose that DPBA's contractile effects are associated with Ca^{2+}-dependent PKC-α in the FPV and with a Ca^{2+}-independent PKC isoform in the FA, likely ε. Supported by NIH HL31704 and HL42293, and by MRC Canada.

Tu-Pos221

THE ELASTIC PROPERTIES OF THE ACTIN-MYOSIN INTERACTION OF VASCULAR SMOOTH MUSCLE. ((**B.G.V.VanHeijst**[1], U.A. VanDerHeide[1], E.L.DeBeer[1], T. Blangé[2] and H.J. Jongsma[1])) 1. Dept. of Medical Physiology, Utrecht University, P.O. Box 80043, 3508 TA, Utrecht, The Netherlands. 2. Dept. of Physiology, University of Amsterdam, the Netherlands.

The myogenic response is one of the important mechanisms of controlling the blood flow to the organs and elicits the increase in active force upon an increase in pressure in a blood vessel. Membrane associated processes are at least partially due to the myogenic response, but it is as yet unknown whether length or load dependent properties of cross bridges lead directly to a myogenic response. The mechanical properties and the binding kinetics of cross bridges are reflected in the elastic properties of muscle preparations. We determined the complex elasticity of the portal vein of the rabbit over a large frequency range (0.02-10,000 Hz).
Strips of the portal vein were skinned by means of Triton-skinning. Length perturbations of 0.5% were imposed and the force response was determined. The complex elastic modulus increases with increasing frequency. At low frequencies the elastic modulus of the activated state, determined at the optimum of the length-tension relationship, is tenfold the elastic modulus in the relaxed state. This indicates a long-lasting increase in the tension upon an increase in length in activated smooth muscle. These data suggest that the actin-myosin interaction of the portal vein does have the elastic properties to contribute to the myogenic response.
This study was supported by the Dutch foundation for Life Sciences (NWO).

Tu-Pos222

Ca^{2+} SENSITIVITY OF TYROSINE PHOSPHORYLATION OF PAXILLIN AND MYOSIN LIGHT CHAIN PHOSPHORYLATION IN ß-ESCIN PERMEABILIZED CANINE TRACHEAL SMOOTH MUSCLE.
((**D. Mehta, M.-F. Wu and S.J. Gunst**)) Dept. Physiology and Biophysics., Indiana University School of Medicine, Indianapolis, IN 46202.

Tyrosine phosphorylation of the dense plaque protein, paxillin, increases during contractile stimulation of canine tracheal smooth muscle (Wang et al. AJP Cell Physiol. : 40, 1996). This suggests that paxillin may play a role in the regulation of airway smooth muscle contraction. The goal of this study was to compare the Ca^{2+} sensitivity of the tyrosine phosphorylation of paxillin to that of myosin light chain (MLC) phosphorylation in tracheal smooth muscle. ß-escin permeabilized strips were stimulated with calcium (pCa 9 to 6) or stimulated with 10^{-4}M acetylcholine (ACh) at pCa 7. Strips were then frozen for measurement of tyrosine phosphorylation of paxillin and MLC phosphorylation under each condition. Both the tyrosine phosphorylation of paxillin and MLC phosphorylation increased when pCa was increased from 9 to 6. At pCa 7, the addition of ACh resulted in an increase in tyrosine phosphorylation of paxillin and increased MLC phosphorylation. Results demonstrate that tyrosine phosphorylation of paxillin and MLC phosphorylation can occur by Ca^{2+}-dependent signalling pathways; however both phosphorylations are potentiated by ACh stimulation. The Ca^{2+} sensitizing effect of ACh on active force might involve the activation of signalling pathways which sensitize both serine-threonine and tyrosine phosphorylation pathways in permeabilized canine tracheal smooth muscle.
Supported by HL-29289 and Am. Heart Assoc. Fellowship to Mehta.

Tu-Pos223

KINETICS OF PHOSPHATE (P_i)RELEASE IN ACTIVATED ISOMETRIC SMOOTH MUSCLE ((**He, H-Z.***, M.A. Ferenczi*, D.R. Trentham*, M.R. Webb*, M. Brune*, A.P. Somlyo, and A.V. Somlyo)) NIMR, London NW7 1AA U.K.* & Univ. Virginia Sch. of Med., Charlottesville, VA 22908, USA.

The rate of appearance of P_i in rabbit portal vein smooth muscle permeabilized with Triton X-100 was monitored at 20° C with the fluorescent P_i-sensitive probe, MDCC-PBP (Ferenczi et al. *Biophysics. J.* 68:191S-193S, 1995) following flash photolysis of caged ATP. In the absence of Ca^{2+}, P_i release, largely attributed to ecto-ATPase, was 37 $\mu M\ s^{-1}$, Data presented are corrected for this ecto-ATPase activity. In muscles in which regulatory light chains (MLC_{20}) were thiophosphorylated, photolytic release of ATP caused monotonic force development and P_i release in two approximately linear phases: a rapid phase, which when converted to take account of the 55 µM myosin head concentration, corresponded to 1.5±0.14 s^{-1} with amplitude of 108±14.8 µM, and a slow phase at 0.25±1.02 s^{-1}. By the end of the fast phase, force had reached 76.0±5.0% of maximum. There was no evidence of a significant "P_i-burst" preceding force development. In non-thiophosphorylated smooth muscle, P_i release following photolysis of caged ATP in the presence of Ca^{2+} had a fast rate of 0.9±0.13 s^{-1}, with amplitude 81±12.6 µM and a slow phase at 0.24±0.04 s^{-1}. We conclude that transition from the rapid to the slow phase of actomyosin ATPase activity occurs within two turnovers, and may result from strain on crossbridges, from a change in the number of cycling heads and/or inhibition by MgADP. The similar rates of P_i release during force maintenance (slow phase) in smooth muscles, whether thiophosphorylated MLC_{20} or activated by Ca^{2+}, indicate that the contribution of pseudo-ATPase (light chain kinase/phosphatase) activity to energy consumption is, at most, equivalent to the possibly greater activation of myosin by thiophosphorylation than by Ca^{2+}. Supported by NIH PO1 HL48807.

Tu-Pos224

CYCLIC STRAIN STIMULATES MONOCYTE CHEMOTACTIC PROTEIN-1 mRNA EXPRESSION IN SMOOTH MUSCLE CELLS. ((M.J. Jiang[1], Y.J. Yu[2] and Y.L. Chen[2])) [1]Department of Anatomy, National Cheng Kung University Medical College, Tainan and [2]Department of Anatomy, National Yang Ming University, Taipei, Taiwan, ROC.
Mechanical forces are important factors for the formation of atherosclerotic plaques. We examined the effects of cyclic strain (-2~-17 kPa, 1 Hz) on monocyte chemotactic protein-1 (MCP-1) mRNA levels of cultured rat aortic smooth muscle cells (RASMC). The MCP-1 mRNA expression levels of RASMC increased as the duration of cyclic strain increased, reaching the maximum at 12 hours. To explore signaling pathways mediating cyclic strain-stimulated MCP-1 mRNA expression, we examined the involvement of tyrosine kinase and protein kinase C. Tyrosine kinase inhibitors, tyrphostin 51 and genistein (50 µM), abolished cyclic strain-stimulated MCP-1 mRNA expression. Phorbol 12-myristate 13-acetate (PMA, 250 nM), a protein kinase C activator, increased MCP-1 mRNA expression after 6-hour incubation. When RASMC were incubated with PMA (1 µM) for 24 hours to down regulate protein kinase C, cyclic strain-induced MCP-1 mRNA expression was further increased.
These results indicate that cyclic strain stimulates MCP-1 mRNA expression in RASMC. The activation of tyrosine kinase(s) is likely to mediate cyclic strain-stimulated increases in MCP-1 mRNA expression levels whereas further studies are required to clarify the roles of protein kinase C. Supported by the National Science Council and Academia Sinica of R.O.C..

Tu-Pos225

PHOSPHORYLATED CPI-17, A NEW PHOSPHATASE INHIBITORY PROTEIN, INCREASES SMOOTH MUSCLE CONTRACTIONS.
((**T. Kitazawa**, M. R. Lee, L. Li and *M. Eto)) Dept. Physiol., Georgetown Univ. Washington, D.C. 20007 & * Chem. Div. Hokkaido Univ., Sapporo, Japan

We have demonstrated that PKC activators increase Ca^{2+} sensitivity of MLC phosphorylation (P) and smooth muscle contraction (F) through inhibition of MLC phosphatase (MLCP; J.G.P. 104, 265-286, 1994). A new protein that regulates MLCP has been recently identified in vascular smooth muscle. This protein named CPI-17 can be phosphorylated by PKC with a resultant potent inhibition of isolated MLCP activity (see the preceding poster). We examined the effects of CPI-17 on F and P in β-escin-permeabilized rabbit femoral artery strips. Thiophosphorylated (P-CPI), but not unphosphorylated (U-CPI) CPI-17 at 800 nM dramatically increased submaximal contractions induced by pCa 6.3 from 5% of maximum contraction to 72%. We also found similar effects of the protein in demembranated strips treated with Triton X-100. P-CPI was capable of increasing the resting F in the Ca^{2+}-free solution. These potentiated contractions were, however, further increased by a MLCP inhibitor microcystin-LR to the maximum force. The EC_{50} of P-CPI was 90 nM while U-CPI had no effect even at 1 µM. MLC phosphorylation was also significantly increased by P-CPI, but not by U-CPI, at constant Ca^{2+}. These results confirm CPI-17's direct regulation of MLCP *in situ* and furthermore suggest that the CPI-17 is a mediator between PKC and its inhibition of MLCP. This study was supported by NIH HL51824.

Tu-Pos226

Calponin controls cycling of dephosphorylated cross-bridges in smooth muscle.
((**U. Malmqvist, K. Trybus, F. Fay.**)) Dep. Physiol. Univ. Massachusetts Med. Ctr, Worcester, MA., Brandeis Univ., Waltham, MA.

It is generally believed that phosphorylation of the regulatory light chain (RLC) on myosin is sufficient and necessary for the initation smooth muscle contraction, but this does not necessary mean that the relaxed state in smooth muscle is merely due to the lack of RLC phosphorylation. We have investigated if calponin, a protein that *in vitro* inhibits the myosin ATPase activity and is present on the thin filament, has any role in the regulation of smooth muscle contraction. We extracted more than 95 % of calponin and the RLC on myosin from isolated skinned single smooth muscle cells from the stomach of the toad Bufo Marinus. Myosin in the extracted cells was reconstituted either with a thiophosphorylated WT RLC (thioRLC) or with a mutant where Thr-18 and Ser-19 had been replaced with a alanine (AARLC). Myosin with the AARLC can not be phosphorylated and has kinetic properties *in vitro* similar to dephosphorylated myosin. Biochemical and morphological analysis showed that the added RLC bound to myosin filaments in the cell. In the absence of calponin, cells reconstituted with the AARLC, had a shortening velocity that was 30 % to that of cells with the ThioRLC. The force of cells reconstituted with the ThioRLC was 3.7 ± 0.5 compared to 2.4 ± 0.7 µN for cells reconstituted with the AARLC when calponin was absent from the thin filaments. These results shows that dephosphorylated myosin can slowly cycle leading to force or shortening of the smooth muscle cells. The shortening velocity of cells with the AARLC was completely inhibited by reconstituting the cell with calponin, whereas the shortening of cells with the ThioRLC was unaffected by the presence of calponin on the thin filaments. The same results were obtained by exchanging the native RLC on myosin with the ThioRLC or AARLC without perturbing the amount of calponin on the thin filaments. When cells reconstituted with theThioRLC, were activated in the presence of calponin on the thin filaments, the force generation of the cells was unaffected. This is in contrast to the finding in cells reconstituted with the AARLC where the cells rigor force after activation dropped to zero, where it remained. Our results suggest that calponin is necessary for keeping smooth muscle relaxed.

Tu-Pos227

ADENOVIRUS MEDIATED EXPRESSION OF MUTANT HUMAN HSP27 IN CULTURED CANINE SMOOTH MUSCLE CELLS. ((W.T. Gerthoffer, J. Larsen, J. Hedges, A. Cook and L. Weber)) Departments of Pharmacology and Biology, University of Nevada, Reno, Reno, NV 89557-0046.

A replication-deficient adenovirus vector (E1 deficient Ad5) was used to manipulate HSP27 expression in cultured smooth muscle cells. Western blot analysis was used to verify expression of smooth muscle phenotype markers in primary cultures of canine colonic and tracheal smooth muscle. Cultured myocytes expressed α-smooth muscle actin, γ-smooth muscle actin, calponin and h-caldesmon. Expression of human HSP27 was induced with an adenovirus vector containing either a cDNA insert coding for full length human HSP27 or a mutant HSP27 in which MAPKAP-2 phosphorylation sites (Ser 15, 78 and 82) were converted to glycine residues by site-directed mutagenesis. The presence of cDNA inserts was verified by Southern blotting of viral DNA. Expression of human HSP27 by canine smooth muscle cells was demonstrated by Western blotting and immunofluorescence using anti-human HSP monoclonal antibodies. Infection of myocytes with an MOI of 10 was sufficient to yield > 90% transfection. Virus infection had no obvious effect on cell structure or viability by phase-contrast microscopy; however, expression of mutant HSP27 was associated with less intense staining of actin fibers with Oregon green-phalloidin. This suggests that, as in nonmuscle cells, phosphorylation of HSP27 may participate in regulating actin filament structure in smooth muscle cells. (Supported by NIH grants HL48183 and DK41315 to WTG).

Tu-Pos228

TELOKIN RELAXES SMOOTH MUSCLE AND IS PHOSPHORYLATED, *IN SITU* ((X. Wu, T.A.J. Haystead, A.V. Somlyo, A.P. Somlyo) Dept. Mol. Physiol. & Biol. Physics, University of Virginia, Charlottesville, VA22903)

8-bromo-cGMP relaxes α-toxin-permeabilized rabbit ileum smooth muscle (Ri) at constant [Ca^{2+}] by accelerating dephosphorylation of MLC_{20} (1). We show that forskolin(10 μM) in intact muscle and 8-br-cGMP(10-100 μM) in permeabilized Ri in Ca^{2+}-free solution increase ^{32}P incorporation into at least three small (22-24 kDa) cytosolic, acidic proteins (pI 4.2-4.4). 2-D gel electrophoresis of a partially purified cytosolic fraction of ileum smooth muscle separated a group of six small, acidic proteins four of which were identifed by mass spectroscopy as telokin. Telokin can be phosphorylated *in vitro* by the catalytic subunit of cAMP- but not cGMP-dependent protein kinase (2). Telokin (~20 μM) purified from turkey gizzard relaxed Ri permeabilized with Triton-X 100 or β-escin at constant [Ca^{2+}], yet did not relax Ri activated by thio-phosphorylating myosin. We are currently testing the hypothesis, supported by preliminary experiments, that telokin accelerates the dephosphorylation of MLC_{20} by enhancing the activity of myosin light chain phosphatase.

1. Wu et al. Biochem. Biophys. Res. Comm. 220: 658, 1996
2. Ito et al. J. Biol. Chem. 264: 13971, 1989

Supported by NIH grant HL 19242

Tu-Pos229

The effects of endogenous MgADP on force-calcium hysteresis in tonic and phasic smooth muscle ((**A.S.Khromov**,A.V.Somlyo, A.P. Somlyo))UVA,Health Sciences Center,Charlottesville,Va,22908, USA.

Triton X-100-permeabilized tonic,(rabbit femoral artery) Rf and phasic (rabbit ileum) Ril smooth muscles were activated at pCa7.2 either by increasing [Ca^{2+}] from pCa<8.0 (ascending protocol) or contracted at pCa 6.0 before lowering [Ca^{2+}] (descending protocol) to pCa7.2 in the presence of **high** [MgATP] (10 mM MgATP+CP+CK) or **low** [MgATP] (2 mM MgATP,0 CP,0 CK).In Rf at **low**,but not at **high**,[MgATP] there was a significant difference in force (hysteresis) at pCa7.2 between ascending or descending protocols (3% vs 54% of F_{max}).MLC_{20} phosphorylation (10±2% vs 9±2%) and V_0 (0.02±0.004 l/s) at pCa 7.2 were not significantly different under either protocol. Addition of CP (10 mM) or MgADP (100 mM) to the pCa 6.0 solution,respectively, reduced or increased (~0.1 vs ~0.8 of F_{max}) the force maintained after lowering [Ca^{2+}] to pCa 7.2.No significant force hysteresis was detected in Ril.We conclude that in tonic smooth muscle high MgADP affinity of cross-bridges [1] and resultant population of AM.ADP states contribute to force maintenance at low MLC_{20} phosphorylation. Supported by HL19242.[1].**J.Muscle Res,Cell,Motil.(1993),15:666.**

Tu-Pos230

EFFECTS OF TYROSINE KINASE INHIBITORS AND ORTHOVANADATE ON TENSION DEVELOPMENT IN RAT MAIN PULMONARY ARTERY. **((P. Choudhury, T. Milburn, D. Hucks, S. Davenport and P.I. Aaronson)) United Medical and Dental Schools, London SE1 7EH, UK.**

There is evidence that vascular smooth muscle excitation-contraction (E-C) coupling mediated by G-protein-linked receptors for agonists such as angiotensin II and serotonin may involve activation of non-receptor tyrosine kinases. We therefore examined the effect on isometric contractures elicited in rat main pulmonary artery by phenylephrine (PE, 10 μM, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 10 μM, and high K^+ solution (80 mM) of the pharmacological tyrosine kinase inhibitors (PTKI) tyrphostin 23 (TYR), genistein (GEN), ST 638 and the tyrosine phosphatase inhibitor orthovanadate (OV, 1-3 mM). Preconstrictions evoked by PE were relaxed in a dose-dependent manner by TYR, GEN and ST 638 (IC_{50} = 72 ± 14, 22 ± 6 and 14 ± 4 μM (mean ± S.E.M.), respectively); similar IC_{50} values were recorded for relaxation of the K+ contracture (74 ± 20, 25 ± 5, and 15 ± 5 μM, respectively). TYR had a less potent effect on the $PGF_{2\alpha}$ contracture (IC_{50} = 138 ± 6 μM). If OV (1 mM) was added in the presence of PE, $PGF_{2\alpha}$, or high K^+, it caused a transient relaxation followed by an additional small contracture. After 1 mM OV had been added, the contractures elicited by these agonists became significantly less sensitive to relaxation by TYR and GEN, but not ST 638. For example the PE contracture was relaxed by 40 ± 5% after 5 min in 100 μM TYR , but the contracture to PE + OV was relaxed by only 21 ± 4%. There were thus interactions between OV and the PTKIs GEN and TYR, but not ST 638, since OV inhibited the vasorelaxing action of TYR and GEN. These results are consistent with the concept that tyrosine kinase activity has a functional role in E-C coupling in this artery.

Tu-Pos231

SMOOTH MUSCLE MYOSIN HEAVY CHAIN ISOFORM RATIOS AND UNLOADED SHORTENING IN SINGLE SMOOTH MUSCLE CELLS. ((**D.P. Meer** and T.J. Eddinger)) Department of Biology, Marquette University, Milwaukee, WI 53233.

The functional significance of the variable expression of the smooth muscle myosin heavy chain (SM-MHC) tail isoforms, SM1 and SM2, was examined in individual permeabilized rabbit arterial smooth muscle cells. The length of untethered single permeabilized smooth muscle cells (SMC) was monitored during unloaded shortening in response to increased Ca++ (pCa 6.0), histamine (1 μM), and phenylephrine (1 μM). Maximal shortening velocities (V_{max}) of the SMCs were measured by fitting a line (least squares) to the steepest region of the length vs. time curve. Subsequent to contraction the relative expression of SM1 and SM2 mRNAs in the individual SMCs was determined by reverse transcription-polymerase chain reaction (RT-PCR) amplification and densitometric analysis. We have previously shown that the SM2/SM1 mRNA ratio gives an acurate and reproducible estimate of the SM2/SM1 protein ratio (Meer and Eddinger, *Am. J. Physiol.* 270:C1819, 1996). Correlation analysis between SM2/SM1 ratio and V_{max} in saponin and α-toxin permeabilized SMCs (n-28) reveal no significant relationship between the SM-MHC tail isoform ratio and maximal shortening velocity. The best correlations between SM2/SM1 ratio and the contraction characteristics of untethered vascular SMCs were with the minimum length attained following contraction (α-toxin, n=20, R=0.72; saponin, n=8, R=0.78). These results suggest that the primary effect of variable expression of the SM1 and SM2-MHC tail isoforms is not on velocity but may be on myosin filament structure. These results are consistent with unique isoform-specific thick filaments which alter cell function. Further studies are examining this possibility.

Tu-Pos232

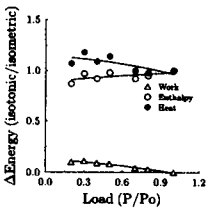
DOWN-REGULATION OF EARLY PEAK BUT NOT STEADY-STATE CONTRACTIONS OF PHASIC BLADDER SMOOTH MUSCLE. ((**P.H. Ratz**, O.Z. Shenfeld, and C.W. Morgan)) Departments of Pharmacology and Urology, Eastern Virginia Medical School, Norfolk, VA 23501

Receptor agonists not only contract vascular smooth muscle, but may also activate a negative feedback mechanism. In particular, KCl-induced contractions are temporarily converted from tonic to phasic in arteries that had been pretreated for 3 to 30 min with a receptor agonist. Thus, we hypothesized that a similar mechanism would *not* exist in a smooth muscle that normally contracts in a phasic manner, such as rabbit urinary bladder (detrusor). Strips of detrusor were secured to isometric force transducers in tissue baths and length-adjusted until K^+-depolarization (110 mM KCl) produced maximum contractions (S_o). Subsequent contractions (S) were normalized to S_o (S/S_o). Contractions (S/S_o) induced by 30 mM KCl in the presence of atropine (0.1 μM) had three phases; fast and slow peak (phasic) responses, FP = 0.59 ± 0.07 and SP = 0.44 ± 0.06, respectively, and a weaker steady-state response, SS = 0.30 ± 0.07. However, in tissues exposed for 30 min to 100 μM bethanechol (BE), then washed for 5 min, FP and SP, but *not* SS responses produced by 30 mM KCl (plus atropine) were reduced by ~40% ($P<0.05$ compared to controls). Down-regulation was reversible, lasting ~60-75 min, and appeared also to be produced by a 100-fold lower [BE]. Interestingly, contractions produced by the L-type Ca^{2+} channel agonist, Bay k 8644, also appeared to be reduced by BE pretreatment, suggesting that BE-induced down-regulation involved inhibition of Ca^{2+} channels. In conclusion, pretreatment of isolated rabbit detrusor, a phasic smooth muscle, with a muscarinic receptor agonist (BE) produced short-term down-regulation of peak, not steady-state, KCl-induced contractions that may have involved inhibition of L-type Ca^{2+} channels.

Tu-Pos233

MECHANICAL EFFICIENCY OF RAT ANOCOCCYGEUS AT 27 °C ((**J.S. Walker**, and R.A. Murphy)) Department of Molecular Physiology & Biological Physics, University of Virginia, Charlottesville, VA 22908

The aim of this study was to evaluate the mechanical efficiency (the ratio of work to total energy expended) of the rat anococcygeus muscle, a fast, tonic, visceral smooth muscle, at 27 °C. Direct electrical stimulation (3s, 10 Hz, 7–10 V, 0.1–0.5 ms) produced a phasic contraction with a myosin regulatory light chain phosphorylation of about 95%. Total heat production, including recovery heat, was measured using a thermopile during afterloaded isotonic contractions. Total heat represents the sum of work and enthalpy. Work was calculated as Δlength × load (*P*). Enthalpy was calculated by subtracting twice the work from the total heat. The mean total isometric energy expenditure in a 3s contraction was about 45 mJ/g. Similar results to those shown were obtained in 12 preparations. The slope of the mean total heat vs load was not significantly different from zero. Peak efficiency under these conditions was about 11.5% under loads of 0.3–0.4 P_o. This estimate is likely conservative, since with increasing contraction duration, more heat is evolved that is unrelated to work production. Shorter duration contractions may be more efficient. *Supported by NIH 5PO1 HL19242 & AHA VA-95-F-6.*



Tu-Pos234

THE IDENTIFICATION OF SPECIFIC PKC ISOFORMS IN PDBU-INDUCED CA^{2+}-SENSITIZATION OF SMOOTH MUSCLE. ((P.E. Jensen, **L.A. Walker**, M.C. Gong, P. Gailly, J. Sando, A.V. Somlyo, and A.P. Somlyo)) Univ. of VA, Dept. of Mol. Phys. & Biol. Physics, Charlottesville, VA 22908 (Spon. by J.M. Gillis)

The multiplicity of Protein Kinase Cs (PKCs) leaves considerable uncertainty about the identity of the isoforms involved in Ca^{2+}-sensitization of smooth muscle by phorbol esters, although recent evidence shows that this pathway plays at most, a very minor role in physiological, G-protein-coupled Ca^{2+}-sensitization (1,2). The purpose of this study was to identify specific PKC isoforms mediating this minor pathway of phorbol ester-induced Ca^{2+}-sensitization. PKC isoforms α, β1, β2, ε, θ and ζ were detected in rabbit portal vein (PV), femoral artery (FA) and ileum smooth muscle. PKC-η was only detected in PV. Contrary to an earlier report, PKC-ε was also present in ferret PV, and γ, and δ were not detected. A ζ antibody also recognized a yet to be identified ~88 kDa band. Treatment (24-96 hrs) with phorbol ester or bryostatin abolished PDBu-induced Ca^{2+}-sensitization and downregulated PKCs α, β1, β2, ε, θ and the ~88 kDa band detected with the ζ antibody, but not η or ζ. Downregulation with PDBu was reversible within 24 hrs: Ca^{2+}-sensitization and the unidentified ~88 kDa ζ-reactive band returned and PKCs β1 and β2 were also present; PKCs α and θ were absent, and ε was unchanged. Treatment of FA with PDBu (16 hr) under Ca^{2+}-free conditions downregulated "conventional" (α and β1) as well as novel (ε and θ) PKCs. We conclude that PKCs α, η, θ and ε are not required for, and PKCε plays no special role in, PDBu-induced Ca^{2+}-sensitization of smooth muscle. Supported by the Danish Nat. Sci. Res.Council and NIH Grants HL19242 and HL07284.

1.Jensen, P. E. et al. (1996) **In press**
2.Hori, Y. (1992) *Kobe J. Med. Sci.* **38,** 79-92

Tu-Pos235

INTERACTION OF MYOSIN PHOSPHATASE WITH PHOSPHOLIPIDS ((F. Jianhua[1], **J. Tanaka**[1], M. Ito[1], K. Ichikawa[1], B. Yamamori[1], M. Inagaki[2], and T. Nakano[1])) [1]Dept. of Int. Med., Mie Univ. School of Med., Tsu 514, Japan, and [2]Lab. Biochem., Aichi Cancer Res. Inst., Nagoya 464, Japan. (Spon. by D.E. Goll)

Contractile activity in smooth muscle is regulated by phosphorylation of myosin. Recently the myosin phosphatase (MP) was purified from several smooth muscles and found to be a heterotrimer of a 38 kD catalytic subunit (PP1cδ) and two regulatory subunits of 130 kD (M130) and 20 kD (M20). The cellular localization of the holoenzyme and/or the component subunits is not known. The M130 was detected in the cytosol, the cytoskeletal fraction and unexpectedly from the membrane fraction in T24 cells using immunoblotting. This study was undertaken to investigate the possible MP/lipid interaction. Interaction between MP and phospholipid vesicles was examined using a sedimentation assay. It was found that MP binds to acidic phospholipids, namely phosphatidylserine (PS), phosphatidylinositol and phosphatidic acid, but not to neutral phospholipids. The amount of MP bound to PS decreased on increasing Mg^{2+} concentration and at 2 mM Mg^{2+} only 20% of MP was bound to PS. The MP/acidic phospholipid interaction inhibited the activity of myosin phosphatase. It was shown, using limited α-chymotryptic digestion and various truncation mutants that phospholipid binding was associated with M20 and with the C-terminal third of M130. M130 and M20 were phosphorylated by protein kinase A (PKA) to 3 mol P/mol and 1 mol P/mol, respectively. Phosphorylation by PKA caused a dissociation of MP from phospholipids and resulted in the recovery of phosphatase activity toward myosin. These results suggest that MP may associate with phospholipids and that this interaction is regulated by phosphorylation by PKA. This mechanism may play an important role in the regulation of myosin phosphatase and its cellular localization.

Tu-Pos236

RHO-ASSOCIATE KINASE (RHO-KINASE), INDUCES MYOSIN LIGHT CHAIN PHOSPHORYLATION AND CA^{2+}-SENSITIZATION OF THE TRITON X-100-PERMEABILIZED RABBIT PORTAL VEIN.

((**Y. Kureishi**[1], S. Kobayashi[2], M. Ito[1], M. Amano[4], K. Kimura[4], M. Fujioka[1], H. Kanaide[3], K. Kaibuchi[4] and T. Nakano[1]))[1]Dept. of Int. Med., Mie Univ. School of Med., Tsu 514, Japan, [2]1st Dept. of Physiol., Yamaguchi Univ. School of Med., Ube 775, Japan, [3]Mol. Cardiol., Faculty of Med., Kyushu Univ., Fukuoka, 812 Japan, and [4]Div. of Signal Transduction, NAIST, Ikoma 630-01, Japan.

Members of the Rho family of guanosine nucleotide-binding proteins (G proteins) are involved in the regulation of the myosin light chain (MLC) phosphorylation and contraction of the smooth muscle. RhoA, in particular, has been shown to be an upstream messenger of Ca^{2+}-sensitization of the contractile apparatus of the smooth muscle. However, the possible cofactor(s) in the 'downstream' of the pathway modulating the Rho-mediated Ca^{2+} sensitization has yet to be identified. Using the membrane permeabilization with Triton X-100, we introduced the recombinant catalytic subunit of Rho-associate kinase (RK), a nobel serine/threonine-protein kinase which is activated by the GTP-bound, active form of RhoA, into the cytosol of smooth muscle of rabbit portal vein (RPV). Cytosolic RK induced a contraction and a concomitant increase in the monophosphorylation of MLC in the Triton X-100-permeabilized RPV at a nominally zero cytosolic Ca^{2+} (buffered with 10mM EGTA) in the absence of calmodulin. The both effects of RK on the force and the MLC monophosphorylation were insensitive to wortmannin (WM), a MLCK blocker. Our results suggest that RK directly induces the Ca^{2+} sensitization of the contractile apparatus of the smooth muscle through the mechanism which is dependent on the levels of the MLC phosphorylation but independent of a Ca^{2+}-calmodulin-MLCK pathway.

MYOSIN

Tu-Pos237

REGULATION OF MYOSIN Iβ ACTIVITY: BINDING OF Ca^{2+} TO CALMODULIN.

((**T. Zhu**, K. Beckingham* and M. Ikebe)) Department of Physiology, University of Massachusetts Medical Center, Worcester, MA 01655, *Department of Biochemistry and Cell Biology, Rice University, Houston, TX 77005

Previously, we have functionally expressed a mammalian myosin Iß with its light chain, calmodulin, in a baculovirus expression system [Biochemistry, 1996, 35:513]. The purified myosin Iß is composed of one heavy chain and three molecules of calmodulin as light chains. One of the three molecules of calmodulin is dissociated from the heavy chain when the calcium concentration is above 1 μM, while Mg^{2+}-ATPase activity of myosin Iß is increased above pCa 6. To further characterize this calcium dependent regulation, we have coexpressed myosin Iß heavy chain with a calmodulin mutant in which the Glu at position 12 in all four of the calcium binding loops is mutated to Gln. We found that the calcium dependent dissociation of calmodulin was abolished in myosin Iß containing this mutated calmodulin. Furthermore, the regulation of Mg^{2+}-ATPase activity by calcium was also lost. These results suggest that the effects of calcium on ATPase activity as well as the binding of calmodulin to myosin Iß heavy chain are due to the binding of calcium to calmodulin. (supported by NIH).

Tu-Pos238

POLARIZATION MEASUREMENTS OF SINGLE FLUOROPHORES ATTACHED TO MOTOR PROTEIN IN AQUEOUS SOLUTION. ((**K. Saito**[1], M. Tokunaga[1], A. Iwane[2], T. Yanagida[1,2,3])) [1]Yanagida Biomotron Project, ERATO, JST, Mino, Osaka, Japan & [2]Department of Biophysical Engineering, [3]Department of Physiology, Medical School, Osaka University, Japan. (Spon. by K. Namba)

In order to measure the orientation and conformation of proteins directly at the single molecular level, we have refined total internal reflection microscopy (*Nature*, **374**, 555-,'95) in order to detect the polarization of a single fluorophore attached to a protein. Two polarized evanescent fields, whose wave vectors were both parallel to the glass surface and intersected, were produced by totally reflecting two s-polarized laser beams passing through an objective lens in a glass-water interface. The polarization direction of the evanescent field was changed with a rate of 10/s by switching the two laser beams in a flip-flop manner. The fluorescence intensities from a single fluorophore that were excited by two polarized evanescent fields were measured with a photon counter (avalanche photodiode). And the orientation of a fluorophore that was bound to a protein was obtained by calculating the polarization and defining as $Q=(I_1 - I_2)/(I_1 + I_2)$.

We measured Q for single myosin subfragment-1s(S-1) bound to the glass surface, which were labeled with TRIA at SH1 or at RLC. These results show that the orientation of TRIA that was bound to RLC was almost random but that bound to SH1 was relatively order. This is consistent with the results obtained from muscle fibers. Our system can therefore resolve the orientation and conformation of protein molecules directly at the single molecular level.