M-PM-E7

THE ISOMETRIC FORCE OF A SINGLE KINESIN MOLECULE. ((C.M. Coppin*, J.T. Finer#, J.A. Spudich# and R.D. Vale*$^))) Departments of *Pharmacology and $Biochemistry, University of California, San Francisco, CA 94143; ^ The Marine Biological Laboratory, Woods Hole, MA 02543; #Departments of Biochemistry and Developmental Biology, Beckman Center, Stanford University School of Medicine, Stanford, CA 94305

The displacements and isometric forces produced by a single kinesin molecule translocating along a stationary axoneme were measured using the optical trap feedback apparatus of Finer et. al. (Finer, J. T. et al. Nature 368: 113-119, 1994). As a kinesin-bound bead moved up the force gradient of the optical trap, it usually exhibited discrete displacements of approximately 8 nm, similar to those described by Svoboda et al. (Svoboda, K. et al. Nature 365: 721-727, 1993). Frequently, the kinesin could move the bead against forces of about 3-5 pN before it released from the microtubule and returned to the trap center. The steps were separated by pauses ranging in duration from a few milliseconds to more than two seconds. A statistical analysis of all the observed pauses is under way.

When the position of the bead was clamped by feedback control of the optical trap's position, kinesin consistently achieved somewhat greater maximal forces than during translocation of the bead. Unlike myosin, which exhibits single force transients, kinesin reached its maximal force in a discontinuous fashion. At 20 μM ATP, the maximal force was often sustained for long periods lasting up to several seconds, but the force transient was sometimes abruptly terminated much sooner. This apparatus is currently being used to further define the characteristics of kinesin's force-generating and elastic components.

M-PM-E8

THE FORCE GENERATED BY A SINGLE KINESIN MOLECULE AGAINST AN ELASTIC LOAD ((E. Meyhöfer and J. Howard)) Dept. of Physiology and Biophysics, University of Washington, Seattle, WA 98195 and Klinische Physiologie, Medizinische Hochschule Hannover, 30623 Hannover, FRG.

To probe the molecular mechanism of force generation we used the kinesin *in vitro* motility assay as a model system and measusured the force single motor molecules can exert against microtubules.We developed a measurement technique that uses tiny glass fibers of calibrated stiffness as force transducers. One end of a biotinylated microtubule is attached via strepavidin to the tip of the glass fiber, the other end is allowed to interact with the surface of a small silica bead sparsely coated with kinesin. The tip of the glass needle is imaged onto a photodiode detector to track the displacements of the microtubule by kinesin with a spatial and force resolution of 1 nm and 1 pN respectively. Fibers were calibrated from the thermal motions of their tips. Their stiffnesses ranged from 0.02 to 0.63 pN/nm. The temporal resolution of the recordings was limited by the time constant of the fibers (0.33 - 5.7 ms).

At low kinesin density we observed stereotyped interactions between motors and microtubules: the speed was initially high, close to the unloaded gliding velocity, and decreased approximately linearly as the maximum force was reached. The repeatability of the events, the low kinesin density (at most 0.5 - 3 kinesin molecules on the surface of the sphere could interact with the microtubule) and the consistent forces (range 4 - 8 pN) suggest that the measurements were from single motors.The maximum force was 5.4 ± 1.0 pN (mean ± SD, n = 16), independent of the stiffness of the fiber, the damping from the fluid, and on whether or not the ATP concentration was high or low.

Supported by the Washington Affiliate of the AHA (EM), and NIH (AR40593) and Pew grants to JH.

K^+ CHANNELS I

M-Pos1

FIXED CHARGE SUBSTITUTION IN K_{Ca} CHANNELS. ((A. Lagrutta, K.-Z. Shen*, R.A. North**, and J.P. Adelman)) Vollum Institute for Advanced Biomedical Research, Oregon Health Sciences University, Portland, OR 97201.

Slowpoke is a large conductance, calcium-activated potassium channel cloned from *Drosophila*. Two glutamate residues in the carboxy terminal portion of transmembrane domain 6 were neutralized (EE335,338QQ) or reversed in charge (EE335,338KK). The double charge neutralization gave a complex phenotype, as analyzed in inside-out patches from *Xenopus* oocytes ($[K^+]_o = [K^+]_i$ = 120 mM; $[Mg^{2+}]_i$ = 2 mM; $[Ca^{2+}]_o$ = 1.8 mM). We determined mean unit currents in single and multi-channel (2-5) patches using all-point amplitude histograms, and then looked at the statistical distribution of these unit currents. Several current levels lower than wildtype were detected at all potentials tested. At +100 mV, these levels were 13.57 ± .34 (n = 4), 11.30 ± .14 (n = 11), 8 ± .14 (n = 17), and 5.35 ± .26 (n = 5) pA, while wildtype unit current was 14.93 ± .13 (n = 8). In some patches that appeared to contain a single channel, 2 or more of these conductance levels were present, with each conductance exhibiting characteristic gating properties. These results suggest that glutamates 335 and 338 are not only contributing to the unit conductance of the open channel, but also to kinetic transitions. To further analyze this phenotype, we introduced point mutations at positions E335 and E338, neutralizing or reversing charge. The E335Q, E338Q and E338K mutants showed a single conductance level (153, 178, and 131 pS, respectively; wildtype conductance: 196 pS), while the E335K mutant displayed two levels (122 and 166 pS). In E335K single channel patches the conductance levels are best described as gating modes. Current work is assessing the mechanistic basis of these discrete conductance levels.

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M-Pos2

BARIUM BLOCKADE OF A CLONED Ca^{2+}- ACTIVATED K^+ CHANNEL (*hSlo*) EXPRESSED IN *XENOPUS LAEVIS* OOCYTES. ((F. Díaz§, M. Wallner*, E. Stefani*, L. Toro* and R. Latorre§)) *UCLA, Los Angeles, CA 90024. §CECS and Univ. Chile, Santiago 9, Chile.

We have expressed the high conductance Ca^{2+}-activated K^+ channel *(hSlo)*, cloned from human myometrium in *Xenopus laevis* oocytes. In cell-attached patches, depolarizing pulses to 100-180 mV elicit ionic currents that increase with time and afterwards remain at a constant value (do not "inactivate"). However, in excised inside-out macropatches, pulses to the same potentials elicit currents that "inactivate"; they increase to a maximum value and then decay with time constants (~200 ms) that become faster for larger depolarizations. Recovery from this process has a time constant of 3 s at 0 mV. The time-dependent current reduction was manifested in single channel records as long-lived closed states triggered by large depolarizations. Internal Ba^{2+} is able to block high conductance Ca^{2+} -activated K^+ channels with high affinity. Addition of a "crown ether" Ba^{2+} chelator ((+)-18-crown-6-tetra-carboxylic acid) to the cytoplasmic side, to a final concentration of 20 μM, removed almost all the current inhibition promoted by depolarizing pulses in the 100 to 180 mV range. The crown ether effect was reversible upon perfusion of the internal side of the macropatch with a crown ether-free solution. These results suggest that the current decay for large depolarizations, in excised patches, is due to contaminant Ba^{2+} in the solutions that blocks *hSlo* channels, and not to an intrinsic voltage-dependent inactivation process uncovered by patch excision. Supported by NIH HL47382, GM50550, Progr. Becas Doc. CONICYT, and FNI 1940227.

M-Pos3

ELECTROSTATIC CONTROL OF MAXI-K CHANNEL TOXIN SPECIFICITY. ((T.J. Mullmann[1],P. Munujos[2], M.L. Garcia[2] and K.M. Giangiacomo[1])) [1]Dept. of Biochemistry, Temple Medical School, Philadelphia, PA 19140 and [2]Dept. of Membrane Biochemistry & Biophysics, Merck Research Laboratories, Rahway, NJ 07065

Iberiotoxin (IbTX) and Noxiustoxin (NxTX) are both highly charged peptide toxins that block the maxi-K channel with high and low affinity, respectively. Electrostatic potential maps calculated from the 3-dimensional structure for IbTX and a model of the NxTX structure reveal striking differences in their distributions of charge that may control their binding specificity. To identify electrostatic elements of the toxin structures that control the rates of toxin association and dissociation, we have examined toxin block of single maxi-K channels incorporated into planar lipid bilayers. Toxin dissociation rates for IbTX and NxTX vary over 80,000-fold. The average duration for NxTX block of the maxi-K channel was ~10 ms (V_m=-20mV), compared to 840 sec for IbTX (V_m=40mV). Removal of positive charges at residues K27 and R34 in IbTX increased the toxin dissociation rates for IbTX 2- and 50- fold, respectively. The rates of association for NxTX and IbTX are differentially influenced by ionic strength. The association rate for NxTX decreased ~ 20-fold as external potassium was increased from 25 to 100 mM. In contrast, the IbTX association rate decreased 3.4-fold. A mutant of IbTX with no net charge, IbTXR34N, exhibited an ionic strength dependence of its association rate similar to that of wild type IbTX. These findings suggest that the rates of toxin association are controlled by the distribution of toxin charge rather than the net charge.

M-Pos4

CLONING OF A MAXI CA-ACTIVATED K-CHANNEL FROM BOVINE AORTIC SMOOTH MUSCLE: SENSITIVITY TO A KUNITZ INHIBITOR. ((G.W.J. Moss, J. Marshall, M. Morabito, E. G. Moczydlowski and J. Howe)) Dept. of Pharmacology, Yale Univ. School of Medicine, New Haven, CT 06520.

Aortic smooth muscle cells express a high density of maxi K(Ca) channels. Previous results have shown that such K(Ca) channels have an internal binding site for Kunitz-type protease inhibitors and dendrotoxins which mediates production of discrete subconductance events in single channels. To study this peptide-channel interaction, the alpha subunit of a K-channel homologous to the *Drosophila slowpoke* K(Ca) channel was cloned from cultured bovine aortic smooth muscle cells. Except for alternatively spliced regions, the deduced amino acid sequence of the cloned cDNA (denoted *bSlo*) is >99% identical to the mouse *mSlo* K(Ca) channel reported by Butler et al. (1993). The isolated *bSlo* clone lacks alternatively spliced insertions A and B previously described in *mSlo* (Butler et al. 1993). *bSlo* also differs from *mSlo* at the C-terminus: 61 C-terminal residues in *mSlo* are replaced by a different 8-residue sequence in *bSlo*. This latter difference may represent a third splicing site that is functionally significant in smooth muscle. The *bSlo* clone was transiently expressed by transfection into cultured human embryonic kidney cells (HEK293) and K(Ca) channels were recorded by the patch clamp technique. The expressed channel exhibits many of the properties of the native channel in vascular smooth muscle including a unitary conductance of ~250 pS in symmetrical 150 mM KCl. To determine whether this cloned K(Ca) channel alpha subunit contains the binding site for Kunitz inhibitors, BPTI (bovine pancreatic trypsin inhibitor) was applied to the intracellular side of expressed channels. BPTI was found to induce subconductance events characteristic of the native channel in smooth muscle cells. This result indicates that the channel-forming alpha subunit of maxi K(Ca) channels contains the internal binding site for BPTI and dendrotoxins. (Supported by Donaghue Foundation, AHA, NIH.)

M-Pos5

ALLOSTERIC INTERACTION BETWEEN AN ACTIVATOR AND A BLOCKER OF CALCIUM-ACTIVATED POTASSIUM CHANNELS. ((M. Sanchez-Fernandez and O.B. McManus)) Univ. of Oviedo, Oviedo, Spain and Merck Research Labs, Rahway, NJ 07065.

Dehydrosoyasaponin I (DHS-I) is a reversible activator of high-conductance calcium-activated potassium (maxi-K) channels that was isolated from a medicinal herb based on its ability to inhibit charybdotoxin (ChTX) binding to this channel. DHS-I increases the open probability of maxi-K channels only when applied to the intracelular side , while ChTX blocks these channels only from the extracellular side. We have examined the interaction between these compounds on smooth muscle maxi-K channels inserted into planar lipid bilayers. Sufficient calcium was added to the intracellular side to maintain a high channel open probability (>0.7). ChTX blocks maxi-K channels by a bimolecular process and the blocked and unblocked event durations were described by single exponential distributions. Subsequent applications of DHS-I to the intracellular side caused decreases in the mean ChTX-induced channel blocked times in a concentration dependent manner with five- to seven-fold decreases observed at maximal DHS-I concentrations. This inhibition of ChTX blocked times by DHS-I could be described by inhibition curves with midpoints of about 10 nM and Hill coefficients of 1-2. At maximal DHS-I concentrations, a single population of ChTX blocked times were observed that are likely to reflect the channel with DHS-I bound. At lower concentrations of DHS-I, more than one component of blocked times could be resolved, suggesting that the kinetics of DHS-I interaction with maxi-K channels occur on a relatively slow time scale. The mean unblocked durations were not significantly altered by DHS-I. Thus DHS-I binds to sites on maxi-K channels that are physically remote from the ChTX binding site; inhibition of ChTX binding to maxi-K channels by DHS-I is entirely due to an increase in the rate of ChTX dissociation.

M-Pos6

CYTOPLASMIC Mg^{2+} MODULATES Ca^{2+}-DEPENDENT ACTIVATION OF MSLO BY BINDING TO A LOW AFFINITY SITE ON THE CHANNEL CORE. ((C.R. Solaro#, C. Nelson#, A. Wei*, L. Salkoff* and C.J. Lingle#*)) #Dept. of Anesthesiology and *Dept. of Anatomy and Neurobiology, Washington Univ. Sch. of Med., St. Louis, MO 63110.

Our previous results suggested that Ca^{2+}-dependent steps leading to activation of a cloned BK-type Ca^{2+}-activated K^+ channel (mSlo) are distinct from voltage-dependent steps. [Wei et. al. (1994), Neuron 13, 674]. Some of the apparent Ca^{2+}-dependence of activation is conferred by a large C-terminal "tail" domain, which may be separated from a "core" domain that resembles voltage-gated K^+ channels. Here, we have extended our analysis by studying mSlo currents activated in inside-out patches by high concentrations (>300 μM) of internal Ca^{2+} or by Ca^{2+} with high internal Mg^{2+}. As $[Ca^{2+}]_i$ is raised to 1 mM, the voltage of half-activation (V_{50}) approaches a limiting value, but at 10 mM Ca^{2+}, activation is shifted leftward by an additional 40 mV. Thus, a plot of V_{50} vs. *p*Ca over the range of 1 μM to 10 mM Ca^{2+} shows a distinct inflection. The 40 mV shift at 10 mM Ca^{2+} is not specific, though, since 10 mM Mg^{2+} in the presence of 100 μM Ca^{2+} produces a similar leftward shift. Mg^{2+} (>100 μM) shifts activation by Ca^{2+} in a concentration-dependent manner, but does not activate channels by itself. Similar shifts were observed in patches containing single mSlo channels. In contrast, activation of a particular splice variant of *Drosophila* slo (dSlo) is not sensitive to Mg^{2+}. Using *mSlo/dSlo* chimeras, we have identified a portion of the Slo "core" which appears to confer shifts in gating by high concentrations of divalent cations. These findings suggest the existence of a low affinity divalent cation binding site that, when occupied, shifts activation of channels already opened by higher affinity, Ca^{2+}-specific binding events. In the presence of 10 mM Mg^{2+}, V_{50} shows little dependence on $[Ca^{2+}]_i$ over 100 μM, suggesting that the high affinity Ca^{2+} site(s) are saturated, while gating remains voltage dependent. These results further support the view that Ca^{2+}- and voltage-dependent steps involved in mSlo activation are distinct. When Mg^{2+} is absent, leftward shifts in activation produced by $[Ca^{2+}]_i$ greater than 100 μM may be caused by binding of Ca^{2+} itself to the low affinity site. Thus, there appear to be multiple kinds of divalent cation sites which influence mSlo gating. (See also Schreiber et. al., this volume).

M-Pos7

THREE ACTIONS OF CALCIUM ON BK-TYPE K^+ CHANNELS. **((M. Schreiber, A. Wei, C. Lingle, and L. Salkoff))** Dept. Anatomy and Neurobiology, Washington Univ. Sch. of Med, St. Louis, MO 63110.

Both calcium and voltage activate the mouse BK-type K^+ channel [mSlo; Butler et al. (1993), Science 261, 221]. Site-directed mutagenesis suggests that regulation by Ca^{2+} may be through three separable mechanisms evoked by three different ranges of $[Ca^{2+}]$ and carried out by three distinct structural domains. The actions of Ca^{2+} at the μM and sub-μM level appear to be localized to the C-terminal "tail" domain [Wei et al. (1994), Neuron 13, 671] while at the mM range there may be a less selective divalent cation interaction with the "core" of the channel [Solaro et al., abstract, this volume]. At site 1, an aspartate-rich domain, sub-μM levels of Ca^{2+} act to "prime" the channel for activity by shifting the activation of the channel into the physiological range of voltages. This site may not be sufficient for activation of the channel. Two functional categories of mutants were produced at this site. Several deletions and substitutions resulted in the same phenotype, a 50 mV depolarizing shift. These appear to be loss-of-function mutations. However, one mutation, a single amino acid substitution, resulted in an intermediate phenotype which behaved as if the Ca^{2+}-affinity of the site was altered. The second site functions in the μM Ca^{2+} range, such that the V_{50} moves to hyperpolarized voltages in a graded fashion saturating at approximately 300 μM Ca^{2+}. Its presence is inferred by the maintenance of Ca^{2+}-dependent gating following loss of site 1 function. Its association with the tail is indicated by mutations outside of site 1 which affect Ca^{2+} sensitivity. Thus, site 2 may be the Ca^{2+} sensor that responds to physiological changes in submembrane $[Ca^{2+}]$. Voltage sensitivity, however, as defined by the slope of the g/v curve, remains the same over a wide range of $[Ca^{2+}]$. In summary, Ca^{2+} may act through more than one mechanism to provide exquisite control over BK channel activity.

M-Pos8

CURRENT RELAXATIONS ASSOCIATED WITH FAST GATING AT SUBZERO TEMPERATURES ((J. Miodownik and W. Nonner)) Department of Physiology and Biophysics. University of Miami, FL 33101. (Spon. by Gerhard Dahl)

At subzero temperatures (0°C to -35°C), elevated $[Ca^{++}]i$ (30-50 μM), and large depolarizations (+30 to +90 mV), individual Ca-activated large-conductance K channels of rat myotubes exhibit long bursts of activity characterized by short closures and substantial open-channel noise. Alignment of opening or closing events with respect to a 50% threshold and averaging revealed transitions that were indistinguishable from a pulse generator transition recorded through the same 8-pole, 2 kHz Bessel filter. Cooling hence did not slow the typical open/shut or shut/open transition itself to a resolvable rate.

On the other hand, when events were selected for minimal intervals in which no threshold crossings occurred before or after the transition itself, slow relaxations of averages spanning tens of ms were detected prior to and after the threshold crossing. Inspection of individual events underlying such relaxations showed a variety of transitional phenomena including ramp-like changes and/or rapid oscillations between fully open or shut and intermediate levels. Evidently, such threshold crossings were preceded and followed by periods of enhanced variability of single channel current. Events embedded between selected minimal intervals free of threshold crossings were equally frequent in the shut/open and open/shut directions and had time-symmetrical averages. Thus microscopic reversibility was not detectably violated.

Our results suggest that the typical opening and closing of the ionic pore are very fast processes unresolvable even in the cold and not detectably affected by the irreversible flux of ions. Slower (tens of ms at -20°C, -30°C) reactions appear to modulate the activity of the fast processes, producing detectable relaxations that lead and trail a threshold crossing. Supported by NIH GM30377 and Lucille Markey Foundation.

M-Pos9

MUTATIONAL ANALYSIS OF A CLONED INWARDLY RECTIFYING K^+ CHANNEL.

((Kugler, J.L., and Nelson, D.J.)) The University of Chicago, Chicago, Illinois, 60637 USA

A family of K^+ channels has been identified which differs from the voltage-gated channels in that they have inwardly rectifying current-voltage relationships. They are also believed to have only two transmembrane segments with limited homology to the fifth and sixth transmembrane domains of the voltage gated channels, as well as a region which is clearly related to the putative pore-forming region of the larger channels. While regions important in forming the inner mouth of the channel have been identified[1,2,3], current models of the structure of the outer vestibule and the pore are based primarily on analogy to the voltage-gated channels. We have identified, by site-directed mutagenesis of IRK1 a residue which may be important in the structural stability of the channel. Mutating Arg148 to either Ile, Met, or Asp results in a null mutant which produces no measurable current when injected into oocytes, whereas mutating Cys149 to Phe produces a channel with wild-type macroscopic characteristics. It is our hypothesis that this residue, Arg148, may be involved in forming a salt bridge with Glu139, thus stabilizing the tertiary structure of the pore.

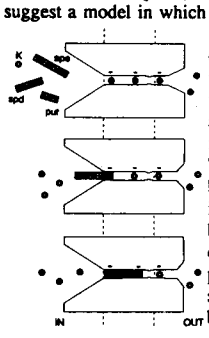
This work supported by NIH RO1 GM36823.

[1]Lu, Z., & MacKinnon, R. *Nature* 371, 243-246. (1994)
[2]Tagliatela, M., Wible, B.A., Caporaso, R., & Brown, A.M. *Science* 264, 844-847. (1994)
[3]Wible, B.A., Tagliatela, M., Ficker, E., & Brown, A.M. *Nature* 371, 246-249. (1994)

M-Pos10

'LONG-PORE PLUGGING' MODEL FOR POLYAMINE-INDUCED RECTIFICATION OF POTASSIUM CHANNELS ((A.N. Lopatin, E.N. Makhina and C.G. Nichols)). Dept. Cell Biol. and Physiol. Washington U. Sch. Med., 660 So. Euclid, St. Louis, MO 63110.

We have shown that, rather than being intrinsic to the channel protein, apparently 'intrinsic' rectification of inward rectifier K^+ channels results from channel block by cytoplasmic factors that we have identified as polyamines, including spermine (spe), spermidine (spd) and putrescine (put) (Lopatin et al. 1994, *Nature*, In press). Based on the linear structure of these substances, we suggest a model in which the long pore of the inward rectifier channel is 'plugged' by polyamines, which bind first at the entrance to the pore, and then undergo further voltage-dependent steps, entering more deeply into the pore. In such a model (Fig. 1), the predicted apparent gating charge is ~2.5, 2.25 and 1.75, respectively for spermine, spermidine and putrescine. The measured steepness of the rectification induced by these agents (Z=2.63, 2.21, 1.69, respectively) is in good agreement with these predictions. Channel block by polyamines is well fit by a Hill coefficient of 1 in each case. The different sensitivity to each polyamine ($k_{1/2,-20mV}$ = 10 μM, 100 μM, 2 mM for spe, spd, put, respectively) is then reflected in the binding at the entrance of the pore.



M-Pos11

Contributions by the C-terminus to Gating and Rectification of Inward Rectifier Potassium Channels (M Pessia, CT Bond, MP Kavanaugh, JP Adelman) Vollum Institute, OHSU, Portland, OR 97201

Clones encoding two distinct subtypes of inward rectifiers have been described, those which are permeable to potassium when the membrane potential falls below E_K, and those which also require the actions of activated G-proteins. In addition to reflief of internal cation blockade, some of these channels display a voltage-dependent gating component. However, the two classes of inward rectifiers differ in their kinetics of activation. A chimeric channel, BG, in which the intracellular carboxyl terminal domain of a G-protein activated inward rectifier (GIRK) replaced the analagous domain of a G-protein independent inward rectifier (BIR10) was constructed. The expressed chimeric channel: 1) passed potassium ions at potentials below E_K without the requirement of activated G-proteins, 2) displayed rectification properties indistinguishable from the GIRK even though the 'rectification residue' in TM2 was provided by BIR10 (glu159), 3) activation kinetics were similar to GIRK, 4) voltage-dependent inactivation at more hyperpolarized potentials, present in BIR10, was lost. For BIR10, activation was well fitted by a single exponential function while GIRK and BG required the sum of two exponential functions. For all three channels, the time constants of activation were decreased by increased external potassium concentrations. However, while the time constant for BIR10 was strongly voltage-dependent, the time constants for GIRK or BG were significantly less voltage-dependent. In addition, the time constants for BG under all conditions were the same as for GIRK. Therefore, residues C-terminal to TM2 are structural components of gating and rectification, but are not sufficient to transduce the effects of activated G-proteins.

M-Pos12

UNILATERAL EXPOSURE TO HYPEROSMOTIC STRESS: EFFECTS ON *SHAKER* B POTASSIUM CHANNELS. ((John G. Strakus[1], Thomas Schlief[2], Martin Rayner[1], Stefan H. Heinemann[2]))
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Inactivation-removed *Shaker* B potassium channels were expressed in *Xenopus* oocytes. Single-channel and macropatch currents were recorded from inside-out and outside-out patches. Unilateral hyperosmotic stress (either internal or external) induced by the addition of sucrose (to ~ 1.6 Osm/kg) produced differential effects on the kinetics, but not on the voltage sensitivity, of activation and deactivation. External osmotic stress slowed only late steps in channel activation. By contrast, internal osmotic stress primarily slowed the early activation steps. External osmotic stress did not alter deactivation kinetics. However, during internal hyperosmolar exposure, tail currents were slowed in the presence of high $[K^+]_o$ (115 mM) but became faster in low $[K^+]_o$ (3 mM).

Additionally, equivalent suppression of both macroscopic and single-channel currents by internal or external osmotic stress indicates that the reduction of macroscopic K^+ current amplitudes arises from changes in single-channel conductance rather than from osmotically-induced changes in equilibrium channel gating kinetics.

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M-Pos13

K^+ CHANNEL PORE STRUCTURE: MUTATIONS CONFERRING Ni^{++}-BLOCK. ((R.G. Vickery and T.L. Schwarz)) Stanford University, Department of Molecular and Cellular Physiology, Stanford , CA 94305.

Histidine and cysteine residues are often found to play a role in metal ion binding sites in metaloproteins and, in concert, can have a high affinity for Ni^{++}. We are using this property in an attempt to engineer a metal binding site into the conduction pathway of K^+ channels to explore pore structure. We screened N-terminal deleted Shaker channels for susceptibility to Ni^{++} block using two-electrode voltage clamp and observed a slowing of the rise time of the current but no blockade of the channel. When thr 449, a residue thought to line the outer mouth of the pore and implicated in C-type inactivation, was replaced with histidine, Ni^{++} reduced the steady state current (ED_{50}= 50uM). This blockade was slow but showed little or no use dependence. Cys had a similar effect, while Tyr, Val, Lys, and Trp did not. Surprisingly however, alanine also conferred sensitivity to Ni^{++} and the block was much faster. Single channel recordings in the presence of Ni^{++} revealed subconductances in the his and cys mutations. The properties of these mutations will be presented in light of a model in which this site restricts access to a Ni^{++}-binding site deeper in the pore.

M-Pos14

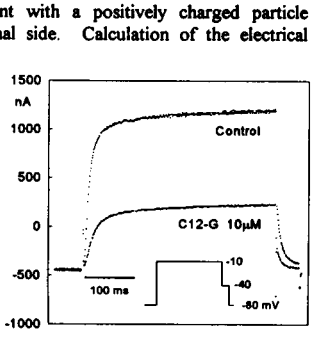
BLOCK OF AN INACTIVATING K^+ CHANNEL BY VERAPAMIL. ((X.-C. Yang[1,2] and W. Tang[1])) [1]CNS Biology, Lederle Laboratories, Pearl River, NY 10965 and [2]Department of Pharmacology, College of Physicians and Surgeons of Columbia University, New York, NY 10032.

The effect of verapamil, a Ca^{2+} channel blocker, on a Kv1.4 K^+ channel clone (RHK1) expressed in *Xenopus* oocytes was studied using a two-electrode voltage-clamp. The transient Kv1.4 current decayed faster with reduced peaks in the presence of verapamil. The decay time constant, τ, was nearly independent of testing potentials from -10 to +40 mV. τ decreased from about 40 ms to 10-15 ms as the concentration of verapamil was increased from 0 to 500 μM. The half block concentration of ~90 μM appeared to be independent of both the testing potential (-10 or +20 mV) and holding potential (-80 and -50 mV). The reciprocal of the decay time constant, τ^{-1}, was a linear function of the drug concentration up to 200 μM. The fraction of block of the peak current, however, was voltage-dependent, decreasing with depolarization. The electrical distance, $z\delta$, of the drug binding site was determined to be 0.35 ± 0.01 (mean ± S.E.M., n=4; 200 μM verapamil) from the outside of the membrane based on the Woodhull model. The drug had slight effects on the activation time constant, the steady state activation and inactivation. The data could be described by an open-channel block model. The apparent discrepancy (τ vs. peak) in voltage-dependence of the block is under investigation.

M-Pos15

BLOCK OF A Kv1.2 CHANNEL BY AN AMPHIPHILIC GUANIDINE. ((F. de Lorenzi, W. Tang and X.-C. Yang)) CNS Biological Research, Lederle Laboratories, Pearl River, NY 10965.

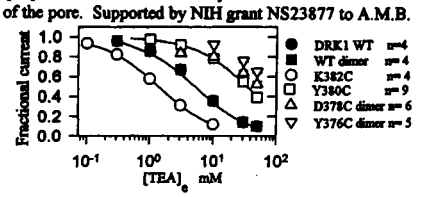
The positively charged guanidine functional group often confers to a drug the ability to inhibit K^+ channels. A delayed rectifier-type K^+ channel (rKv1.2) was expressed in *Xenopus* oocytes and currents were recorded using the two-electrode voltage-clamp technique. Dodecylguanidine ($C_{12}H_{25}$-G) blocked the steady-state current in a voltage-dependent manner. The block decreased steeply with depolarization, consistent with a positively charged particle entering the channel from the external side. Calculation of the electrical distance ($z\delta$) yielded 1.43±0.07 (n=6), which may suggest cooperativity. Activation was slowed down in a similar voltage-dependent manner (by 50% at 0 mV), consistent with a fast development of block upon current activation (see Figure). Because $C_{12}H_{25}$-G also interacted with the block by dendrotoxin, it is proposed that $C_{12}H_{25}$-G, whilst embedded in the membrane, is an open-state blocker acting from the extracellular side of the channel.



M-Pos16

MULTIPLE CHARGED AND AROMATIC AMINO ACIDS CONTRIBUTE TO BLOCK BY TETRAETHYLAMMONIUM IN POTASSIUM CHANNELS ((J.M. Pascual, C.C. Shieh and A.M. Brown)) Department of Molecular Physiology and Biophysics. Baylor College of Medicine. Houston, Texas 77030.

A ring of four tyrosines (one per channel subunit) was proposed to constitute part of the external binding site for tetraethylammonium (TEAe) in *Shaker* potassium channels (Heginbotham *et al. Neuron* 8, 1992). We substituted this (Y380) and neighboring residues in Kv2.1 by single cysteines. Mutation of some of the evolutionarily conserved residues was tolerated only in dimeric constructs containing one wild type repeat. In addition to Y380C, we found that K382C, D378C and Y376C significantly affected TEAe sensitivity without altering Na^+/K^+ selectivity or macroscopic gating kinetics. Except for Y376C, all of these residues were available for conductance modification by methanethiosulfonate derivatives (MTSX). In contrast, I379C did not affect TEAe sensitivity but could be modified by MTSX. No other residue from I369C to T383C affected TEAe affinity or was accessible to MTSX. We propose that in Kv2.1 block by TEAe involves numerous residues at the outer mouth of the pore. Supported by NIH grant NS23877 to A.M.B.



M-Pos17

EXPRESSION OF RECOMBINANT α-DENDROTOXIN: A BLOCKER OF VOLTAGE-DEPENDENT POTASSIUM CHANNELS. ((R.G. Sorensen)) Department of Medicine, Div. of Environmental Medicine and Toxicology, Jefferson Medical College, Philadelphia, PA 19107.

The dendrotoxins are a family of homologous polypeptides isolated from snake, Dendroaspis, venom that block voltage-dependent potassium channels. We have prepared a synthetic gene that encodes the structure of α-dendrotoxin, a polypeptide component of D. angusticeps venom. We now show that the expressed recombinant toxin blocks potassium channels in a manner similar to that of the native toxin. The synthetic gene, 200 bp that included restriction enzyme sites at both the 5' and 3' ends to facilitate subsequent cloning, was prepared from 6 complimentary pairs of oligonucleotides that encompassed the full DNA coding length, as derived from the amino acid sequence (59 amino acids) of the native α-dendrotoxin polypeptide. The synthetic gene was cloned into the expression vector, pFLAG-1 (IBI-Kodak), and over-expressed in bacteria (XL1-blue, Stratagene). The purified recombinant polypeptide was tested, by the two-microelectrode voltage clamp technique, for the ability to block cloned mammalian brain potassium channels, $K_v1.1$ and $K_v1.6$, expressed in Xenopus oocytes. Both the native and recombinant dendrotoxins blocked the expressed K currents at nM concentrations, and block of the currents was not voltage-dependent. Mutant dendrotoxins are currently being prepared, using the synthetic α-dendrotoxin gene as template, to be used as probes for identifying the structural features of the dendrotoxin polypeptide involved in binding to, and thereby blocking, voltage-dependent potassium channels. (Supported by grant NIH NS31670).

M-Pos18

A MUTANT VOLTAGE-GATED K^+ CHANNEL WITH ALTERED Mg^{++} BINDING. ((*R.E. Harris and E.Y. Isacoff*)) Dept. of Mol. and Cell Biol., University of California at Berkeley, Berkeley, CA 94720.

Intracellular Mg^{++} blocks voltage-gated K^+ channels by binding to a site deep within the permeation pathway. We are interested in identifying residues that form the Mg^{++} ion binding site in voltage-gated K^+ channels. We have found that the voltage-gated channels ShH4(Δ6-46) and Kv3.1 differ markedly in their block by Mg^{++}. The affinity [$(Ki\ OmV)^{-1}$] of ShH4(Δ6-46) was 17-fold less than Kv3.1. The electrical distance to the Mg^{++} binding site was .43 for ShH4(Δ6-46) and .34 for Kv3.1. The blocking kinetics also differed between these two channels. Mg^{++} blocking and unblocking events of Kv3.1 were too fast to resolve full channel openings or closings and were observed as a flickery block of open channel current at depolarized potentials. In contrast, blocking and unblocking events of ShH4(Δ6-46) were on the msec time scale and produced resolvable channel openings and closings. Replacement of the H5 domain of ShH4(Δ6-46) with that of Kv3.1 introduced flickery block to the chimeric channel which suggested that H5 residues that differed between ShH4(Δ6-46) and Kv3.1 were responsible for the difference in Mg^{++} binding. One such residue is L401 in Kv3.1. Mutation of this residue to a valine decreased affinity for Mg^{++} by ten-fold, changed δ from .34 to .44, and slowed both the Mg^{++} on and off rates enough to abolish flickery block. As observed previously (Aiyar *et al, Biophys. J. abs.*, 64:(2)A197, 1993), single channel conductance and K^+/Na^+ selectivity were unaltered by this mutation. These data are consistent with an alteration of the Mg^{++} ion binding site in this mutant.

M-Pos19

H-ERG, A MEMBER OF THE *EAG* FAMILY OF K^+ CHANNELS, ENCODES AN INWARD RECTIFIER. ((Matthew C. Trudeau, Jeffrey W. Warmke#*, Barry Ganetzky# and Gail A. Robertson)) Dept. of Physiology and #Laboratory of Genetics, University of Wisconsin, Madison, WI, 53706, and *Dept. of Genetics and Molecular Biology, Merck Res. Lab., Rahway, NJ.

Eag-related channels are similar to *Sh*-related channels in their overall body plan. In addition, they are functionally similar, gated by depolarizing voltages and selective for K^+ ions. We have recently expressed *h-erg*, an *eag* homolog from human hippocampus, and found that it has the hallmark properties of inwardly rectifying K^+ channels. Like other inward rectifiers, *h-erg* channels are activated at voltages below E_K and preferentially conduct inward K^+ currents. External Cs^+ blocks the channel in a voltage-dependent manner. These results are surprising, both because of the extensive sequence homology between *h-erg* and other *eag*-related channels, and because *h-erg* channels are structurally so different from other recently-identified inward rectifiers in mammals. The mechanism by which inward rectification arises in *h-erg* channels is the subject of present investigation.
Funded by the American Heart Association of Wisconsin.

M-Pos20

ACTIVATION PROPERTIES OF WILD-TYPE AND S4 MUTANTS OF A PLANT INWARD RECTIFYING POTASSIUM CHANNEL (KAT1). ((Paul C. Zei, Alan G. Miller, and Richard W. Aldrich)) Dept. of Mol. and Cell Physiol., HHMI, Stanford Univeristy School of Medicine, Stanford, CA 94305.

Activation properties of the plant inward rectifying potassium channel KAT1 were studied using the patch clamp and the cut-open oocyte clamp techniques. The time course of wild type (WT) macroscopic activation is sigmoidal and voltage dependent. Deactivation kinetics are also voltage dependent and can be fit by a single exponential. The instantaneous tail IV is linear between -200 mV and +20 mV. Single channel analysis reveals that open time distributions can be fit by a single exponential, while closed time distributions are fit by at least two exponentials. After first opening, KAT1 channels exhibit bursting behavior. Both the first latency distribution and the mean burst duration are voltage dependent over the range of activation, while the open time and closed time distributions within bursts exhibit little voltage dependence. The single channel conductance is 7 pS, with a linear iV between -180 mV and -80 mV. KAT1 runs down in excised patches, resulting in longer first latencies and shorter mean burst durations at a given voltage. Rundown is not significant in the cut-open clamp configuration. Four individual neutralizations introduced into the S4 region of KAT1 formed functional channels. Steady-state GV relationships were fit with a fourth power of a Boltzman:

$$G = G_{max}[1/(1 + e^{-(V-V_{1/2})zF/RT})]^4$$

with G_{max}, $V_{1/2}$ and z as free parameters. R177Q shifts $V_{1/2}$ by +100 mV with respect to WT and decreases z by a factor of two. Deactivation kinetics are slower, while activation kinetics are faster than WT. R184Q shifts $V_{1/2}$ by +30 mV, with little change in z, deactivation, nor activation kinetics. K187Q and R174Q both shifted $V_{1/2}$ in the negative direction. Deactivation kinetics in K187Q are faster than WT.

M-Pos21

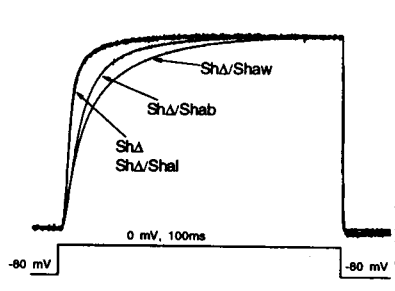
SUBSTITUTION OF THE S4 REGION IS NOT SUFFICIENT TO CONFER THE VOLTAGE-DEPENDENT PROPERTIES OF SHAW ON SHAKER ((J.L. Ledwell, C.J. Smith-Maxwell, and R.W. Aldrich)) Dept. of Molecular and Cellular Physiology and Howard Hughes Medical Institute, Stanford University, Stanford CA 94305

Several studies have suggested that the S4 region plays a role in voltage-dependent activation. We have studied the behaviour of two potassium channels from *Drosophila, Shaw and Shaker*, and of a chimeric construct with the S4 region of *Shaw* substituted into a *Shaker* background. All studies in *Shaker* and the chimera are in a truncated form of *Shaker* B in which fast N-type inactivation has been removed. *Shaw* does not exhibit fast inactivation. Currents were measured using on-cell and excised patch clamp configurations. The conductance-voltage relationship of *Shaw* is very shallow and the conductance does not saturate in the range of voltages up to +200mV. In contrast, the conductance-voltage relationship of *Shaker* is steeply voltage -dependent and does saturate. The conductance-voltage relationship of the chimera is greatly shifted in the depolarized direction with respect to *Shaker* and is more shallow than that of *Shaker*; however, the conductance-voltage relationship of the chimera is much more steeply voltage-dependent than that of *Shaw*. Activation kinetics of *Shaker* are voltage-dependent and the time course of activation is sigmoidal. The time course of activation of *Shaw* is described well by a single exponential, generating time constants of 2-4 ms that are remarkably voltage-independent over the range of -80mV to +80mV. The time course of activation in the *Shaw* S4 chimera is single-exponential with no evidence of the sigmoidal delay observed in *Shaker*; but the time constants are voltage-dependent like *Shaker* and are much slower than the time constants of either parent channel. These results indicate that while the chimeric substitution of the S4 region profoundly alters the activation properties of *Shaker*, this substitution does not confer *Shaw*-like characteristics on *Shaker*.

M-Pos22

ROLE OF THE S3-S4 LINKER IN ACTIVATION OF *SHAKER* K^+ CHANNELS. ((R. Mathur, J. Zheng, Y. Yan and F. J. Sigworth)) Cell. and Molec. Physiology, Yale School of Medicine, New Haven CT 06510. (Spon. by W. K. Chandler)

Although dissimilar among subfamilies of voltage-gated channels, the putative extracellular linker formed by *Sh*B residues 333-357 might be expected to be functionally important because it would participate in motions of the S4 region during voltage activation. 13 mutations in this region of N-terminal truncated *Sh* 29-4 were characterized, including deletions, charge reversals, substitutions of prolines, etc. Most had no effect on voltage dependence, kinetics or selectivity. Of interest however are chimeras in which the short (7-9 residue) S3-S4 linkers from *Shab, Shal* and *Shaw* were substituted for the 25-residue *Shaker* linker. Analogously to some chimeras in Ca^{++} channels (Nakai et al, *PNAS* 91:1014, 1994), *Shaw* and *Shab* linkers conferred slower activation kinetics; *Shab* also gave an activation shift of -20 mV. There were no apparent changes in the limiting slope of voltage dependence.



M-Pos23

C-TYPE INACTIVATION DETERMINED BY MULTIPLE SUBUNITS. ((D.I. Levy, G. Panyi, Z.F. Sheng, L. Tu, and C. Deutsch)) Dept. of Physiology, University of Pennsylvania, Philadelphia, PA 19104-6085.

Kv1.3 lacks N-type inactivation, but undergoes C-type inactivation (Grissmer and Cahalan, 1989), which has been postulated to involve a conformational change near the outer mouth of the channel (Yellen *et al.*, 1994). We have cloned a Kv1.3 mutant in which alanine 413 has been mutated to valine (Kv1.3A413V), similar to the Shaker mutant studied by Hoshi *et al.* (1991). This mutant produces an n-type current in mouse cytotoxic T cells (CTLL-2) with 50-fold enhancement in inactivation rate over wild-type Kv1.3 and markedly slower recovery from inactivation. The steady-state inactivation versus voltage curve is shifted to very negative membrane potentials compared to wild-type Kv1.3. The C-type inactivation time course is reversibly slowed when external [K^+] is increased, and external TEA decreases peak current amplitude and inactivation kinetics. To establish whether one or more subunits is sufficient to determine C-type inactivation, we have co-tranfected CTLL-2 with a double-gene plasmid containing Kv1.3A413V and a surface marker gene, CD20, and a plasmid containing wild-type Kv1.3. Heteromultimeric currents predominated, manifesting inactivation kinetics intermediate between Kv1.3A413V (τ = 3.7±0.4 msec, n=5) and wild-type Kv1.3 (τ = 270±33 msec, n=8). The currents from co-transfected cells had inactivation time constants for a step to +50 mV from -130 mV that ranged from 4 to 60 msec. These results are used to distinguish between two models for C-type inactivation of a tetrameric channel: a cooperative model and a model in which each subunit contributes equally and acts independently. (Supp. GM41467; FO5 TWO 5079).

M-Pos24

CORRELATION BETWEEN CHARGE MOVEMENT AND IONIC CURRENTS DURING C-TYPE INACTIVATION IN SHAKER-IR POTASSIUM CHANNELS ((R. Olcese, L. Toro, F. Bezanilla & E. Stefani.)) Dept. of Anesthesiology and Physiology, UCLA School of Medicine, Los Angeles, CA 90024.

In previous study, performed in a non-conducting mutant of Shaker K^+ channel lacking the ball peptide (*ShH4IR-W434F*) and expressed in *Xenopus* oocytes, we found that charge movement voltage dependence was shifted to more negative potential during prolonged depolarization (type-C inactivation). We now extended this study by comparing the time course of installation and recovery, and the steady state properties of the ionic current in the conducting clone *ShH4IR* during C-inactivation, with the changes in charge movement properties in the respective non-conducting clone. Records were made using the cut-open oocyte voltage clamp technique. We found a good correlation between the changes in the ionic currents and charge movement during C-inactivation. The time course of the slow inactivating ionic current during 1 min pulse at 0 mV and the time course of the charge inactivation at the same potential were well fitted simultaneously with the sum of two exponential functions with the same time constants (τ): 4.1 and 24.0 s for the fast and slow component, respectively. Recovery at -90 mV after a one minute inactivating pulse at 0 mV was fitted to a double exponential function for both ionic and gating currents with the same τ: 0.01s and 1.1s. The voltage dependence of the fraction of the channels that reaches the open state after the prolonged depolarization had the same voltage dependence, suggesting that activation pathway was unaltered. The data could be fitted to the sequential model for Shaker K^+ channels (Bezanilla et al., 1994), but with the addition of a series of parallel states that becomes populated during prolonged depolarization.(Supported by NIH grant GM50550).

M-Pos25

MODELING OF *SHAKER* H4IR IONIC AND GATING CURRENTS. ((M.J. Roux[#], D. Sigg[#*], R.S. Hurst[#], F. Bezanilla[#*] & E. Stefani[#])) Departments of Anesthesiology[#] and Physiology[*], UCLA, Los Angeles, CA 90024.

Steady-state and kinetic properties of gating currents (*Shaker* H4IR W434F) and ionic currents (*Shaker* H4IR) of the *Shaker* K^+ channels were studied after expression in *Xenopus* oocytes and recorded with the COVG and patch-clamp techniques. The number of channels were determined by variance analysis from the ionic current data and ranged from the limiting charge movement assuming 12-16 electronic charges per channel. Ionic and gating currents were simultaneously fitted to a strictly sequential cooperative model (Bezanilla et al 1994, BPS model) or to a model with four independent subunits with two conformational changes in each of the four subunits (Schoppa et al. 1994, Zagotta et al. 1994, referred to as ZA model). The fitted traces predicted by such models were compared to the experimental data for critical protocols like fast reopening after depolarizing prepulses, deactivation tails at different post-pulse potentials and Cole-Moore effect. With the same set of parameters, the ZA model accounts well for the ionic currents but does not fit properly to the ON and OFF gating currents, while the BPS model can account for most of the features of ionic and gating currents. The BPS model also predicts most of the characteristics of the gating current noise, such as the delay of the ON variance in respect to the mean currents, the absence of negative correlation around the covariance spike and the overall time course of the OFF variance. This analysis supports the view that the four subunits of the channel protein function in a coordinated and non-independent way.(Supported by NIH grants GM50550 to ES and GM30376 to FB)

M-Pos26

TRANSMEMBRANE ACIDIC RESIDUES CONTRIBUTE TO VOLTAGE-DEPENDENT ACTIVATION OF SHAKER K^+ CHANNELS. ((Sang-Ah Seoh and Diane M. Papazian)), Department of Physiology, UCLA School of Medicine, Los Angeles, CA, 90024

Previous studies have identified the S4 segment as part of the voltage sensor in Shaker K^+ channels. We have now tested the possibility that conserved acidic residues in putative transmembrane segments also contribute to activation. Charge-conserving and charge-neutralizing mutations were made at positions E283 and E293 in S2 and D316 in S3. After expression in *Xenopus* oocytes, the voltage-dependence of activation in the mutant channels was determined in inactivation-removed (IR) constructs using macroscopic tail current measurements. Data were analyzed using both model-dependent and model-independent methods. Our results support the hypothesis that D316 is a voltage-sensing residue in Shaker channels. In addition, E293 plays a key role in gating kinetics, because the mutation E293Q slows activation and dramatically slows deactivation. The last opening transition is not the rate-limiting step for activation in Shaker-IR or E293Q-IR channels, as shown by double pulse experiments. Therefore, E293Q-IR affects earlier (presumably charge-moving) steps in the activation pathway.

M-Pos27

KINETIC VARIABILITY IN INACTIVATION-REMOVED *SHAKER* B K^+ CHANNELS

((M. Andres, M.D. Rayner, P.C. Ruben, M. Henteleff, M. Hermosura and J.G. Starkus)) Békésy Lab, University of Hawaii, Honolulu, HI 96822.

We have assessed the kinetic stability of inactivation-removed *Shaker* B K^+ channels expressed in *Xenopus* oocytes in cell-attached, inside-out, outside-out and "crammed-in" patches. We observed the following changes over time: 1) a decrease in the rate of activation, 2) a depolarizing shift in midpoint voltage, and 3) a decrease in the rate of deactivation. Whereas most of on-cell patches are stable, inside-out and outside-out patches show significant parameter changes within the first 10 minutes at room temperature. The changes outlined above occur independently of each other and are partially reversible by cramming the patch back into the intracellular milieu of the oocyte. Using on-cell recordings, we found that there are variations in both the kinetics and voltage-sensitivity between different patches from the same oocyte. Our observations imply that both the voltage-sensitivity and rates of activation and deactivation may be separately and reversibly regulated by metabolic processes within the non-homogeneous intracellular environment of the oocyte.

(Supported by PHS grants #NS-21151 and #NS-29204, NIH RCMI grant #RR-03061, and the American Heart Association, Hawaii Affiliate)

M-Pos28

EFFECTS OF AN AROMATIC AMINO ACID MUTATION IN THE S4 SEGMENT IN *SHAKER* K CHANNEL ((Z. Lu, H. Bao, M. Rayner, P.C. Ruben, J.G. Starkus)) Békésy Lab, Pac. Biomed. Res. Ctr., Univ. Hawaii, Honolulu, HI 96822

We previously investigated effects of mutant L860F in RBIIaNa channel (Fleig et. al., 1994), which changes a flanking residue from FKL^{860} to FKF^{860} at the 4th charge in S4 of domain II. The 4th charge in wild type *Shaker* K S4 segment has the same FKL^{363} sequence. Mutation L363F in *Shaker* K channel was made by site-directed mutagenesis and expressed in *Xenopus* oocytes for comparison with both the L860F mutant in Na channel and the V1 (L363V) mutant in *Shaker* K channel (McCormack et al., 1991). Channels in on-cell and inside-out patches were recorded in symmetric K^+ solution. The F(V) curve is right-shifted about 24 mV in L363F of K channel. This is well matched with L860F in Na channel (about 20mV right-shifted). Apparent activation valence is reduced by about 1.8*e* in the mutant, whereas the reduction is about 0.9*e* in Na channels. Our results are different from the effect of the hydrophobic V1 mutant in that the L363F mutant is less right-shifted and its valence is less dramatically reduced.

(Supported by PHS grants NS21151 and NS29204, RCMI grant #03061, and the American Heart Association, Hawaii Affiliate).

M-Pos29

MUTATIONS IN S4 OF A DELAYED RECTIFIER K CHANNEL (DRK1) SLOW ACTIVATION AND PROMOTE SUBSTATES. ((ML Chapman, HMA VanDongen and AMJ VanDongen)) Department of Pharmacology, Duke University Medical Center, Durham NC 27710. (Spon. by L.S. Beese)

Voltage dependent K channels are formed by four identical subunits surrounding a central pore. Each subunit contains a voltage sensor (S4) and contributes to the pore lining. Channel activation is a sequential mechanism involving movement of the S4 segments, followed by a concerted conformational change of the pore. Single channel analysis of drk1 has revealed the existence of short-lived subconductance states during open-close transitions, suggesting a new role for subunits in gating and permeation (VanDongen *et al*, this meeting). To test this model we have attempted to alter the activation properties of drk1. Site directed mutagenesis was employed to alter the N-terminal region of S4 and mutant channels were expressed in *Xenopus* oocytes. Whole voltage clamp analysis showed that for some S4 mutants activation kinetics were slowed dramatically. In addition, activation time constants and steady-state open probabilities were much less voltage-dependent. At the single-channel level, a notable degree of long-lived subconductance behavior was seen, with many openings gradually reaching the full conductance level in a staircase manner. Heteromeric channels were produced by engineering a tandem construct containing one wildtype and one mutant subunit. The activation and steady state properties of this dimer were intermediate to those of the two parent channels. Single channel recordings also revealed numerous substates but with different characteristics than the mutant parent. Many partial openings were seen, especially early in the pulse, but staircasing was rare. These data support a new model for activation and permeation which is based on the conformational state of individual channel subunits.

M-Pos30

VOLTAGE DEPENDENCE OF TWO OPEN STATES IN CLONED DELAYED RECTIFIER K^+ CHANNELS EXPRESSED IN *XENOPUS* OOCYTES. ((Scott N. Russell, Fivos Vogalis, Burton Horowitz, and Nelson G. Publicover)) Universtiy of Nevada, Department of Physiology (352), Reno, NV 89557 (Spon. by J. Hume)

The voltage-dependence of activation and inactivation of $cK_v1.2$ and $cK_v1.5$ delayed rectifier K^+ currents expressed in *Xenopus* oocytes was examined using the double-electrode voltage clamp and patch clamp techniques. Whole-cell current responses to voltage clamp steps were fit by the sum of 2 exponential functions during both the activation and inactivation phases. At positive potentials, activation rate constants were linearly dependent on membrane potential. The slope of the voltage dependence was the same for $cK_v1.2$ and $cK_v1.5$, averaging 11 and 2.4 $sec^{-1}mV^{-1}$ for the fast and slow activation rate constants respectively. Single-channel, dwell time histograms of both open and closed states were fit by the sum of 2 exponentials. Mean open and closed dwell times were not voltage dependent. The primary voltage dependence in single channel records appeared in the latency between the voltage clamp step and the first channel opening (median 1^{st} latency) which decreased e fold per 51mV. Inactivation was voltage independent in both $cK_v1.2$ and $cK_v1.5$, and there was no significant difference in the fast and slow inactivation rates in the two clones, averaging 0.89 and 0.06 sec^{-1}. At all membrane potentials, the amplitude of current associated with faster inactivation was less than that associated with slower inactivation. The primary difference between the 2 clones was in the fraction of current associated with the faster inactivation rate constant (approximately 40% for $cK_v1.2$ versus 20% for $cK_v1.5$ at positive potentials). Responses of both $cK_v1.2$ and $cK_v1.5$ channels can be described by a kinetic model that consists of at least 2 distinct open states. (Supported by NIDDK)

M-Pos31

THE S2 AND S3 TRANSMEMBRANE SEGMENTS ARE ADDITIONAL COMPONENTS OF THE VOLTAGE SENSOR IN VOLTAGE-GATED POTASSIUM CHANNELS.

((*R. Planells-Cases, †A. V. Ferrer-Montiel, ‡C. D. Patten and †M. Montal)) Departments of *Neurosciences, ‡Physics and †Biology. University of California San Diego. La Jolla CA 92093-0357.

The S4 transmembrane segment of voltage-gated potassium channels is considered a key component of the voltage-sensing structure. S4 segment alone, however, can not account for the disparate voltage sensitivity expressed by different members of the potassium channel family. We used site-directed mutagenesis and heterologous expression in *Xenopus* oocytes and show that S2 and S3 are additional components of the voltage sensor. Mutations that change conserved-negatively charged or polar residues on S2 and S3 affect channel gating without altering ion selectivity or single channel conductance (γ) (Table). Aspartic acid at position 258 on S3 was intolerant to any attempted change. Mutation of other residues on S2 and S3 affected both the voltage-dependence of activation (Va) and of inactivation (Vi).

	Va (mV)	Vi (mV)	γ (pS)
wt	-30	-46	15
E225D	-10	-30	15
E235K	-45	-65	14
D258E			
T268D	-15	-35	15
E272R	-17	-36	14

The profile and magnitude of these changes resemble those reported for mutations of the conserved-positively charged residues on S4. These results, therefore, indicate that S2, S3 and S4 segments are essential components of the voltage-sensing structure of voltage-gated potassium channels. (Supported by NIH).

M-Pos32

ANALYSIS OF GATING AND IONIC CURRENT FLUCTUATIONS BY MONTE CARLO SIMULATION ((D. Sigg, F. Bezanilla)) Department of Physiology, UCLA, Los Angeles, CA, 90024.

Electrophysiological recordings of voltage-dependent ion channels measure either the rate of flow of conducting ions (ionic current) or, if the channel is rendered non-conducting, the much smaller and transient movement of the voltage-sensor (gating current). In either case, fluctuations in the macroscopic current records allow one to infer single channel quantities such as the unitary conductance and the elementary gating charge movement in a model-dependent way. Generally the non-stationary variance and autocovariance are measured, though higher order moments about the mean can be used to obtain a more complete characterization. In order to estimate the mean and variance of macroscopic currents predicted by a stochastic model one can either explicitly calculate expectation values of the required moments, or use a Monte Carlo technique in which random sampling produces sums of ionic and gating singles that are collected as an ensemble from which sample estimates are obtained. The main advantage to using the Monte Carlo technique is that the computer-generated data can be processed in the same way as is experimental data from real channels. This allows the effects of filtering, rate changes, background noise, digitizing error, sample size, and data processing to be studied in a more simple and natural way than is possible by analytical calculations of the expected moments. We have measured fluctuations in ensembles of macroscopic ionic and gating currents from a *Shaker* H4 K^+ channel and a non-conducting counterpart, respectively. The Monte Carlo technique was useful in distinguishing between competing kinetic models in terms of the voltage- and temperature-sensitivity of channel activity (see accompanying abstracts by B. Rodriguez and M. Roux). Supported by USPHS GM30376.

M-Pos33

VOLTAGE DEPENDENT PROPERTIES OF A S4-CHIMERA OF THE h-DRK1 CHANNEL ((R. Koopmann[1], K. Benndorf[1]*, C. Lorra[2] and O. Pongs[2])) [1]Institut für Vegetative Physiologie, Universität zu Köln, 50924 Köln, Germany. [2]Zentrum für molekulare Neurobiologie, Martinistr. 52, 20251 Hamburg, Germany *Heisenberg-fellow of the Deutsche Forschungsgemeinschaft. (Spon. by P. Honerjäger)

The role of the S4-segment in the gating process of voltage dependent potassium channels has been previously investigated by introducing various point mutations. In this work we compared properties of the native h-DRK1 channel with that of a chimeric construct in which the S4 of the original h-DRK1 channel was replaced by the S4 of the much faster and steeper activating RCK5 channel. The single-channel conductance was unaltered. Steady state activation of the chimera was shifted by 55 mV in the positive direction leaving the slope unchanged. Activation kinetics of the native and the chimeric channel were compared by relating all voltages to the midpoint of activation. Doing this, deactivation was not significantly different. The time to half maximal activation was longer in the chimera, which means that the chimera activates slower at equal relative voltages. The amount of this additional shift was approximately 10 mV. C-type inactivation was conserved in the chimera and shifted by the same amount as activation leaving the slope preserved. The time course of recovery from inactivation as compared by relating all voltages to the midpoint of the steady state inactivation was slowed in the chimera. This slowing can also be interpretated as a shift by approximately 10 mV. The data suggest that C-type inactivation is strictly coupled to activation and that the kinetics of conformational changes of the channel near the open state are mainly conserved. It is concluded that voltage dependence cannot exclusively be assigned to the S4-segment as structural element, but that activation kinetics must be determined mainly by other elements, possibly by rearrangements associated with the pore opening.

M-Pos34

NON - STOCHASTIC PROCESSES IN ION CHANNEL GATING? ((Y. Palti, Y. Mika, G. Omri, Y. Wasserman & R. Schatzberger)) Dept. of Physiol. & Biophys., B. Rappaport Faculty of Medicine & B. Katz Minerva Center for Cell Biophysics, Technion, P.O.B. 9697, Haifa 31096, Israel.

It is generally accepted that the ion channel gating process involves a series of conformational state-transitions of the channel molecule. These are energy barrier limited and therefore stochastic in nature. We have recently presented evidence, based on measurements of single channel gating currents, that the gating process includes elements of deterministic nature. We present here single channel measurements of ionic currents, that together with the corresponding gating currents, seem incompatible with channel statistical behavior and channel current reconstructions which are based on multi-state stochastic processes. These exptl. results are however consistent with a combined stochastic - deterministic reaction scheme. Such a scheme is also compatible with new data, on the Delay in the onset of the ionic currents (Cole-Moore Effect) and the corresponding physical model of the gating process. Furthermore, the temperature dependency of the Delay and its voltage dependence indicates that it involves two processes: one with a Q_{10} of approximately 1.2 and the other of 3-4. This evidence is interpreted as an indication of the possible coexistence of physical (deterministic) and chemical (stochastic) processes at the initial stages of gating.

M-Pos35

ENERGETICS OF hKv1.5 GATING ((T. C. Rich and D. J. Snyders))
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The human delayed rectifier hKv1.5 exhibits fast activation kinetics followed by slow inactivation. The sigmoidal activation time course indicates that the activation proceeds through a series of closed states. The voltage dependent transition rates reflect the energy barriers between the states. To gain further insight into the activation process we have modeled the temperature dependence of the activation kinetics using the Gibbs free energy equation. The whole cell voltage clamp technique was used to measure hKv1.5 currents expressed in a clonal L-cell line. As temperature was increased from 22°C to 32°C the peak current increased 41±3% (n=4) and the midpoint of activation shifted -15±4 mV. Exponential fits of activation at +50 mV showed that the dominant time constant decreased with temperature (τ = 1.8±0.1 ms at 22°C, and 0.51±0.1 ms at 32° C (n= 3)). The high temperature dependence of activation kinetics (Q_{10}=3.85) indicates that the activation energy contains a significant enthalpic component (ΔH=22.4 kcal/mol). Biexponential fits of inactivation at 22°C gave time constants of $\tau_{i,s}$=250, 2100 ms. At 32°C an additional fast component of inactivation was revealed ($\tau_{f,i}$=20±4 ms, 290±30 ms at 32°C (n=3)). The appearance of this fast exponential component indicates that additional kinetic states can be resolved at higher temperatures. A possible explanation of the enthalpic contribution to the activation energy is hydration of the ion channel in the open state. Supported by NIH Grants GM08452 and HL47599.

M-Pos36

BLOCK OF A G-PROTEIN ACTIVATED POTASSIUM CHANNEL CLONED FROM RAT ATRIUM BY PEPTIDES APPLIED FROM THE CYTOSOLIC SIDE. ((W. Schreibmayer[1], N. Dascal[2], N. Davidson[3] and H.A. Lester[3])) [1]: Institute for Medical Physics and Biophysics, University of Graz, Harrachgasse 21/4, A-8010 Graz, Austria. [2]: Department of Physiology and Pharmacology, Tel Aviv University, Ramat Aviv 61390, Israel. [3]: Biology Division, California Institute of Technology, Pasadena, CA 91125, U.S.A..

The G-Protein activated potassium channel from rat atrium (GIRK1/KGA) possesses a large carboxylic terminus, unique for the family of inward rectifying potassium channels. In order to get insight into the functional role of this putatively cytosolic part of the ion channel, we have designed a set of peptides, homologous to the GIRK1/KGA primary structure. Their effects on isolated patches from *Xenopus laevis* oocytes, expressing this channel and mutants thereof, were tested.

A peptide, comprising 17 amino acids from the very C-terminal end (DS6, aa 485-501), blocked the GIRK1/KGA channel, previously activated by addition of either GTP-γ-S or purified G-protein β/γ-subunits (IC_{50}: 8.4 ± 1.6 mg/l). DS6 lead to a disappearance of GIRK1/KGA activity without profound changes in single channel kinetics. Mutant channels in which the last 20, 40 and 160 C-terminal aa's had been deleted were also blocked by DS6. Another peptide from the C-terminal part (DD3, aa 447-464), blocked GIRK1/KGA activity with different kinetics: Burst duration was significantly shortened by DD3, resembling basal levels, where the channel had not been stimulated by the G-protein.

The data are compatible with the hypothesis that the C-terminal part of the GIRK1/KGA channel acts as some inhibitory blocking particle of the ion permeation pathway and might interact and/or compete with G-protein β/γ -subunit binding.

Supported by HFSP, US-Israel BNSF and Austrian National Bank.

M-Pos37

MUTATIONS AT D447 IN THE P REGION OF SHAKER K^+ CHANNELS ACCELERATE C-TYPE INACTIVATION. ((Sang-Ah Seoh and Diane M. Papazian)), Department of Physiology, UCLA School of Medicine, Los Angeles, CA 90024-1751. (Spon. by T. Mirzabekov)

We have studied the effects of mutations at D447 in the P region on the rate of C-type inactivation in Shaker K^+ channels using constructs lacking N-type inactivation (IR). Conservative (D447E-IR) and neutralization (D447N-IR) mutations accelerated the rate of C-type inactivation more than 1000-fold compared to the IR control. In D447E-IR, 30 mM external TEA enlarged the peak current amplitude by about 2- or 3-fold, in contrast to its effect on IR, which was 50% blocked by this concentration. High external K^+ (59 mM) also increased the peak current amplitude in D447E-IR. These effects of external TEA and K^+ can be explained by a "foot in the door" mechanism, or by C-type inactivation from closed states. When TEA and high K^+ were applied simultaneously, the peak current amplitude of D447E-IR was smaller than that obtained when TEA or high K^+ was applied alone. This suggests that position 447 contributes to a binding site for which both cations compete. In addition, external TEA slowed the rate of C-type inactivation in D447E-IR by 14-fold, and in IR by 2-fold. In contrast, external TEA had little effect on the current amplitude or inactivation kinetics of D447N-IR, suggesting that this mutation reduces or eliminates TEA binding. The role of subunit interactions in the mechanism of C-type inactivation is being investigated by co-expressing IR with D447E-IR or D447N-IR in defined proportions.

M-Pos38

DEVELOPING A MEMBRANE QSAR TO DETERMINE MECHANISM OF ACTION AND INCREASE POTENCY OF MINK CHANNEL BLOCKERS. ((Leo Herbette[1], Michelle Vecchiarelli[1], Mark Calcagno[2]))
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A series of cationic amphiphiles that block the minK channel with various potency were examined for their potential to interact with membranes. The primary strategy was used to develop a QSAR that would allow an assessment of what portions of the drug structure result in a greater membrane interaction which could lead to a more potent minK channel blocker. Fifty compounds choosen for study were placed in three different groups based on a parent structure: Group I contained analogs where the "grease" moiety was varied in chemical composition; Group II had different "linker" chemical moieties; and Group III contained analogs with different cationic "headgroup" structure. Differential scanning calorimetry was used to quantitate the change in cooperative unit size (ΔCUS) upon melting of a dimyristoyllecithin lipid preparation containing different amounts of each drug. It appears that the size and extent to which the "grease" moiety penetrates the lipid bilayer structure correlates with a greater membrane interaction. QSAR's against IC50's for minK channel activity are being established to allow a complete redesign of each segment of these drugs to increase overall membrane interaction and channel activity.

M-Pos39

Cl^- - CHANNEL BLOCKERS ACTIVATE ISK - CHANNELS ((S.Waldegger, T. Herzer, F. Schmidt, G. Raber, C. A. Wagner, E. Gulbins, A.E.Busch and Florian Lang)) Dept. of Physiology, University of Tübingen, D-72076 Tübingen (Spon. by P. Dietl)

Blockers of Cl^--channels, such as fenamates, are widely used to filter out Ca^{2+}-activated, endogenous Cl^--channels for a 'clean' study of expressed ion channels in *Xenopus* oocytes. However, their use could be restricted if these compounds also alter other ion conductances. Indeed, fenamates are known to inhibit cation channels and preliminary data indicate an effect on Ca^{2+}-activated K^+ channels. Further, fenamates are known to develop nephrotoxic effects via an undefined mechanism. The aim of this study was to study putative effects of such blockers on slowly activating, voltage-gated I_{sK} channels, originally cloned from kidney and expressed in *Xenopus* oocytes. cRNA encoding the human I_{sK} protein was injected into *Xenopus* oocytes and the induced I_{sK} channels were investigated using the two-microelectrode voltage-clamp method. Niflumic acid, mefenamic acid, flufenamic acid and DIDS which are commonly used in *Xenopus* oocytes to suppress endogenous Ca^{2+}-activated chloride channels were tested for their effects on I_{sK} channels. At low concentrations (10 μM) all compounds increased I_{Ks} amplitude up to 41 % and decreased their rate of deactivation. Under Cl^--free conditions I_{sK} had somewhat altered activation properties, but niflumic acid produced similar positive regulatory effects on I_{sK}. Under niflumic acid the voltage needed to evoke halfmaximal I_{sK} activation ($V_{1/2}$) was shifted by about -20 mV without altering the activation kinetics. In water-injected oocytes these compounds inhibited partially endogenous Ca^{2+}-activated chloride channels, but did not activate any ion conductances. The mechanism by which Cl^--channel blockers increase I_{sK} seems to be related to the activation of I_{sK} by organic cross-linkers.

An activation of this renal brush-border potassium current could be of clinical relevance. The influence of Cl^--channel blockers on I_{sK} could contribute to the renal action of these drugs.

M-Pos40

L-3-HYDROXYBUTYRATE BLOCKS THE TRANSIENT K^+ OUTWARD CURRENT IN ISOLATED VENTRICULAR CELLS OF MICE. ((B. Doepner, S. Thierfelder and H. Hirche)) Zentrum für Physiologie, Universität zu Köln, Germany. (Spon. by J. Hescheler)

In enzymatically isolated ventricular mouse heart cells, transient K^+ outward currents (I_{to}), which mediate the fast repolarisation of the action potential, were measured in whole cells using a patch clamp technique. These rapidly activating currents are large (amplitudes up to 20 nA at +40 mV) and activate at potentials between -30 and -20 mV. Exposure to L-3-hydroxybutyrate in the concentration range of 0.1 to 20 mM caused an immediate reduction of I_{to} whereas the D-isomer and the racemate were ineffective. This block of I_{to} was fully reversible with respect to its regular run-down. Washing in and out the drug allowed to repeat this experiment in the same cell up to five times. The decrease of I_{to} was concentration dependent. The dose-response-relationship yielded a dissociaton constant K_D of 2.1±0.1 mmol/l and a Hill-coefficient of 0.96±0.03 (mean±SD; n=37). Inactivation kinetics of I_{to} was altered by L-3-hydroxybutyrate in the sense that the slow exponential of the inactivation time course was more affected than the fast exponential. The same result was obtained for the recovery of inactivation, which could also be described by two exponentials under control conditions. We conclude that the blocking effect of L-3-hydroxybutyrate on the transient K+ outward current is most likely mediated by a direct affect on the channel rather than via a metabolic cascade.

M-Pos41

CLOFILIUM IS A BLOCKER OF RAPIDLY ACTIVATING OUTWARD CURRENT IN CULTURED ATRIAL TUMOR MYOCYTES (AT-1 CELLS). ((Mohit L. Bhattacharyya, Shukla Sarker))Department of Physiology, Meharry Medical College, Nashville, TN.

In atrial tumor myocytes derived from transgenic mice (AT-1 cells) the major repolarizing current reported is resembling I_{kr}. Unlike in guinea pig, this current can be observed without subtracting other outward currents. Clofilium, has been reported to have potent C3A action in Purkinje fibers but its mechanism of action is not clear. In guinea pig myocytes, it blocks I_x with IC_{50} of 50 µM. In this study we report a potent blocking effect of clofilium on I_{kr} type currents in AT-1 cells. Overlapping inward currents were eliminated by holding at -40 mV and using Cd_2Cl. Clofilium blocked the outward current in a dose dependent manner. Dose response curve could be fitted to a Hill equation with an IC_{50} of 0.66 µM. Percent block of peak current (I_{peak}) and current at the end of 1 s pulse (I_{1sec}) were 28.4 ± 6.4 % and 59.5 ± 16.8 % whereas the tail current was reduced by almost 100 % (n = 8) for 40 mV depolarization. I-V relationship suggests that the current peaks between 10-20 mV. Percent reduction of I_{1sec} by clofilium at 0, 10, 20, 30, and 40 mV are 73.8 ± 15.5 %, 74.2 ± 8.5 %, 74.5 ± 12.4 %, 68.1 ± 15.4 % and 59.9 ± 16.2 % respectively. Since largest block occurs at the maximum activation of this current we suggest that clofilium blocks this channel in the open state.
Supported by NIH grants SO6 GM 80372 and HL02480

M-Pos42

RELAXIN EFFECTS ON ATRIAL POTASSIUM CURRENTS ((E. S. Piedras-Rentería, O. D. Sherwood and P. M. Best.)) Dept. Molecular & Integrative Physiology, University of Illinois, Urbana, IL 61801.

Relaxin is a reproductive hormone that has chronotropic and inotropic effects in the rat heart, where specific relaxin receptors are found only in the atria. Our previous data suggest the elongation of the atrial action potential by relaxin is coupled to protein kinase A but not mediated by β-adrenergic receptors. We studied the effect of rat relaxin on the outward K^+ currents of enzymatically dispersed atrial cells from 4.5-8 week-old Fischer rats (both sexes). The bath solution contained Tyrode +50 µM TTX and 500 µM $CdCl_2$; the pipette solution contained (mM):100 KCl,10 K_2EGTA, 8 $MgCl_2$,1 $CaCl_2$,10 HEPES,0.2 Na_2GTP,7 Na_2ATP,15 phosphocreatine and also 40 µg/ml creatine phosphokinase. Atrial cells showed two components of K^+ currents, a transient component and a time-independent current. Relaxin addition slowed down the transient component kinetics of activation (time to peak was 63% longer than controls). In addition, steady state of activation analysis showed a shift in the V_x value of 12 mV to the right. These effects could explain the elongation in the action potential caused by relaxin, and might account for the inotropic effect of the hormone observed in the cardiac atrium.
Supported by AHA, Illinois (P.M.B. and E.S.P-R.) and N.I.H. (O.D.S.).

M-Pos43

CELL SWELLING POTENTIATES THE SLOW COMPONENT OF DELAYED RECTIFIER POTASSIUM CURRENTS IN GUINEA PIG VENTRICULAR MYOCYTES. ((Siân A. Rees, Jamie I. Vandenberg, Anthony R. Wright, Atsuya Yoshida & Trevor Powell)) University Laboratory of Physiology, University of Oxford, Oxford, UK.

Cell swelling, as occurs during ischemia/reperfusion has been shown to cause alterations in delayed rectifier current (I_K) in guinea pig ventricular myocytes. The aim of the present study was to further characterise the effect of swelling on K^+ currents in ventricular cells. I_K was measured, using the whole-cell voltage clamp technique, during a 1 sec voltage step from -40 to +40mV and also via tail current measurements (steps from -40 to +40mV for durations between 50 msec and 2.9 sec). Pipette solution contained (in mM), 140 K-aspartate, 5 $MgCl_2$, 5 K_2ATP, 10 EGTA and 5 HEPES. In isotonic solution (140mM NaCl; 37°C) the amplitude of I_K was 0.82±0.05nA (mean±s.e.m, n=36; measured at the end of the 1 sec pulse). Switching to hypo-osmotic solution (100mM NaCl) caused rapid cell swelling and an increase of I_K to 1.33±0.09nA (n=36; p<0.05). Reducing osmolarity (300 to 200 mOsm) also altered the I-V relationship (-80 to +60mV), causing a significant increase in the outward current flowing between +10 and +60mV. In further studies using the voltage ramp protocol, swelling-induced I_K was blocked almost completely by the selective I_K blocker UK66,914 (10µM). A far lesser degree of inhibition of swelling-induced I_K was seen on application of 0.2µM dofetilide, another blocker of I_K which only blocks the rapidly activating component, I_{Kr}. Tail current amplitudes (n=20) were significantly increased by hypo-osmotic solution only when the duration of the step pulse exceeded 150 msec. For example when the step duration was 50 msec the amplitudes in iso- versus hypo- solution were 0.42±0.03 versus 0.40±0.03 nA (p:NS), however, when the duration was 1.4 sec values were 0.48±0.04 versus 0.54±0.04 nA, respectively (p<0.05). These results together with the pharmacological evidence indicate that cell swelling preferentially activates the slow component of I_K (I_{Ks}) with minimal effects on the rapid component (I_{Kr}).

M-Pos44

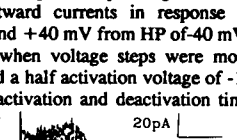
EFFECTS OF ZATEBRADINE ON hKv1.5 CHANNELS. ((C. Valenzuela, E. Delpón, O. Pérez, J. Tamargo, D.J. Snyders)). Universidad Complutense, Madrid, SPAIN and Vanderbilt Univ. Nashville, TN, USA.

Zatebradine (ZT) is a bradycardic agent that inhibits I_f in the sinoatrial node and prolongs the action potential duration in ventricular cardiac preparations. To test whether this effect was due to cardiac K^+ channel block and to explore possible mechanisms of block we analyzed the effects of ZT on a cloned human cardiac K^+ channel (hKv1.5) expressed in a stable *Ltk*$^-$ cell line using the whole-cell configuration of the patch-clamp technique. ZT, 10 µM, did not modify the activation time course of the hKv1.5 current, but induced a subsequent slow decline with a time constant of 109.2±16.3 ms. Steady-state block was measured at the end of 500 ms depolarization steps to +60 mV. ZT inhibited hKv1.5 with an apparent K_D of 1.8 µM and a Hill coefficient of 1.1. Block induced by ZT was voltage-dependent, increasing, at 2 µM, from 43.7±3.4% at 0 mV to 52.9±3.3% at +60 mV ($p<0.01$) and it was consistent with a binding reaction sensing 18% of the applied electrical field ($\delta=0.177\pm0.003$). Compared to control, ZT reduced the tail current amplitude elicited upon return to -30 mV and slowed the deactivation time course (from 53.2±13.4 ms to 97.2±17.2 ms; $p<0.05$, n=6) resulting in a "cross-over" phenomenon. These results indicate that: 1) ZT acts as an open channel blocker of hKv1.5, most likely in the internal mouth of the ion pore, 2) unbinding is required before the channel can close, 3) hKv1.5 block induced by ZT suggests that hKv1.5 block may be clinically relevant. Supported by Salud 2000, CICYT SAF92-0157 and NIH HL47599 Grants.

M-Pos45

THE FAST COMPONENT OF DELAYED RECTIFIER K^+ CURRENT (I_{Kr}) IN FETAL MOUSE VENTRICULAR MYOCYTES. ((L. Wang, H.J. Duff)) University of Calgary, Calgary, Alberta, Canada

Although the genetics of mammalian cardiac K^+ channels have been intensively studied in the mouse, there are limited electrophysiological data in mouse heart. In this study, we characterized dofetilide (a specific I_{Kr} channel blocker) sensitive K^+ current in fetal mouse (18 gestation days) ventricular cells by using whole-cell patch-clamp technique. Figure shows rapidly activating and noninactivating outward currents in response to depolarizing pulses (500msec) between -30 and +40 mV from HP of-40 mV, at 22°C. This current rectified inwardly when voltage steps were more positive than 0 mV. The activation curve had a half activation voltage of -16 mV and a steep slope of +6.3 mV. Both activation and deactivation time constants of the current were strongly voltage dependent. Longer depolarizing pulses (2sec) did not activate I_{Ks} in this preparation. Dofetilide (1 µM), completely abolished the tail currents. Action potentials in fetal mouse ventricular cells have distinct plateau with relative long duration, which were significantly prolonged by dofetilide and EADs occurred in some recordings. These findings indicate that I_{Kr} is an important K^+ current during plateau potentials and plays an important role in repolarization in fetal mouse heart.



M-Pos46

ACCUMULATION OF INACTIVATION ON RAT BRAIN K+ CHANNELS
((O. Moran, A. Bertoli and F. Conti)) ICB–CNR, I-16146 Genova, Italy

We studied the phenomenon of cumulative inactivation in the voltage dependent K^+ channels RCK4, cloned from rat brain and expressed in *Xenopus* oocytes. Trains of stimulations with pulse intervals shorter than 20s produce cumulative inactivation, even by brief stimuli during which K^+ currents do not show any significant decline. Protocols with two identical pulses with amplitude V_P and duration T_P, separated by an interval T_H, were used to study the time course of the process. By varying T_H we find that inactivation increases while the channels deactivate, reaches a maximum after ≈30 ms, and subsides with two time constants τ_1 ≈0.3 s, and τ_2 >3 s, indicating the existence of (at least) two different inactivated states: I_s, with recovery time τ_2 and I_f, with shorter recovery time τ_1. Varying T_P we find that the number of channels activable by the second pulse decreases with the cumulative integral of the open channel probability during the first pulse and the interpulse, implying that the inactivated states are mainly reached from the open state. The rate at which the channels enter either I_s or I_f states is voltage independent. For a fixed T_H the current elicited by the second pulse decreased with increasing T_P according to what expected if inactivation proceeds from the open state or from activated states that are in fast equilibrium with the open state. The simplest kinetic scheme accounting for our observations involves two inactivated states, I_f and I_s, that can be both reached from the open state, O, and that are coupled also sequentially. Transition from I_f to I_s can occur also during the early repolarisation period and are the cause of the much stronger cumulative inactivation in RCK4 channels compared with other clones lacking N-type inactivation (RCK1, RCK2, RCK3, RCK7, RCK4–Δ–100).
[Supported by P.F. Ingegneria Genetica, CNR]

M-Pos47

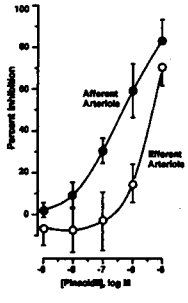
BLOCKADE OF THE HUMAN CARDIAC K+ CHANNEL Kv1.5 BY THE NONSEDATING ANTIHISTAMINE ASTEMIZOLE
((D. Rampe[1], A. E. Lacerda[2], B. Wible[2], A.M. Brown[2])). [1]Marion Merrell Dow Research Institute, Cincinnati, Ohio 45215, [2]Dept. Molecular Physiology and Biophysics, Baylor College of Medicine, Houston, Texas 77030.

Administration of the nonsedating antihistamine astemizole (Hismanal®) has on rare occasions been associated with altered cardiac repolarization including QT prolongation and torsades de pointes. Since these adverse effects can be associated with blockade of cardiac K^+ channels, we examined the effects of astemizole on the delayed rectifier K^+ channel Kv1.5 which we have cloned from human heart and expressed in human embryonic kidney cells. Astemizole inhibited Kv1.5 current under whole-cell (test potential = +30 mV) and inside-out patch (test potential = +50 mV) conditions with IC_{50} values of 1.7 and 1.5 μM, respectively. The main effect of astemizole was to accelerate current decay during step depolarization consistent with blockade of the open channel state. Astemizole (3 μM) had no effect on Kv1.5 current activation but significantly slowed current deactivation as measured by tail current time constants upon return to -45 mV. Astemizole inhibited Kv1.5 current over a wide range of test potentials with block increasing at higher voltages. These data indicate that astemizole acts as an open channel blocker of Kv1.5 and that its binding site likely lies at the intracellular face of the channel. Such interactions may underline the cardiac toxicity associated with the administration of astemizole in man.

M-Pos48

EFFECTS OF PINACIDIL ON RENAL MICROVASCULAR REACTIVITY TO ANGIOTENSIN II
((Martina Reslerova and Rodger Loutzenhiser)), The University of Calgary, Calgary, AB, Canada T2N 4N1 (Spon. by M. Kargacin).

Renal afferent (AA) and efferent (EA) arterioles regulate pre-and post-glomerular resistances and are demonstrated to have differing activating mechanisms. For example, calcium antagonists block AA responses to agonists such as Ang II but have no effect on the EA; suggesting that L-type Ca channels mediate vasoconstriction only of the AA. If K-channel openers modulate arteriolar reactivity by hyperpolarization-induced inhibition of Ca channel activity, Ang II-induced EA responses would not be affected. We examined the renal microvascular effects of pinacidil (Pin), KATP opener, using the perfused hydronephrotic rat kidney (Circ Res 74:861-, 1994). Ang II (0.1 nM) reduced AA diameters from 15.2±0.7 μm to 6.1±1.1 μm and EA diameters from 11.1±1.3 μm to 4.4±0.3 μm. Pin preferentially inhibited AA responses at 0.01-1.0 μM, but blocked both EA and AA at 10 μM (right). In each case, glibenclamide reversed the inhibition. Pin also inhibited EA responses in the presence of 5 μM diltiazem. This inhibition was reversed by 40 mM KCl. These findings indicate that KATP activity preferentially affects AA responses at lower concentrations (<1.0 μM). At 10 μM, Pin inhibits EA responses through a mechanism which requires a K-gradient but is independent of L-type Ca channel activation. (Supported by the MRC, AHFMR & The Kidney Foundation of Canada).



M-Pos49

FREQUENCY-DEPENDENT EFFECTS OF NE-10064 (AZIMILIDE) ON CARDIAC REPOLARIZATION: BLOCK OF DELAYED RECTIFIER POTASSIUM AND L-TYPE CALCIUM CURRENTS.
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The Class III antiarrhythmic agent NE-10064 (NE) has been described as a potent blocker of the slowly activating delayed rectifier potassium current (I_{Ks}), but preliminary reports suggest that it also inhibits several other ionic currents in cardiac tissue. We studied the effects of NE in ferret papillary muscle and guinea pig ventricular myocytes to assess better its electrophysiologic actions. NE (0.3-10μM) increased effective refractory period (ERP) but decreased isometric twitch tension of papillary muscle. Increases in ERP were reverse frequency-dependent; greater at 1 Hz than at 3 Hz. NE (0.3-3.0 μM) prolonged action potential duration (APD) of ventricular myocytes at 1 Hz but not 3 Hz. Higher concentrations of NE (10 μM) shortened APD and lowered AP plateau level at 3 Hz. NE potently blocked the rapidly activating component of the delayed rectifier, I_{Kr}, (IC_{50}: 0.4 μM) and inhibited I_{Ks} (IC_{50}: 3 μM) but ~10x less potently. NE (10 μM) did not block the inward rectifier K^+ current (I_{K1}). NE (10 μM) blocked L-type Ca^{2+} current (I_{Ca}) in a use-dependent fashion; block was greater at 3 Hz than at 1 Hz. We conclude that 1) NE's block of potassium current in ventricular myocytes is relatively selective for I_{Kr} over I_{Ks} 2) NE inhibits I_{Ca} use-dependently 3) the effects of NE on ERP and tension in papillary muscle as well as APD and plateau level in ventricular myocytes may be explained by its K^+ and Ca^{2+} channel blocking properties.

M-Pos50

ECTOPIC EXPRESSION OF THE RAT BRAIN DELAYED RECTIFIER POTASSIUM CHANNEL rKv1.1 IN THE HEARTS OF TRANSGENIC MICE.
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Many different K^+ channels are expressed in the mammalian heart. The regulation of these channels and the contribution of any single channel to the cardiac action potential are not currently understood. The ability to manipulate the mouse genome provides a novel approach to the study of this problem.

To clarify the cardiac role of delayed rectifier K^+ channels, we have engineered transgenic mice that ectopically express a rat brain K^+ channel in the heart. The flutag, a 9 amino acid antigenic epitope, was added to the carboxy-terminal end of rKv1.1. This modified channel, rKv1.1f, was transcribed *in-vitro* and cRNA was injected into Xenopus oocytes; the delayed rectifier type current was indistinguishable from that of the native channel. rKv1.1f was also transfected into cos cells; antigenicity of the flutag was confirmed by cellular immunofluorescence and Western blot. We then injected into the pronucleus of fertilized mouse eggs a transgenic construct consisting of the α-myosin heavy chain promoter, rKv1.1f, and the SV-40 poly-A tail. Genomic Southern blot analysis of tail DNA demonstrated 4 founder mice with variable copy numbers of the transgene. Northern blot analysis of the F1 generation confirmed abundant cardiac-specific expression of channel mRNA in one of the transgenic lines.

High resolution electrocardiograms to date show no differences between transgenic mice and littermate controls. Studies to detect channel protein and to confirm the presence of functional channels are currently underway. We will also study the effect of the transgene on native channel expression, histology, and cellular electrophysiology.

M-Pos51

A QUANTITATIVE DESCRIPTION OF THE RAPID REPOLARIZATION CURRENT (I_{Kr}) IN RABBIT HEART CELLS.

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Single ventricular myocytes from rabbit hearts were used to study the rapidly activating repolarization current I_{Kr} using the whole cell patch-clamp technique. Holding potentials > -50 mV were used to inactivate the transient outward current in these cells, so that I_{Kr} could be measured in isolation from other time dependent currents. Voltage steps positive to -30 mV revealed an instantaneous negative slope conductance (I_{K1}), and a time-dependent component (I_{Kr}) which was completely blocked by 1μM E-4031. I_{Kr} was fully activated at +10 mV, and the fully-activated IV relation displayed marked inward rectification with negative slope > -40 mV. Moreover, the current was ~ 0 for V > +20 mV. The E_{rev} for I_{Kr} was 10-15 mV positive to that of I_{K1} with 5 mM K_o. This result and the sensitivity of E_{rev} to K_o in the 2.5 to 15 mM range suggest that I_{Kr} is carried only in part by K^+. The kinetics and the current-voltage relation for I_{Kr} are consistent with a role for this component in the latter phase of repolarization, as revealed by the effects of E-4031 on the action potential waveform. (Supported by MRC, Canada).

M-Pos52

VOLTAGE- AND STATE-DEPENDENT BLOCK OF DELAYED RECTIFIER K^+ CURRENT BY 4-AMINOPYRIDINE IN RABBIT CORONARY SMOOTH MUSCLE CELLS. ((C.V. Rémillard, and N. Leblanc)) Dept. of Physiology, U. of Montréal, and Research Centre, Montréal Heart Institute, Montréal, Québec, Canada H1T 1C8.

Delayed rectifier K^+ channels (K_w) play an important role in regulating resting membrane potential in smooth muscle cells. Such a conclusion was to a large extent based on the use of 4-aminopyridine (4-AP) as a relatively specific inhibitor of these channels in smooth muscle. The mechanisms involved in the 4-AP-induced block of K_w were studied in whole-cell voltage-clamped rabbit coronary myocytes. The contribution of Ca^{2+}-dependent ion channels (K_{Ca}, $I_{CI(Ca)}$), ATP-sensitive K^+ channels and L-type Ca^{2+} channels was minimized by dialyzing the cells with 5 mM EGTA and ATP, and by the use of 0.5 mM TEA and 1 μM nifedipine in the bathing medium. 4-AP inhibited slowly inactivating K_w (20 sec steps to +20 mV from HP = -60 mV) in a dose-dependent manner (K_D = 1.4 mM), and shifted the steady-state activation and inactivation curves of K_w by +11 and +16 mV, respectively. The time constant of activation (250 ms steps to +20 mV from HP = -60 mV) was significantly increased by 2 mM 4-AP; deactivation kinetics at -40 mV were unaffected. 4-AP (0.5 mM) did not alter the time constant of inactivation of K_w at +20 mV (20 sec steps) nor its reactivation kinetics. Use-dependent unblock of K_w was revealed at potentials $\geq$ -10 mV from analyses of the voltage-dependence of 4-AP-sensitive currents and the frequency-dependent changes of K_w during the application of repetitive steps to 0 or +20 mV (250 ms steps from HP = -60 mV; 0.1-0.25 Hz). The time course of current recovery during washout suggested that 4-AP preferentially binds the channel in the close state, and unbinding is promoted by transitions to the open state. We conclude that closed-state binding of 4-AP on K_w depolarizes vascular smooth muscle cells by shifting the activation curve of these channels to more positive potentials. C.V.R. is a pre-doctoral fellow of the HSFC. Supported by MRC grant to N.L. (MT-10863).

M-Pos53

AN ACETYLCHOLINE-SENSITIVE K^+ CURRENT IN RAT CULTURED CEREBELLAR GRANULE NEURONS ((Christopher Watkins and Alistair Mathie)) Department of Pharmacology, Royal Free Hospital School of Medicine, London NW3 2PF U.K.

Cerebellar granule neurons (CGNs) are known to possess both transient & delayed rectifier-type voltage-activated K^+ conductances. Here we present evidence for another component to their K^+ conductance. Whole cell K^+ currents were recorded from neurons cultured for up to 14 days, using the perforated patch clamp technique. The resting membrane potential (RMP) of CGNs became more hyperpolarised with days in vitro (-30 ± 2 mV, n = 14 at 2 d.i.v cf. -79 ± 3 mV, n = 13 at 7 d.i.v.) Concomitantly, a standing outward current at a holding potential of -30 mV (314 ± 32 pA, n = 14) became evident. There was a highly significant correlation between RMP and the magnitude of this standing outward current (r = 0.8893, $p < 0.0001$) in CGNs.

The current was selective for K^+ ions, was voltage-dependent & exhibited outward rectification; activation was not dependent on Ca^{2+} influx. The kinetics of activation & deactivation were very rapid, with a deactivation time constant of 0.55 msec, being much faster than a conventional M current. Application of 10 μM ACh reversibly inhibited this current (79 ± 2 %, n = 8), with the inhibition being antagonised by 0.1 μM atropine (n = 3). 10 μM muscarine inhibited the current by 61 ± 3% (n = 12). Inhibition of this current by muscarinic agonists increased the input resistance at the RMP from 0.48 ± 0.08 GΩ in control conditions to 1.21 ± 0.17 GΩ (n = 7) which demonstrates that inhibition of this current will have profound effects on the excitability of CGNs.

This work was supported by the MRC.

M-Pos54

ACTIONS OF THE BENZOPYRAN COMPOUND RP62719 ON TRANSIENT OUTWARD K^+ CURRENT IN RAT VENTRICULAR MYOCYTES. ((L. Xu and J.G. McLamon)) Department of Pharmacology & Therapeutics, Faculty of Medicine, The University of British Columbia, Vancouver, B.C. V6T 1Z3, Canada. (Spon. by P.C. Vaughan)

Whole-cell transient outward potassium current (Ito) in rat ventricular myocytes has been studied in the presence of the benzopyran agent RP62719. The compound, in a dose-dependent manner over the concentration range from 1-50 μM, diminished the time course of inactivation of the macroscopic current. The time constant of decay was 75% of control value with 5 μM RP62719 and was 20% of control with 30 μM of the agent. With drug concentrations in excess of 10 μM, the peak amplitude of Ito was also decreased. The voltage-dependence of both channel activation and inactivation were not significantly altered from control in the presence of the benzopyran compound. In addition, RP62719 showed little evidence for block of the resting state of the Ito channel. Drug effects were consistent with interactions with the open channel state; however, trains of depolarizing stimulus pulses did not lead to evident use-dependence of drug action. The onset and wash-off times of drug were slow, suggesting an internal membrane site of action. At concentrations in excess of 10 μM, RP62719 also diminished the inactivation time course of inward sodium currents and decreased the amplitude of inward rectifier potassium currents. Inward calcium currents recorded from the rat myocytes were not altered in the presence of the benzopyran compound.

Supported by a grant from the Heart & Stroke Foundation of British Columbia and Yukon.

M-Pos55

4-AMINOPYRIDINE (4-AP) ALTERS THE VOLTAGE-DEPENDENCE OF MACROSCOPIC K^+ CURRENTS IN VASCULAR MYOCYTES. ((R. H. Cox, N. J. Dietz, AND S.-Y. Shi)) Bockus Research Institute, Graduate Hospital, and Dept. of Physiology, University of PA, Phila., PA 19146.

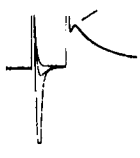
In preliminary studies, we observed that 4-AP appeared to alter the voltage-dependence of macroscopic K^+ currents (I_K) in vascular myocytes. Experiments were performed to study this observation in detail using single myocytes isolated by collagenase and elastase from rabbit portal vein. I_K was recorded by whole cell methods using a pipet solution containing (mM) 140 KCl, 10 NaCl, 5 MgATP, 20 HEPES, and 10 BAPTA at pH 7.2. Cells were superfused at room temperature with a solution containing (mM) 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES and 10 glucose at pH 7.4. I_K recorded from a holding potential (HP) of -100mV activated at about -40mV, exhibited fast activation which increased with voltage, and slow inactivation which was voltage-independent. At voltages above +20mV, a high noise component was superimposed (K_{Ca}). From a HP of -20mV, only the high noise component (inhibited by iberiotoxin, charybdotoxin, and low [TEA]) was present. Difference currents (ΔI) calculated with I_K recorded from HPs of -100 and -20mV, peaked at +50mV with a half maximal activation at about +10mV. The ΔI was inhibited by 1mM 4-AP (50%) and 10μM tedisamil (90%). With 1mM 4-AP, I_K recorded from a HP of -20mV was significantly increased at voltages above +10mV (C = 1140±138 pA; 4-AP = 1574±230 pA @ +60mV). The ΔI was inhibited by 1mM 4-AP but its voltage dependence was not altered. The voltage dependence of I_K availability, determined at a test voltage of +10mV from conditioning potentials of -120 to +10mV, was shifted to the left by 1mM 4-AP ($V_{0.5}$: C = -43mV; 4-AP = -68mV). These results demonstrate that in addition to inhibiting the K_{dr} current amplitude, 4-AP shifts the voltage dependence of K^+ channels in the hyperpolarizing direction.

M-Pos56

REDUCTION OF EXTERNAL SODIUM DOES NOT REDUCE I_{TO1} IN CANINE VENTRICULAR MYOCYTES

((Andrew C. Zygmunt, and Charles Antzelevitch)), Masonic Medical Research Lab, Utica, NY

TTX or removal of external Na has been shown to reduce the transient outward current (I_{TO1}) in rat ventricular myocytes. Because I_{Na} and I_{TO1} overlap over a wide range of potentials, we tested whether a similar effect of changing external Na could be found in canine ventricular myocytes if I_{Na} was first inactivated by a prepulse. Shown in the figure below are the whole cell currents recorded in a cell initially bathed in 150 mM NaCl and then switched to an external solution containing 5 mM NaCl. The cell was held at -80 mV, stepped to -50 mV for 5 ms and then to +10 mV to record I_{TO1} (arrow). Low external Na reduced I_{Na} recorded during the prepulse but had no effect on the transient outward current. The prepulse used to inactivate I_{Na} had considerable effect on the I-V relation for I_{TO1}, shifting threshold from -30 to -20 mV and reducing the amplitude of I_{TO1} at all potentials. I_{TO1} at 0 mV was between 21 and 34% smaller following a prestep, when compared to the current recorded at 0 mV after a step directly from the holding potential of -80 mV. If this reduction in I_{TO1} results from inactivation of I_{TO1} during the prestep, increasing the duration and potential of the prestep should further reduce I_{TO1}. However, I_{TO1} was insensitive to prepulse duration over the range 5-50 ms. Instead, the reduction in I_{TO1} exactly corresponded with inactivation of I_{Na}. **Conclusion:** Our data indicate that reduction of external sodium produces no effect on I_{TO1} and suggest that activation of I_{Na} may result in loss of voltage control and overestimation of I_{TO1} when the test potential is not preceded by a prepulse.



M-Pos57

DELAYED POTASSIUM CURRENTS IN AN INSECT CUTICULAR MECHANORECEPTOR NEURON

((P.H.Torkkeli and A.S. French)) Department of Physiology, University of Alberta, Edmonton, Alberta, Canada.

Tactile spines on the exoskeleton of insects are mechanoreceptors that allow the animal to perceive its external mechanical environment. We used current- and voltage-clamp recordings from the bipolar neuron in the femoral tactile spine of the cockroach (*Periplaneta americana*) to reveal the ionic currents that cause the phasic behaviour of this neuron. Here, we present the results of kinetic analysis of the slowly inactivating outward potassium currents of this neuron. More than half of the steady outward current was sensitive to the Ca^{2+}-channel blockers Co^{2+} and Cd^{2+}. This current ($I_{K(Ca)}$) activated in less than 1 ms, reached an amplitude of about 5 nA at strong depolarizing voltages, and did not inactivate during a stimulation of 500 ms duration. Current-clamp recordings indicated that this current is an important component of the rapidly adapting behaviour of the tactile spine neuron. TEA blocked a delayed rectifier type of K^+-current (I_K) and also a small part of the $I_{K(Ca)}$. I_K is the sole component of action potential repolarization in this neuron, turning on before the onset of Na^+-current inactivation and inactivating slowly during a maintained stimulation.

Supported by the Medical Research Council of Canada, the Alberta Heritage Foundation for Medical Research and the Academy of Finland.

M-Pos58

VOLTAGE GATED DELAYED RECTIFIER TYPE K^+ CURRENT IN HUMAN MEGAKARYOCYTES AND IT'S ABSENCE IN HUMAN MEGAKARYOBLASTIC TUMOR CELLS AND MEGAKARYOCYTES FROM MYELOGENOUS LEUKEMIA PATIENTS ((L. Kapural and A. Fein)) Dept. of Physiology, Univ. of Conn. Health Center, Farmington, CT 06030

Using the patch clamp whole cell recording technique we examined human megakaryocytes, two megakaryoblastic tumor cell lines (DAMI, CHRF-288-11) and a human erythroleukemia cell line for the presence of voltage-gated membrane currents. In human megakaryocytes we found a voltage-gated outward current, activated for depolarizations above -40 mV, which resembles the delayed rectifier K^+ current present in other cell types. This voltage-gated outward current was not detected in any of the three tumor cell lines. The functional absence of the outward current in the three megakaryocytic tumor cell lines may somehow be related to their inability to differentiate and produce platelets.

We also examined 29 megakaryocytes from the bone marrow of 6 patients diagnosed with various acute forms of myelogenous leukemia. Three were diagnosed as being in a blast crisis of chronic myelogenous leukemia, two with acute myelogenous leukemia (AML) and one with acute promyelocytic leukemia. From 5 out of 29 megakaryocytes a normal large amplitude delayed rectifier K^+ current was present, while in the remaining 24 cells the current was greatly suppressed. Additionally, we examined voltage activated membrane currents of megakaryocytes from 7 patients with acute lymphocytic leukemia. Out of 23 cells examined only 1 megakaryocyte exhibited suppression of the delayed rectifier while the remaining 22 cells had an apparently normal current. The suppression of the delayed rectifier may be a contributory factor to the dysregulation of thrombopoiesis and the resultant thrombocytopenia in myelogenous leukemia. Exposure to the adenylate cyclase activator, forskolin, caused the appearance of a delayed rectifier-like current in the megakaryocytes of patients with AML. This finding suggests that the K^+ channels underlying the current are present, but somehow suppressed in megakaryocytes from these patients.

M-Pos59

IDENTITY AND DEVELOPMENTAL ROLE OF THREE VOLTAGE-DEPENDENT POTASSIUM CHANNELS EXPRESSED BY MURINE $CD4^-CD8^-$ THYMOCYTES. ((B.D. Freedman*, B.K. Fleischmann+, J. Punt*, Y. Hashimoto*, G. Gaulton*, M.I. Kotlikoff*)) Depts. of Pathology and Laboratory of Medicine* and Animal Biology+, University of Pennsylvania, Philadelphia, PA 19104. (Spon. by M. Civan)

Using the nystatin permeabilized patch clamp technique and reverse transcription polymerase chain reaction (RT-PCR), we have determined that the whole cell K^+ current of murine $CD4^-CD8^-$ thymocytes is comprised of the functional products of at least three voltage-dependent potassium channel genes. KV1.3 (n-channel), the predominant K^+ conductance, was previously identified on all major thymocyte subpopulations. We have identified two additional voltage-dependent K^+ currents in $CD4^-CD8^-$ thymocytes; these currents exhibited pharmacologic and electrical behavior consistent with KV1.1 and KV1.2 gene products. RT-PCR analysis of RNA from thymocytes provided confirmation of the molecular identity of KV1.1 and KV1.3. In order to assess the functional importance of KV1.1, KV1.2 and KV1.3, we evaluated the effect of K^+ channel blockade on development in intact fetal thymic organ explants. Blockers of each of these three channels decreased thymocyte yields from cultured organs at concentrations corresponding to blockade of each of the three conductances. Moreover, K^+ channel blockers exhibited a similar effect on stimulated proliferation of $CD4^-CD8^-$ fetal thymocytes in suspension culture. These findings are consistent with the hypothesis that specific K^+ channels play an important role in thymocyte development. (Supported by NIH HD-01056)

M-Pos60

THE NEW ACTIVATORS OF Ca^{++}–DEPENDENT K^+ CHANNEL OF ERYTHROCYTES. ((A.V. Gyulkhandanyan)) Institute of Biochemistry, National Academy of Sci., Yerevan, 375044, ARMENIA.

Using ion-selective electrodes, it is shown that tetrazolium salts such as monotetrazolium blue (TB–0.6mM), m-nitrotetrazolium chloride (0.035mM), and £-naphtol (0.3–0.4mM) induce K^+ and Ca^{++}-dependent K^+ conductivity of human erythrocytes. In its turn, 0.1mM £-naphtol inhibits Ca^{++}-dependent K^+ channel induced by the electron donor system ascorbat+PMS and propranotol. At those concentrations of TB and £-naphtol that do not cause K^+ efflux, the addition of the-se compounds to erythrocytes, which were preincubated with protonophore CICCP, electron transport inhibitor antimycin, anion trahsport inhibitor DIDS, or anionic detergent SDS leads to a sharp rise in K^+ conductivity and hyperpolarization of membranes. The influence of the energetic processes inhibitors on the activity of Ca^{++}-dependent K^+ channel induced by TB and £-naphtol in combination with the above compounds is studied. The increase in the cation conductivity may be attributed to the formation of complexes of tetrazolium salts and £-naphtol with K^+ and Ca^{++} ions and/or to the structural changes of erythrocytes' membrane.

M-Pos61

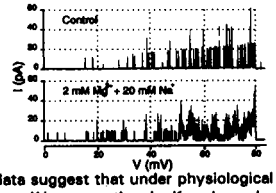
ACTIVATION OF LARGE-CONDUCTANCE, CALCIUM-DEPENDENT K CHANNELS IN MICROVESSELS OF RAT MESENTERY BY NITROVASODILATORS. ((G.O. Carrier and R.E. White)) Medical College of Georgia, Augusta, GA and Wright State University, Dayton, OH.

Vascular endothelial cells modulate the tone of the underlying smooth muscle by releasing a number of factors including nitric oxide (NO). NO released from the endothelium or generated by the metabolism of nitrovasodilators causes relaxation of vascular smooth muscle which is associated with increasing levels of cGMP. Recent evidence suggests nitrovasodilators can activate large-conductance, calcium-activated potassium channels (K_{Ca}) to promote vasodilation in several vascular beds. In the present study we have examined the effects of sodium nitroprusside (SNP) and s-nitroso-N-acetyl-DL-penicillamine (SNAP) on the large conductance, calcium-and voltage activated K channels in dissociated muscle cells from microvessels (> 100 μm) of rat mesentery. Both SNP (10μM) and SNAP (10μM) increased the outward macroscopic K^+ current in whole-cell recordings using the perforated-patch approach. TEA (1mM) caused a 70% inhibition of the macroscopic K^+ current and prevented the effects of SNP and SNAP. A membrane permeant analog of cGMP also product a marked enhancement of the outward macroscopic K^+ current which was blocked by TEA. In single K channels recordings, SNP activated a large-conductance K channels (110pS) in the cell-attached configuration by increasing the open probability (measured as NPo). The conductance of this channel is in accordance with reported values for the large-conductance Ca-activated K channel. These results suggest that SNP and SNAP promote vasodilation of microvessels by activating the large-conductance, Ca-activated K channel possibility linked to a cyclic-GMP-dependent process.

M-Pos62

REDUCED UNITARY CONDUCTANCE OF BIG Ca^{2+}-DEPENDENT K^+ CHANNELS IN CELL-ATTACHED PATCHES OF VASCULAR SMOOTH MUSCLE CELLS MAY BE DUE TO BLOCK BY Mg^{2+} AND Na^+. **((E. Morales*, W.C. Cole*, C.V. Remillard, and N.R. Leblanc)), *Smooth Muscle Research Group, U of Calgary, Calgary, Alta, and Montreal Heart Institute, U of Montreal, Montreal, Que, Canada.**

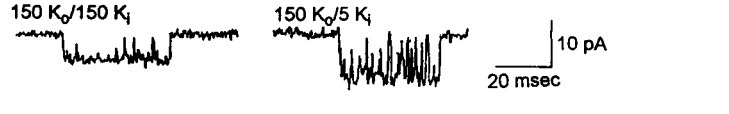
Large Ca^{2+}-dependent K^+ channels (K_{Ca}) are present in high density (1 to 3x 10^4 per cell) in smooth muscle cells. Because of their high density and large conductance, K_{Ca} should contribute substantially to macroscopic K^+current. However, their contribution within the physiological voltage range (-80 to +20) at normal levels of internal Ca^{2+} is relatively much smaller than would be expected (pA rather than nA). This study shows that K_{Ca} have a lower unitary conductance, particularly with larger depolarizations, in cell-attached configuration with 5.4 mM external K^+ than expected based on measurements made in symmetrical K^+ conditions. Iberiotoxin-sensitive (20 nM) openings of less than 1 pA were observed versus an expected current of 5 pA at 0 mV. The ionic basis for this disparity was explored by exposing the internal face of excised patches to solutions of varied Mg^{2+} and Na^+ under asymmetrical K^+ ($140_i/5.4_o$) simulating the physiological gradient for K^+. As Mg^{2+} was increased from 0 to 5 mM at 100 nM Ca^{2+} (buffered with EGTA) unitary conductance declined from 116 to 78 pS . Mg^{2+} block had a Kd of 9.7 mM. In addition to its effect on conductance Mg^{2+} also increased open probability. However, in order to account for the marked rectification, a combination of Mg^{2+} and Na^+ was required (see figure). The data suggest that under physiological conditions, both Mg^{2+} and Na^+ may regulate K^+ permeation in K_{Ca} channels. Supported by MRC grants to WCC (DG-N025) and NRL (MT-10863).



M-Pos63

EFFECTS OF INTRACELLULAR K^+ ON GATING OF EMBRYONIC RAT TELENCEPHALON MAXI-K CHANNELS. ((J.-M. Mienville and J. R. Clay)) NIH, Bethesda, MD 20892. (Spon. by D.E. Goldman)

The effects of changes in K_i on single, maxi-K channels from embryonic rat telencephalon were studied using the inside-out configuration of the patch-clamp technique. Ionic conditions were either 150 K_o/150 K_i, or 150 K_o/5K_i,145 NMDG. Bursts of openings (O_b) separated by relatively long lasting closed time durations (C_L) were observed in both conditions at -30 mV (see below). No effect of K_i was observed on the durations of either O_b or C_L. The primary effect was a change of probability of occupancy, p_c, of a short lived closed state (C_S). In particular, p_c was substantially increased by the reduction in K_i. This result was not altered by a change of cytoplasmic pCa. Moreover, no effects of K_o on gating were observed in outside-out patches. The results with K_i suggest that the channel does not have to open for C_S to be occupied and that the effects of K_i may not be related to ion permeation.



M-Pos64

Ca^{2+}-DEPENDENT K^+ CURRENT AND INDO-1 SIGNAL DURING DEPOLARIZATION IN SINGLE SMOOTH MUSCLE CELLS OF THE GUINEA-PIG VAS DEFERENS. ((Y. Imaizumi, N. Nagano, K. Muraki and M. Watanabe)) Dept. of Chem. Pharmacol. Faculty of Pharmaceut. Sci., Nagoya City Univ., Nagoya, 467, Japan (Spon. by N. Anderson)

Changes in intracellular Ca concentration ($[Ca^{2+}]_i$) were monitored by Indo-1 fluorescence signal under whole cell voltage-clamp in single smooth muscle cells of the guinea-pig vas deferens at 35°C. The pipette solution contained mainly 140 mM KCl and also 100 μM Indo-1. When a cell was depolarized from -60 to 0 mV for 150 msec under voltage-clamp, an initial inward current through Ca channels and a following outward current were recorded. The outward current consisted of two components; a fast transient component which had a peak about 30 msec from the start of depolarization and a following sustained one having smaller amplitude. Both components were greatly reduced by 100 μM Cd^{2+}, 2 mM TEA or 10 nM charybdotoxin, indicating that Ca^{2+}-dependent K^+ current ($I_{K\text{-}Ca}$) through BK channels substantially contributes to them. The increase in Indo-1 signal started ~20 msec and reached the peak after 100-200 msec from the beginning of depolarization, respectively. Occasionally, a small notch of Indo-1 signal was recorded at the same time of the peak of fast transient component of outward current. Application of 30 μM ryanodine, 5 mM caffeine or 10 μM cyclopiazonic acid greatly reduced two outward current components and the increase in Indo-1 signal, suggesting that Ca induced Ca release (CICR) from Ca storage sites is involved in these changes. A short depolarization of 10 msec induced a substantial $I_{K\text{-}Ca}$ and its large tail current but did not increase Indo-1 signal. These results suggest that the increase in $[Ca^{2+}]_i$, which activates a large transient $I_{K\text{-}Ca}$ upon depolarization, is mediated by Ca^{2+}-influx through Ca channels and subsequent CICR, but can not be clearly detected as a peak of Indo-1 signal. CICR which activates $I_{K\text{-}Ca}$ may occur locally just beneath the cell membrane and could be functionally resolved from that elicits a large indo-1 signal and contraction.

M-Pos65

SODIUM AND POTASSIUM CURRENT IN EMBRYONIC MUSCLE CELLS CONTACTED BY SPINAL NEURONS. ((F. Moody-Corbett and S. Hancock)) Div. Basic Med. Sci., Memorial University, St. John's, NF A1B 3V6.

In adult muscle the distribution and type of Na channels are regulated by the nerve. The purpose of the present study was to examine the expression of Na and K channels in embryonic muscles following nerve contact compared with age-matched muscle cells grown without neuronal contact. *Xenopus* muscle cells from 1 day old embryos were grown in culture in the presence or absence of spinal cord neurons. Whole cell currents were recorded from nerve-contacted and non-contacted muscle cells using a List patch clamp and standard recording conditions (Moody-Corbett and Gilbert, Dev. Br. Res.,1989,55:139-142). We have found that 76% (n=25) of nerve-contacted muscle cells displayed a Na current while only 23% (n=17) of non-contacted muscle cells expressed Na^+ current. Furthermore, muscle cells which were contacted by nerve and which expressed a Na current had a higher density of inactivating outward K current (41 pA/pF, n=12) compared with cells which were neither contacted by nerve nor had a Na current (32 pA/pF, n=5). In contrast, the current density of the inward rectifier K current and the delayed rectifier-type K current were similar regardless of the presence of nerve contact and Na current. The results suggest that innervation can up-regulate the expression of Na channels in embryonic muscle cells and that innervation or the subsequent expression of Na channels influences the expression of some, but not all, K channels in these cells.

M-Pos66

VOLTAGE-DEPENDENT- AND CA-ACTIVATED-POTASSIUM CHANNELS IN H9C2 CLONAL CELL LINE FROM RAT HEART. ((W. Wang, T. Nakamura and R. Ochi)) Dept. Physiology, Juntendo Univ. Sch. of Med., Tokyo, 113, Japan

We examined the developmental changes of outward currents in myoblast and myotube of a clonal cell-line (H9c2) derived from embryonic rat heart. Both in the myoblast and in the myotube, there existed voltage-dependent K^+ currents (IKv) which rose rapidly and decayed slowly during 500 ms test pulses at large positive potentials. The IKv was little affected by superfusion with Ca^{2+}-free solution. In the differentiated myotube, there also existed K^+ currents (IKCa) with slow activation and deactivation kinetics and were completely suppressible by external Ca^{2+}-free solution or internal 10 mM EGTA solution. The IKCa was inhibited by CTX (100 nM) by 60% and by apamin (100 nM) by 40%, suggesting the existence of two different IKCas. The density of IKv was by 10% larger in the myotube but IKCas shared 60% of the total K^+ currents at positive potentials. We conclude that a voltage-dependent K^+ channel is expressed during the myoblast stage and in addition to this channel two different types of Ca^{2+}-activated K^+ channels are expressed during the myotube stage.

M-Pos67

ACTIVATION OF BK_{Ca} CHANNELS IN ISOLATED CEREBRAL ARTERIAL SMOOTH MUSCLE CELLS BY THE BENZIMIDAZOLE NS1619. ((Holland,M., Boyle,J.P. and Standen,N.B.)) Department of Cell Physiology and Pharmacology, University of Leicester, Leicester, LE1 9HN, U.K.

We have studied the ability of the benzimidazole, NS1619, to open large conductance Ca^{2+}-activated potassium (BK_{Ca}) channels, using arterial smooth muscle cells isolated enzymatically from rat basilar artery. In perforated patch whole cell recordings NS1619 (10μM) activated a noisy outward current from cells held at 0 mV in 6mM K^+ (see Fig. 1).

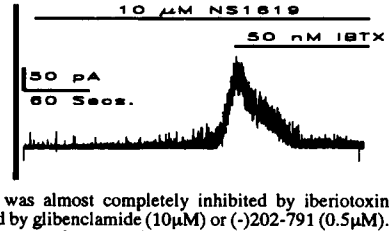


Figure 1. Whole-cell amphotericin perforated patch recording from isolated rat basilar arterial myocyte at 0 mV in presence of 0.5 μM (-)202-791 and 10 μM glibenclamide.

This steady-state current was almost completely inhibited by iberiotoxin (50nM) but was unaffected by glibenclamide (10μM) or (-)202-791 (0.5μM). In inside-out patches, a large conductance channel was activated at holding potentials positive to -40 mV. NS1619 increased the open probability of this channel, while current amplitude was not significantly altered. We conclude that NS1619 activates BK_{Ca} channels in cerebral arteries.

This work was supported by The British Heart Foundation.

M-Pos68

ADENOSINE-ACTIVATED, TEA-SENSITIVE I_K IN CORONARY SMOOTH MUSCLE FROM EXERCISE-TRAINED PIGS ((D.K. Bowles, M.H. Laughlin and M. Sturek)) Vascular Cell Biophysics Lab, Dalton Cardiovascular Research Center and Departments of Physiology and Veterinary Biomedical Sciences, University of Missouri, Columbia, MO 65211. (Spon. by C.C. Hale)

Previous studies from our lab have shown that adenosine (ADO)-stimulated relaxation is inhibited by TEA (1 mM) in coronary arteries from exercise-trained (EX), but not sedentary (SED), pigs. Thus, we tested the hypothesis that 1) whole-cell K^+ current (I_K) during ADO would be increased by exercise training, and 2) this increase would be inhibited by TEA. I_K was measured in the presence and absence of ADO (10^{-5} M) using the perforated patch technique on freshly dispersed coronary smooth muscle cells from EX and SED pigs. Cells were loaded with fura-2/AM for simultaneous microfluorometry and voltage-clamp in symmetrical K^+ solutions. Holding potential was -60 mV. During short (220 ms) ramp depolarizations (-100 to +50 mV) ADO increased I_K in cells from EX, but not SED at positive test potentials ($p < 0.05$ at +20, +40 and +50 mV). This increase in ADO-stimulated I_K was not observed in the presence of TEA (1 mM). ADO did not affect either the holding current at -60 mV or basal myoplasmic Ca^{2+} in either group. Cromakalim (5×10^{-5} M) activated a large inward current (~ 80 pA) at -60 mV in both groups. These data support the hypothesis that exercise training uncovers an ADO-activated, TEA-sensitive I_K in coronary smooth muscle, probably Ca^{2+}-activated K^+ current. (Supported by NIH HL09086, HI36531, HL41033 and RCDA HL02872)

M-Pos69

MECHANISM OF ACTIVATION OF ATP-SENSITIVE K^+ CHANNELS BY INTRACELLULAR PROTONS AND K-CHANNEL-OPENERS.

((C. Forestier, J. Pierard & M. Vivaudou)) Laboratoire de Biophysique Moléculaire et Cellulaire, CEA-DBMS-URA CNRS 520, Grenoble, France

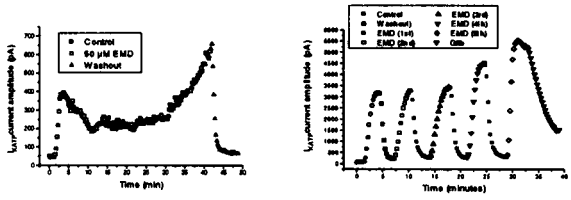
The molecular mechanisms underlying the actions of K-Channel-Openers (KCO) and protons on K-ATP channels were studied with the patch clamp technique using excised inside-out patches from frog skeletal muscle fibers. Benzopyran KCOs (levcromakalim and SR47063) opened channels partially blocked by ATP, ADP, or ATPγs, with and without Mg^{2+}. This activation arose from a competitive interaction between KCOs and nucleotides as we observed that ATP decreased the apparent affinity for KCOs and that, conversely, KCOs decreased ATP or ADP sensitivity. Activation by protons relied on a similar competitive mechanism. Acidification reduced ATP sensitivity and ATP reduced the pK value of activation by protons. Similar effects were observed when ADP was used as the inhibitory nucleotide. Unlike KCOs, protons could activate K-ATP channels in absence of nucleotides. Protons also reduced channel conductance by a mechanism compatible with neutralization by protonation of negative surface charges at the pore entrance. Furthermore, protons antagonized the effects of KCOs by enhancing their dissociation rate. This effect was observed with both benzopyran (Forestier et al., *FEBS Lett.* 325:276,1993) and non-benzopyran KCOs (pinacidil, aprikalim) suggesting a common binding site for all KCOs.

These results are in quantitative agreement with a structural model of the K-ATP channel where **(a)** the nucleotides ATP and ADP share the same binding sites, **(b)** the various classes of KCOs share the same binding sites, **(c)** nucleotide and KCO sites are separate but competitively linked, **(d)** protons can bind first to 2 sites competitively linked to the KCO sites but not to the nucleotide sites, and **(e)** protons can bind subsequently to 2 additional sites which are competitively linked to both KCO and nucleotide sites.

M-Pos70

POTENTIATION OF THE CARDIAC ATP-SENSITIVE K^+ CHANNEL DURING PROLONGED AND MULTIPLE ACTIVATIONS BY BIMAKALIM. ((WM Kwok, AT Martinelli, ZJ Bosnjak)) Dept. of Anesthesiology, Medical College of Wisconsin, Milwaukee, WI 53226

We have investigated the effects of prolonged and multiple activations of the K_{ATP} channel in guinea-pig ventricular myocytes by a potassium channel opener, bimakalim (EMD 52692). Whole-cell K_{ATP} current (I_{KATP}) in response to a 100 msec test pulse to +10 mV from a -40 mV holding potential was monitored every 15 seconds. We report that the ability of bimakalim (50 μM) to activate I_{KATP} is enhanced during prolonged application. The bimakalim-induced I_{KATP} initally reached steady-state within 8 minutes, but after 35 minutes, current amplitude gradually increased by 120±20% compared to steady-state levels (left figure). This potentiation of I_{KATP} over time is also observed during multiple activations by bimakalim (right figure). Current amplitude showed increases of 15±8 and 45 ±18% compared to steady-state levels after a fourth and fifth exposure to 50 μM bimakalim, respectively. Present experiments are designed to determine whether bimakalim induces intrinsic modification of the K_{ATP} channel's sensitivity to intracellular ATP.



M-Pos71

LOW CONCENTRATION OF ARACHIDONIC ACID DIRECTLY ACTIVATES THE ATP-SENSITIVE K^+ CURRENT IN DOG CORONARY SMOOTH MUSCLE CELLS. ((Xiaoping Xu and Kai S. Lee)). Upjohn Laboratories, Kalamazoo, MI 49007

ATP sensitive K^+ channels play a major role in controlling blood vessel tone, particularly in coronary vasculature. Arachidonic acid (AA) exhibits potent vasorelaxant effects of the coronary vessels with unknown mechanism. We examined the activity of AA on the K_{ATP} channel in dog coronary smooth muscle cells using whole-cell voltage-clamp technique. In the absence of K_{ATP} current in cells dialysed with 1 mM ATP pipette solution, external application of AA activated the K_{ATP} current with a bell-shaped concentration-response curve. The K_{ATP} current at -50 mV was increased from 1.0 ± 1.1 to 4.4 ± 3.1, 25 ± 9, 43 ± 13, 26 ± 10 and 23 ± 16 pA by 1, 2, 4, 8 and 16 μM AA, respectively. In contrast, if the K_{ATP} current was activated by dialysing the cell with ATP-free pipette solution to begin with, external application of AA at concentrations from 2 to 16 μM concentration-dependently inhibited the K_{ATP} current (IC_{50}=3.8 μM). Pretreatment of the cells with indomethacin (10 μM) or nordihydroguaiaretic acid (10 μM), a blocker of the cyclooxygenase and lipoxygenase pathway, respectively, did not prevent activation or inhibition. The nonmetabolizable analogue of AA, eicosatetraynoic acid (ETYA), also produced dual effects on the K_{ATP} current which, at -50 mV, was increased by 4 μM ETYA from 2.9 ± 1.2 to 37 ± 7 pA in 1 mM intracellular ATP (ATP_i), and 8 μM ETYA inhibited the K_{ATP} current in 0 mM ATP, by 71 ± 3 %. These results suggest that at high concentration, AA inhibits the K_{ATP} current, via a unknown mechanism; at low concentrations, they may compete with ATP for binding to the ATP inhibition site, thereby relieving the ATP block to increase the ATP-sensitive current and coronary vasorelaxation.

M-Pos72

Actin microfilament-disrupters anatagonize ATP-dependent gating of cardiac ATP-sensitive K^+ channels.

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Stretch induces activation of cardiac ATP-sensitive K^+ (K_{ATP}) channels through an unknown mechanism. To assess whether actin-filaments regulate K_{ATP} channel gating, currents were measured in the inside-out configuration and cytoskeletal disrupters applied to the internal side of patches excised from cardiomyocytes. When intracellular ATP (ATP_i) suppressed K_{ATP} channels, DNase I (10-200 μg/ml), which forms complexes with G actin and prevents filament formation, enhanced K_{ATP} channel activity. After boiling, DNase I lost this ability. Once DNase I (100 μg/ml) diminished the ability of ATP_i to inhibit K_{ATP} channels, addition of purified actin subunits (200 μg/ml) partially restored the sensitivity of K_{ATP} channels toward ATP_i. Cytochalasin B (10 μM), an other actin filament disrupter, also enhanced channel activity in the presence of ATP_i. By contrast, taxol (30-100 μM), an agent acting on microtubules, did not significantly affect K_{ATP} channels. Hence, agents known to act on actin-filaments regulate cardiac K_{ATP} channel gating which suggests that actin-filaments may couple mechanosensitive stimuli to channel activity.

M-Pos73

ATP-SENSITIVE AND INWARD RECTIFIER K^+ CHANNELS OF DEVELOPING RAT SKELETAL MUSCLE FIBERS. ((D. Tricarico, R. Mallamaci, R. Petruzzi and D. Conte Camerino)) Dept. of Pharmacobiology, Faculty of Pharmacy, University of Bari, Via Orabona n° 4, 70125 Bari, ITALY (Spon. By D. Conte Camerino)

The resting K^+ conductance (gk) of rat skeletal muscle, at birth, dominates the membrane conductance than decreases with development. To asses whether the changes in gk occurring during develpment are due to alterations in K^+ channels populations or properties, we surveyed the K^+ channels of the sarcolemma of developing rats by patch clamp technique. Single fibers were isolated enzymatically at 30° C from flexor digitorum longus muscle of *15, 21-23, 56 and 82 days old* rats. Continuous recording of the channel activity was performed in cell-attached (C-A) and inside-out (I-O) configurations at -60 mV (Vm), at 20° C, in symmetrical 150 mM KCl. C-A recordings, revealed inward currents flowing throught multiple channels. In I-O configuration, the K^+ channel activity was higher in *15 and 21-23 days old* rats decreasing at *52-56, and 82 days* of age; the mean current of the patches was -10.1±0.14 pA, -13.29±2.4 pA, -8.93±1.2 pA and -8.13±0.37 pA respectively for *15, 21-23, 56 and 82 days old* rats. The application of MgATP and Na_2ATP (1 μM-4 mM) to the cytoplasmic face of the patches reduced the mean current, suggesting that most of the channels were ATP sensitive. At *15 and 21-23 days* of age, four types of K^+ channels were routinely found; two types of 40 pS and 60 pS, were blocked by internal ATP (K_{ATP}), while the others were inward rectifier (IRK) of 26 pS and 38 pS. The 38 pS channel was also MgATP activated . At *56 and 82 days*, the K_{ATP} channel of 40 pS disappear, while the frequency of occurring of the 60 pS channel was 60%. At this age, the IRK of 26 pS and 38 pS were found in only 15% and 17% of the patches examined. Ca^{2+} activated K^+ channels of 260 pS were rarely observed in these fibers. Our results suggest that the high gk of developing rat skeletal muscle is due to an high density of K_{ATP} and IRK channels. (CNR 94-2368; TELETHON 579)

M-Pos74

Pharmacological Comparison of Inward Rectifier Potassium Currents in RBL-1 Cells and Stimulated Human Monocytes

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Central Research Division, Pfizer Inc, Groton CT 06340

We have performed a pharmacological comparison of inward rectifier potassium (K_{IR}) currents recorded in RBL-1 cells and LPS-stimulated human monocytes. In whole-cell patch-clamp studies, the antiviral agents amantadine and rimantadine, and capsaicin produced a reversible use-dependent block of peak inward K^+ currents with IC_{50} values in the 10-30 μM range. We are not aware of previous reports identifying these agents as K_{IR} inhibitors. In contrast, dendrotoxin, which was recently reported to block K^+ inward rectifier currents in *Vicia* guard cells at 30 nM (J. Membrane Biol. 137:249(1994)), had no effect on K_{IR} currents in either human monocytes or RBL cells at a test concentration of 100 nM. Charybdotoxin (100 nM) and resiniferatoxin (50 μM) also had no effect on these two inward currents. K_{IR} currents in the two cell types were blocked by barium and TEA with IC_{50} values of approximately 15 μM and 60 mM, respectively. In summary, we have identified compounds that inhibited K_{IR} currents in both RBL cells and human monocytes, and have been unable to distinguish these two currents with a variety of pharmacological agents. Studies are in progress to compare the GTP dependence of these K_{IR} currents.

M-Pos75

INWARD RECTIFIER POTASSIUM CURRENTS IN CORONARY RESISTANCE ARTERIES FROM PIG ((J.M. Quayle, C. Dart, & N.B. Standen.)) Ion Channel Group, Dept. Cell Physiol. & Pharmacol., Leicester University, Leicester, U.K.

Small arteries regulate local coronary blood flow. We have studied whole cell potassium currents in enzymatically isolated single smooth muscle cells from these resistance-sized coronary arteries. In cells isolated from 4th order branches of the left anterior descending coronary artery, an inwardly rectifying potassium current was identified. This current was inhibited by extracellular barium ions (0.5 mM), which block K_{IR} channels. The barium-sensitive current reversed at a potential close to the calculated potassium equilibrium potential when the extracellular potassium concentration was 6, 60 or 140 mM (6 cells). As extracellular potassium changed, outward current remained small at membrane potentials positive to the new potassium equilibrium potential. These features are typical of a K_{IR} channel.

K_{IR} currents have not been reported in previous studies of potassium currents in large coronary arteries. The density of K_{IR} currents was therefore compared in cells isolated from coronary vessels taken from different points along the vascular tree. In 4th order branches of the left anterior descending coronary artery (artery diameter 93 to 290 μm) there was 22.7 ± 6.5 pA/pF (15 cells) of K_{IR} current at -140 mV (140 mM $[K^+]_o$/140 mM $[K^+]_i$, assessed as the 0.5 mM barium-sensitive current). In comparison, in the LAD (artery diameter 1850 to 2520 μm), there was 0.7 ± 0.2 pA/pF (9 cells) of K_{IR} current. Outward current through voltage dependent potassium channels measured at +30 mV (6 mM $[K^+]_o$/140 mM $[K^+]_i$) was 12.4 ± 2.2 pA/pF (11 cells) in 4th order branches and 18.4 ± 3.8 pA/pF (9 cells) in the LAD.

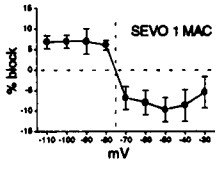
We conclude that the K_{IR} current is at higher density in smaller coronary arteries. The presence of an enhanced K_{IR} current may have functional consequences for the regulation of cell membrane potential and therefore tone in coronary resistance arteries.

Supported by the British Heart Foundation & MRC.

M-Pos76

SEVOFLURANE INHIBITS THE INWARD RECTIFIER POTASSIUM CURRENT IN GUINEA-PIG VENTRICULAR MYOCYTES. ((A. STADNICKA, W.M. KWOK, Z.J. BOSNJAK)) Department of Anesthesiology, Medical College of Wisconsin, Milwaukee, WI 53226.

Sevoflurane (SEVO), a novel fluorinated methyl-isopropyl-ether anesthetic, produces myocardial depression by an undetermined mechanism. To establish whether SEVO may affect resting potassium conductance in myocardial cells we examined the effects of 0.6, 1.2 and 1.8 mM SEVO, equivalent to 1, 2 and 3MAC (minimum alveolar concentration of anesthetic), on the inward rectifier K+ current (I_{KIR}) in enzymatically isolated guinea-pig ventricular myocytes using the whole cell configuration of the patch clamp technique. At potentials negative to the potassium reversal potential (E_K) SEVO produced a concentration-dependent block: 6.0± 1.8% at 1MAC, 13.0±1.3% at 2MAC and 16.8± 1.8% at 3MAC SEVO. No block (3MAC SEVO) or a slight enhancement of the current (1 and 2MAC SEVO) was evident at voltages positive to E_K (Figure). Block of I_{KIR} by SEVO at hyperpolarizing potentials and the lack of block at depolarizing potentials is consistent with a voltage-dependent mechanism. Since SEVO is uncharged at pH 7.4 these results are unexpected and suggest that modulation of I_{KIR} by SEVO may involve a complex regulatory pathway.



M-Pos77

PROPERTIES OF THE INWARDLY RECTIFYING CONDUCTANCE ACTIVATED BY ACETYLCHOLINE IN GUINEA PIG COCHLEA. ((A.P.Nenov, C.Norris* and R.Bobbin)) Kresge Hearing Research Lab., LSUMC, New Orleans, Tulane University School of Medicine, New Orleans*

Acetylcholine (ACh) is the major neurotransmitter mediating efferent output to the OHCs through a novel "suberyldicholine" receptor. The activation of a calcium dependent potassium conductance has been suggested to be the final ACh induced event leading directly to hyperpolarization of guinea pig OHCs. We propose that in guinea pigs ACh operates a channel with properties similar to those of inwardly rectifying potassium selective conductances. We used patch clamp technique in the whole cell configuration to test the hypothesis. Increase in the intracellular concentration of magnesium caused a pronounced inward rectification of the ACh induced currents. Extracellular application of 200 μM barium in the presence and absence of ACh blocked currents with similar characteristics to the ACh induced currents and reduced the ACh response in a voltage dependent manner. The maximal conductance of the channel and its half activation voltage were dependent on the difference of the voltage and the reversal potential for potassium. The channel was highly selective for potassium as determined by the shift of the reversal potential of the response upon a tenfold increase in the concentration of extracellular potassium. In the absence of the agonist the channel was slightly active. Since ACh receptor in OHCs appears to be nicotinic - like and the subsequent mechanisms of the ACh response could be viewed as muscarinic - like it is possible that cholinergic inhibition in cochlea is mediated through an unique novel "suberyldicholine" system.
Supported by NIH grants (PO1-DC00379, RO1-DC00722), DAMD 17-93-V-3013, Kam's Fund for Hearing Research, and the Louisiana Lions Eye Foundation.

M-Pos78

ACTIVATION OF NOVEL K^+-CHANNELS BY NO AND NO DONORS IN CANINE COLONIC SMOOTH MUSCLE CELLS. ((S.D. Koh, J.D. Campbell, A. Carl, K.S. Kim* and K.M. Sanders)) Department of Physiology, University of Nevada School of Medicine, Reno, NV 89557 and *Department of Phyiology, School of Medicine, Hanyang University, Seoul, 133-791, Korea

Nitric Oxide (NO) and NO donors activate potassium currents and induce hyperpolarization in canine colonic smooth muscle (*Biophys.J.*64:A386, 1993). To identify specific K^+ channels activated by NO and NO donors, smooth muscle cells were dispersed and the activities of single K^+-channels were recorded with cell-attached and cell-free configurations of the patch clamp technique. Pipette solutions contained 140 mM K^+ and 200 nM charybdotoxin to suppress Ca^{2+}-activated K^+-channels. Symmetrical and asymmetrical potassium gradients were used with bath solutions containing 140 and 5 mM K^+, respectively. In cell-attached patches, sodium nitroprusside (SNP, 10 μM) increased the open probability (P_o) of a 90 pS channel from 1.0 to 3.2 ± 0.8 (n=12, normalized P_o) and a <4 pS K^+-channel. The effects of SNP persisted for 20 minutes after washout of the drug. The reducing agent, dithiotreitol (2 mM) and the alkdylating agent, N-ethylmaleimide (5 mM) blocked SNP-induced channel openings. Dibutyryl cGMP (1 mM) added during cell-attached patch recording also increased the normalized P_o of the 90 pS channel (to 4.2 ± 0.6, n=7) and the <4 pS channel. After patch excision, the P_o of the 90 pS and <4 pS channels were unchanged by the addition of 0.2 - 1 mM cGMP and dibutyryl cGMP. Direct application of NO (~1 μM) or S-nitroso-N-acetylpenicillamine (10 mM), however, significantly increased the normalized P_o of 90 pS (to 6.0 ± 2.1, n=7) and <4 pS K^+-channels. We conclude that the hyperpolarization response to NO in smooth muscles may be mediated by multiple K^+-channels. These channels may be activated by a cGMP-mediated pathway or by NO directly. (Supported by DK41315)

M-Pos79

ION PERMEATION IN DELAYED RECTIFIER AND M-TYPE POTASSIUM CURRENTS IN BULLFROG SYMPATHETIC NEURONS. ((Brian M. Block and Stephen W. Jones)) Departments of Neurosciences and Physiology & Biophysics, Case Western Reserve University, Cleveland, OH 44106.

Ion permeation and conduction were studied for M-current (I_M) and delayed rectifier (I_{DR}), two K^+ currents that differ greatly in gating kinetics and neurotransmitter modulation. Isolated bullfrog sympathetic neurons were patch clamped in the whole cell configuration with 88 mM $[K^+]_i$. Permeability ratios for monovalent cations were determined from reversal potentials in biionic conditions. The permeability sequence was generally similar for I_M and I_{DR} (see Table), but Na^+ carried detectable inward current for I_{DR} but not I_M. Although P_{Rb}/P_K was near 1.0 for both channels, G_{Rb}/G_K was 1.06 ± 0.01 for I_{DR} and only 0.41 ± 0.02 for I_M (chord conductances for inward current, 25 mM $[Rb^+]_o$ or $[K^+]_o$). Extracellular Cs^+ carried detectable inward current for both I_M and I_{DR}, but blocked I_M with higher affinity (K_D = 81 mM at 0 mV for I_M, K_D = 580 mM at 0 mV for I_{DR}). For both currents Cs^+ block was strongly voltage dependent (δ ~ .9), consistent with a site of action in the pore. These studies demonstrate that two K^+ currents in the same neuron can have significantly different permeation properties, notably Na^+ permeation in I_{DR}, and variations in G_{Rb}/G_K and in Cs^+ block.

P_X/P_K	2.5 Rb^+	15 Rb^+	25 Rb^+	NH_4^+	Cs^+	Na^+	$CH_3NH_3^+$	Li^+
I_M	1.08 ± 0.07	0.99 ± 0.05		0.09 ± 0.004	0.09 ± 0.007	< 0.004	< 0.004	< 0.004
I_{DR}	0.97 ± 0.02		0.91 ± 0.05	0.10 ± 0.003	0.07 ± 0.002	0.008 ± 0.0002	< 0.004	< 0.004

Comparisons (±SEM) are from 115 mM $[X^+]_o$ vs. 2.5 mM $[K^+]_o$, except $[Rb^+]_o$ = $[K^+]_o$.

M-Pos80

REAL-TIME ANALYSIS OF SINGLE CHANNEL CURRENTS. ((S. Jovanovic, K. Lynn, X. Wu, R.S. Eisenberg*, R.A. Levis*)) Dept. of Physics, Brookhaven National Lab., *Dept. of Physiology, Rush Medical College.

A new instrument for real-time analysis (RTA) of single channel currents is presented. The RTA, coupled with a software running on a PC host computer in a Windows environment, detects single channel events, measures their duration and amplitude, and performs analysis in real time of the incoming data stream. For detecting transitions between open and closed states of one or more ionic channels, the RTA utilizes threshold-crossing circuitry able to distinguish up to 8 levels. This instrument operates in two basic modes, continuous mode and pulse mode. Pulse mode is used for studying voltage activated channels. In the learning phase, the amplitude and number of channels are automatically determined and the thresholds are set according to a 50% threshold criterion. During the collection period, correction for baseline drift is performed. Relevant results are displayed in real time, including amplitude information, mean open and closed time, amplitude histogram and histograms of open and closed durations. In addition, a display of time dependent amplitude histogram, called a stategram is provided. It consists of a collection of amplitude histograms arranged in an array, with bin values encoded by colors. This provides qualitative observation of baseline stability, channel activity and signal contamination by noise.

M-Pos81

THE SUBCONDUCTANCE HINKLEY-DETECTOR: AUTOMATED SINGLE-CHANNEL ANALYSIS EVEN WITH ARBITRARY CURRENT LEVELS. ((S. Draber)) Inst. f. Angewandte Physik, 24098 Kiel, Germany.

Many types of channels, for instance the K^+channel in the tonoplast of *Chara corallina*, show subconductance levels. The automated event detection becomes more complicated then. Standard detectors like the half-amplitude threshold algorithm cannot be used because the jumps from the actual level can have different amplitudes. The solution for arbitrarily spaced current levels is the "Subconductance Hinkley-Detector" SHD. It is a new algorithm for jump detection that takes all possible jump magnitudes into account. It calculates several test values in parallel, one for each possible jump. After a jump has taken place, the maximum velocity of increase of the test values is used to identify the correct jump. The result of the analysis is a reconstructed noise-free time series jumping between the given levels of current, which can then be used for any kind of statistical analysis. This presentation shows an exemplary data set from *Chara* K^+channels. The main question in that case was whether the observed jumps are indeed an evidence for subconductance or simply the result of a superposition of a second channel type having a smaller unitary conductance. This question is answered by means of the so-called transition matrix, in which the SHD counts the transitions between the levels. For the *Chara* data the high number of transitions between the sublevel and the full level provide strong evidence for a subconductance conformation of the K^+channel.

M-Pos82

DETERMINATION OF INDIVIDUAL CHANNEL OPEN PROBABILITIES FROM MULTICHANNEL DATA. ((R.J. Bauer, A. Carl, C.L. Kapicka, K.F. Korver, and J.L. Kenyon)) Department of Physiol., Univ. of NV School of Medicine, Reno, NV 89557, and Xoma Corp. Berkeley CA.

Amplitude histograms for current records generated by n single channels can be fit by a Gaussian function with n+1 peaks using the parameters: p_i = open probabilities for channels 1 to n, m_i = current amplitudes for 0 to n open channels (pA), and s_i = standard deviations for the n+1 peaks (pA). We developed and tested a program, GAUSS, that fits amplitude histograms using a maximum likelihood method to allow statistical comparisons of specific models. For the analysis, we establish that the current observations are statistically independent, i.e. that they are separated by intervals long relative to the kinetics of channel gating. Then GAUSS obtains values for p_i, m_i, and s_i, first with no constraint on the open probabilities (i.e. $p_1 < > p_2 < > p_3$ ...,etc.) followed by increasing constraints ($p_1 = p_2 < > p_3$..., $p_1 = p_2 = p_3$..., etc.). We next compare the χ^2 values of the fits and accept the most constrained model that is not statistically different from the unconstrained model. Single channel data provide limited information for distinguishing among such models. For example, we analyzed 5000 points of theoretical data derived from $p_1 = 0.2$, $p_2 = 0.4$, and $p_3 = 0.6$. The best fit models ($p_1 = 0.22$, $p_2 = 0.42$, $p_3 = 0.58$) and ($p_1 = p_2 = 0.3$, $p_3 = 0.6$) were not distinguishable. However, models with 3 identical open probabilities could be excluded. NIH DK-41315.

M-Pos83

LOWERING EXTERNAL PH INCREASES A POTASSIUM CURRENT IN RABBIT CORNEAL EPITHELIAL CELLS ((A. Rich, C. Bartling, G. Farrugia, and J.L. Rae)) Mayo Foundation, Rochester, Minnesota 55905.
The effects of pH on a K^+ conductance in freshly dispersed rabbit corneal epithelial cells were measured using the amphotericin perforated patch whole cell voltage clamp technique. The bath solution was a standard Ringer's solution containing (in mM) 4.74 KCl, 149.2 NaCl, 2.54 $CaCl_2$ and 5 HEPES. NaOH was used to adjust pH. Perfusion with pH 6.00 Ringer's solution following standard Ringer's solution (pH=7.35) resulted in an increase in a delayed-rectifier type outward K^+-selective current (I_K) from 120±29 to 312±64 pA (mean±SEM, n=15, steady-state current at +60mV). This was accompanied by a membrane hyperpolarization from -52±5 to -62±3 mV (n=15). The pH-stimulated increase in I_K did not reverse during a 15 minute wash at pH 7.35. The time course of activation was not changed significantly. In a separate series of experiments, when bath pH was decreased from 7.35 to 7.0, 6.5, and 6.0, I_K increased from 153±26 to 270±53, 385±99, and 555±100 pA (n=6), respectively. The resting membrane potential also decreased from -48±11 to -50±10, -55±11, and -61±7 mV, respectively. Single channel studies in perforated outside-out vesicles showed that a decrease in bath pH from 7.35 to 6.00 was accompanied by an increase in the single channel open probability (NP_o) from 0.55 to 0.69 at a membrane potential of 15mV. NP_o was also increased in excised inside-out patches. The unitary conductance, measured from -60 to +40 mV, was not changed. These results suggest that a decrease in bath pH directly increases single channel open probability resulting in an increase in I_K and a membrane hyperpolarization. (supported by grants EYO3282, EYO6005, and DKO8677-03)

M-Pos84

EFFECT OF PROTEINS ON THE MEMBRANE POTENTIAL OF ISOLATED HEPATOCYTES. ((F.J. Burczynski and G-Q. Wang)) Department of Pharmacology, University of Manitoba, Winnipeg (Spon. by R. Bose)

The electrical potential difference across the plasma membrane (PD) of hepatocytes has been shown to affect the uptake of long-chain fatty acids). Since albumin (ALB) is known to enhance the overall uptake of lipophilic organic anions, it was of interest to determine if the enhanced uptake was related to an interaction of ALB with the hepatocyte surface (an event that may change the PD). **METHODS** Hepatocytes were incubated for ≈4 hr in serum-free culture medium. Monolayers were washed with protein-free buffer. During PD measurements, using intracellular microelectrodes in the presence and absence of ALB, hepatocytes were not perfused. ALB was added directly to the culture plate. Total volume of buffer on the culture plate was 1 ml. **RESULTS** ALB caused a dose-dependent depolarization of hepatocytes. The reduction in PD ranged from 1.3±0.6 to 20.6±2.7 mV using 52 and 643 μM ALB, respectively (n=11). The t_K^+ was 0.46±0.03 (n=6) and 0.20±0.01 (n=8) at 0 and 643 μM ALB, respectively. **CONCLUSION** ALB affects the hepatocyte membrane potential by interacting with the cell surface. This interaction leads to altered K^+ conductance. (Supported by the Canadian Liver Foundation and Manitoba Health Research Council.)

M-Pos85

ISOSMOTIC INTRACELLULAR Ca^{2+}-DEPENDENT VOLUME LOSS AND KCl EFFLUX IN INTERNALLY PERFUSED BARNACLE MUSCLE CELLS. ((D. Ferguson, K. McGruder, C. Peña-Rasgado, and H. Rasgado-Flores)) Dept. Physiol & Biophys. FUHS/Chicago Medical School.N.Chicago,IL 60064.

The effect of isosmotic increases in the concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$) on cell volume and the efflux of osmolytes and water was studied in internally perfused barnacle muscle cells. Intracellular perfusion with solutions containing low $[Ca^{2+}]$ (i.e. 0.01 μM) for >1 hr permits a subsequent increase in $[Ca^{2+}]_i$ (i.e. 1-20 μM) without producing contraction. The free $[Ca^{2+}]$ was monitored with a Ca^{2+}-selective electrode. An increase in $[Ca^{2+}]_i$ (i.e 1.0-20 μM) produced a reduction in cell diameter due to net loss of water and KCl. Net loss of water was demonstrated by a Ca_i-dependent increase in the perfusate's concentration of a radiolabelled, non-permeant solute (i.e. ^{14}C-sucrose) as compared before and after perfusing the cell. Support for loss of KCl was provided by a Ca_i-activated unidirectional efflux of ^{86}Rb and ^{36}Cl. The Ca_i-activated ^{86}Rb efflux was larger (~40%) than the expected fluxes calculated from the measured volume reduction; the Cl^- efflux was only ~10% larger. Replacement of intracellular Cl^- by the non-permeant anion, methanesulfonate, abolished the Ca_i-activated volume loss but only partially inhibited the ^{86}Rb efflux. This indicates that high $[Ca^{2+}]_i$ promotes net loss of KCl and osmotically obligated water as well as unidirectional Rb^+ efflux through a pathway not accompanied with Cl^- (likely $^{86}Rb/K^+$ exchange through Ca_i-activated non selective cation channels).

M-Pos86

CHARACTERIZATION OF K^+ AND Cl^- CHANNELS FROM CANINE DIAPHRAM MUSCLE SARCOPLASMIC RETICULUM MEMBRANE. ((E. Rousseau, C. Michaud, S. Proteau and A. Decrouy)) Dept. of Physiology and biophysics, Univ. of Sherbrooke. Sherbrooke. Quebec. Canada.

SR vesicles were prepared from dog diaphragm muscles and fused into planar lipid bilayers to study the monovalent ionic channels controlling the counter charge movements during rapid Ca^{2+} release and uptake by the SR. The channel activities were recorded either in asymmetrical KCl , KGluconate or CsCl buffer systems. A 148 pS cation selective channel was consistently recorded in 50 mM/ 250 mM Kgluc (n=7). This channel displays a typical subconducting level at 50% and a ohmic behavior between -60 to 60 mV. Its K^+ selectivity was ascertained under various ionic conditions (Nernstrian behavior). The mean Po/voltage relationship is sigmoidal with a half maximum activation at 15 mV and a low Po at negative voltages.
In CsCl buffer, the activity of a large conducting (103-180 pS) anion selective channel was recorded. Its gating behavior is characterized by high Po values and a kinetics heterogeneity leading to various bursting patterns. The time analysis is complicated by the presence of several conduting levels. Most of the recordings result in multiple channel activities which agues in favor of a high density of this pathway in the SR membrane.
Both channel are functionally co-expressed in asymmetrical KCl solutions furthermore they are both insensitive to Ca changes (Pca=7 to 3).
Supported by a MRC grant. ER is a senior-scholar of the FRSQ.

M-Pos87

GLIAL EXPRESSION OF Kv1.5 CHANNELS AND THEIR ALTERATION BY ANTISENSE OLIGODEOXYNUCLEOTIDE TREATMENT. ((M.L. Roy, D. Saal, T. Perney, and L.K. Kaczmarek)) Depts. of Neurology and Pharmacology, Yale University School of Medicine, New Haven, CT 06520.

Delayed rectifying K^+ channels have been shown to underlie basic physiological properties of cells. To date, it has been difficult to directly associate a specific K^+ channel transcript with an expressed ionic current. We have evidence demonstrating the expression of Kv1.5 in adult rat hippocampus, cerebellum, hypothalamus, and cultured spinal cord astrocytes (CSPA) using a polyclonal antibody rasied against an intracellular portion of the protein. CSPA were treated with antisense oligodeoxynucleotides designed designed to block Kv1.5 mRNA translation into protein. These antisense-treated astrocytes differed from their nonsense-treated and control counterparts in that their whole cell current inactivation, sensitivity to TEA block, and delayed rectifier K^+ current density were altered, whereas cell capacitance, initial resting membrane potential, and 50% activation voltage were not affected. Based upon these results, we conclude i) that Kv1.5 channel protein is expressed in adult rat CNS glia as well as CSPA, ii) that antisense oligodeoxynucleotide treatment of cultured cells effectively alters K^+ channel expression, and iii) that Kv1.5 channel protein directly influences some basic biophysical and pharmacological characteristics of delayed rectifier K^+ current in cultured spinal cord astrocytes.

M-Pos88

ALTERED ION CHANNEL EXPRESSION DURING PMA-INDUCED DIFFERENTIATION OF THP-1 MONOCYTES. ((S.Y. Kim, T.E. DeCoursey, V.V. Cherny and M.R. Silver)) Dept. of Molecular Biophysics & Physiology and Dept. of Medicine, Rush-Presbyterian-St. Luke's Medical Center, Chicago, IL 60612.

THP-1 is a human monocytic leukemia cell line that can be induced to differentiate into macrophage-like cells by treatment with phorbol esters. We have examined the changes in ion channel expression resulting from differentiation of THP-1 cells by phorbol myristate acetate (PMA, 10 ng/ml, 3 days). Whole-cell and single-channel patch clamp techniques were used to identify four types of ion channels in undifferentiated THP-1 cells and three types of ion channels in PMA-treated cells. **Undifferentiated cells expressed** *1)* an outward inactivating K current which activated above -50 mV, inactivated with a time constant of ~330 msec, and was blocked by TEA and charybdotoxin, *2)* a nonselective cation current which activated at positive potentials, was time-independent, and was carried by K, Cs, Na but not TMA, *3)* a Ca-activated inwardly rectifying K current, observed in the presence of micromolar internal Ca, which was voltage-independent, inhibited by charybdotoxin and TEA, and blocked in a voltage-dependent manner by Ba and Cs, and *4)* a H^+ current which activated upon depolarization with a very slow time course, was inhibited by Zn and Cd, and whose H^+ selectivity was confirmed by shifts in reversal potential over a range of pH's. **PMA-treated cells expressed** *1)* an inwardly rectifying K current which activated below E_K with activation dependent on $[K]_o$, was blocked in a voltage-dependent manner by Ba and Cs, but was insensitive to charybdotoxin, *2)* a large conductance Ca-activated K current which activated at positive potentials, was inhibited by TEA, and had a single channel conductance of 200-300 pS, and *3)* a H^+ current which was similar to that in undifferentiated THP-1 cells but was about five-fold smaller in magnitude.

M-Pos89

TRUNCATED K^+ CHANNEL DNA SEQUENCES SPECIFICALLY SUPPRESS LYMPHOCYTE K^+ CHANNEL GENE EXPRESSION. ((L. Tu, V. Santarelli, and C. Deutsch)). Depts. of Physiology, University of Pennsylvania and Jefferson Medical College, Philadelphia, PA 19104-6085.

We have constructed a series of deletion mutants of Kv1.3, a Shaker-like voltage-gated K^+ channel, and examined the ability of these mutants to form channels and to specifically suppress full-length Kv1.3 currents. These constructs were expressed heterologously in both *Xenopus* oocytes and a mouse cytotoxic T cell line. Our results show that a truncated Kv1.3 must contain both the amino terminus and a transmembrane spanning segment in order to suppress full-length Kv1.3 currents. The S1-amino terminus construct is among the most effective in suppression, however, other transmembrane segments substituted for S1 can also suppress. Truncated DNA sequences suppress expression of K^+ channels only within the same subfamily. To test the ability of truncated Kv1.3 to suppress endogenous K^+ currents, we constructed a plasmid that contained both a truncated Kv1.3 and a selection marker gene (mouse CD4). Although constitutively expressed K^+ currents in Jurkat (a human T cell leukemia line) and in GH3 (an anterior pituitary cell line) cells cannot be suppressed by this double-gene plasmid, stimulated (up-regulated) endogenous Shaker-like K^+ currents in GH3 cells can be suppressed. (Supp. GM 41467).

M-Pos90

ETHANOL MODIFIES THE GATING OF LARGE CONDUCTANCE, Ca^{++}-ACTIVATED K^+ CHANNELS (mslo) EXPRESSED IN XENOPUS LAEVIS OOCYTES. ((Alejandro M. Dopico, Vellareddy Anantharam and Steven N. Treistman)) Department of Pharmacology, University of Massachusetts Medical School, Worcester, MA 01655. (Spon. by A.R. Rittenhouse)

We have previously found that the activity of Ca^{++}-activated K^+ (CAK) channels is increased by 25-100 mM ethanol (EtOH) in both PC12 cells and neurohypophysial terminals, although the mechanism(s) underlying this activation is not clear. To work with a homogenous population of CAK channels, we have studied the effects of EtOH on the activity (NPo) of mouse CAK channels (mslo; Butler et al., 1993) expressed in Xenopus laevis oocytes. EtOH (50 mM and up) reversibly increased NPo of mslo channels when applied to the internal surface of inside-out patches, without changing either the unitary conductance or the zero current potential (245 pS and 0 mV, respectively, in symmetric 145 mM K^+). The voltage-sensitivity of the channel (approximately 21 mV for an e-fold change in NPo) was also unaffected. We are currently determining whether the Ca^{++}-sensitivity of mslo channels is modified by EtOH. Both openings and closures distributions were best fitted by double exponential functions. The greatest effect of EtOH was to decrease both the duration and the contribution to the fit of long closures.
Supported by NIH grant AA08003.

M-Pos91

A SATURABLE GENERAL ANESTHETIC SITE IN A CLONED K^+ CHANNEL. L. Escobar, T.B. Vyas, C. Choe and M. Covarrubias. Dept. of Anatomy, Pathology and Cell Biology, Jefferson Medical College, Philadelphia, PA 19107.

To study the molecular basis of general anesthesia, we studied the interactions of *n*-alkanols (C2-C6) with a cloned K^+ channel expressed in *Xenopus* oocytes. This channel is encoded by *Drosophila Shaw2* and has been found to be selectively inhibited by clinical concentrations of ethanol (Covarrubias and Rubin. *Proc Nat. Acad. Sci. USA 90,* 6957-60, 1993). To investigate the presence of a saturable site for *n*-alkanols, we conducted equilibrium dose-inhibition experiments. Clear evidence of saturation was observed, and curves were well described assuming a simple binding isotherm with Hill coefficients ranging between 0.9 to 1.5. Long-chain *n*-alkanols were more potent inhibitors. Between ethanol and *n*-hexanol $K_{0.5}$ decreased over more than two orders of magnitude (180 mM and 1 mM). The change in binding free energy was found to be -703 cal/mol per methylene group. Thus, suggesting the presence of a hydrophobic pocket. We also conducted single channel studies to further understand this interaction. In control experiments, open time histograms were best described with the sum of two exponential components, suggesting the presence of two open states. In the presence of 44 mM *n*-butanol, opening frequency decreased by ~30% and the fraction of brief openings increased from 60 to 90% without affecting single channel conductance. These changes can explain inhibition of the macroscopic current by *n*-alkanols. We are currently conducting mutagenesis experiments to identify the site of action.

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M-Pos92

CHARACTERIZATION OF THE MUSCARINIC POTASSIUM CURRENT IN THE PRESENCE OF VOLATILE ANESTHETICS ((J. Magyar, Y. Li and G. Szabo)) University of Virginia, Charlottesville, VA 22908. (Spon. by M. K. Al-Shawi)

We studied the influence of halothane and isoflurane on G-protein coupled signal transduction using the muscarinic agonist activated K^+ current ($I_{K(ACh)}$) in bullfrog atrial myocytes as a rapid and specific indicator of G protein (G_K) function. Rapid applications of acetylcholine (ACh, 10^{-5} M) in the absence and presence of 0.5 to 5 vol % of anesthetics were used to examine differential anesthetic effects. Both halothane and isoflurane decreased the rate of muscarinic $I_{K(ACh)}$ activation. For halothane the decrease was from 18.5 ±1.7 s^{-1} to 11.9±0.7 s^{-1}; for isoflurane it was from 17.2±1.2 s^{-1} to 15.2±1.1 s^{-1}. Halothane and isoflurane also significantly decreased the peak value of $I_{K(ACh)}$, to 76%±4.5% and 65%±5% of control respectively. The steady-state component of $I_{K(ACh)}$ was increased by halothane to 116%±2.5% but remained unaltered by isoflurane. In agreement with this observation, halothane increased the receptor independent, GTPγS induced current while isoflurane had no effect. Anesthetic effects on the desensitization of $I_{K(ACh)}$ were evaluated by fitting the decay of $I_{K(ACh)}$ by the sum of two exponentials. Both anesthetics reduced the relaxation amplitudes (the fast amplitude decreased to 20%±5% and 40%±8% while the slow amplitude decreased to 40%±8% and 60%±10% by halothane and isoflurane respectively) without effecting time constants. Our data reveal similarities as well as differences in the action of these anesthetics on G protein coupled signal transduction and may account for some actions of these volatile anesthetics *in vivo*.
Supported by NIH Grant GM 47525

M-Pos93

ESTROGEN RELAXES CORONARY ARTERIES BY OPENING BKCa CHANNELS THROUGH cGMP-DEPENDENT PHOSPHORYLATION. ((D.J. Darkow, J. Falvo-Lang, T. Howard, K.A. Daugherty, and R.E. White)) Dept. of Physiology & Biophysics, Wright State Univ. Sch. Med., Dayton OH 45435.

Only 10% of women suffer significant cardiovascular dysfunction before menopause; however, postmenopausal women experience cardiovascular disease at the same rate as men of comparable age. Interestingly, estrogen replacement therapy reduces a woman's chance of suffering myocardial infarction by 50-70%. Estrogen increases cardiac blood flow by relaxing coronary artery smooth muscle (CSM) through an undefined mechanism. The present study has identified a novel molecular mechanism for estrogen-induced CSM relaxation. Contractile studies of intact porcine coronary arteries revealed that estrogen induces endothelium-independent relaxation of CSM that involves potassium efflux. Perforated patch recordings from metabolically intact CSM cells revealed that 5-10 μM 17β-estradiol more than doubles steady-state outward currents, which are comprised mainly of currents through BKCa channels. Furthermore, estrogen increases the open probability of single BKCa channels by two orders of magnitude. Blocking BKCa channels with 10 nM iberiotoxin contracts intact coronary artery preparations and inhibits estrogen-induced relaxation by ~90%. Cyclic GMP also relaxed intact arteries and stimulated BKCa currents in single CSM cells. Moreover, estrogen stimulation of BKCa channel gating was reversed or blocked by inhibitors of cyclic GMP-dependent protein kinase. Therefore, we propose that a significant portion of the cardiovascular benefit derived from estrogen reflects relaxation of coronary artery and other vascular smooth muscle. The vasodilatory response to estrogen involves stimulation of BKCa channel gating through cyclic GMP-dependent phosphorylation mechanisms. *(Supported by the American Heart Association, Ohio Affiliate, WSU Office of Geriatric Medicine, and WSU 662144)*

M-Pos94

PROTEIN PHOSPHORYLATION PREVENTS BK CHANNEL ACTIVATION BY PHYSIOLOGICAL Ca^{2+} AND VOLTAGE IN RAT PITUITARY TUMOR CELLS. ((Sarah K. Hall and David L. Armstrong)) Lab. Cellular & Molecular Pharmacology, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709.

We have investigated the effects of phosphorylation on the activity of native, large-conductance, fast gating, TEA- and charybdotoxin-sensitive (BK) potassium channels in inside-out membrane patches from GH_4C_1 cells. We used BAPTA and its derivatives (Tsien, R.Y. 1980 *Biochemistry* 19: 2396) to buffer Ca^{2+} at the intracellular surface and avoid artifacts associated with the slow, H^+ and Mg^{2+}-dependent buffering by EGTA.

	Stimulus required for $P_O \geq 0.5$		
BK channels	at 0 mV	at 0.1μM Ca^{2+}	at 1μM Ca^{2+}
dephosphorylated	1 μM Ca^{2+}	+50 mV	0 mV
phosphorylated	>10 μM Ca^{2+}	>80 mV	+80 mV

Activation of the cAMP-dependent protein kinase with 0.1 mM 8-chloro-phenyl-thio-cAMP reduced BK channel activity in cell-attached patches to $P_O < 0.01$ at 0 mV. When patches were excised subsequently into ATP-free solutions, channel activity recovered fully within ten minutes (*dephosphorylated*). This recovery was reversed by addition of 1 mM Mg-ATP and prevented by 1 nM okadaic acid, a specific inhibitor of the serine/threonine-directed protein phosphatase 2A. Under these conditions (*phosphorylated*), ten-fold higher concentrations of Ca^{2+} or 80 mV larger depolarizations were required to elicit half-maximal channel activity. Thus, BK_{Ca} channel activity can be regulated across the entire range of physiological $[Ca^{2+}]$ and voltage by endogenous enzymes closely associated with the channels in the patch.

M-Pos95

PHOSPHORYLATION STATE AND CALCIUM SENSITIVITY OF THE LIVER 'MAXI-K' CHANNEL. ((Ceredwyn E. Hill & Darlene C. Pon)) Dept. Physiology and GI Disease Research Unit, Queen's Univ., Kingston, ON, Canada K7L 3N6.

Large conductance Ca^{2+}-activated K^+ channel (K_{Ca}) activity is dependent, minimally, on the membrane potential, cytosolic $[Ca^{2+}]$, and cAMP-dependent kinase (PKA) phosphorylation. We have used modulators of protein phosphatase and PKA activities to alter the phosphorylation state of the embryonic chick hepatocyte K_{Ca}, and to study the relationship between the degree of phosphorylation and Ca^{2+}-sensitivity. Whole-cell dialysis with PKA reveals a K^+-selective current that is insensitive to $[Ca^{2+}]$ above.1 μM. In cell-attached patches dibutyryl-cAMP or okadaic acid does not shift the open probability x potential relation. When the patch is excised (inside-out) into 1 mM Ca^{2+} the P_OxV curve shifts negatively and, unlike in the absence of cAMP, remains there following washout of the Ca^{2+}. The results suggest that the hepatocyte Maxi-K channel may be controlled in a similar manner as other regulatory proteins of the liver (e.g., glycogen phosphorylase). The potential integration of the activities of K_{Ca}, PKA, phosphorylation-dependent protein phosphatases and cytosolic $[Ca^{2+}]$ is discussed. Supported by NSERC, Canada.

M-Pos96

MODULATION OF M-CURRENT AND M-CHANNELS BY PHOSPHATASE 2B (CALCINEURIN) IN VERTEBRATE SYMPATHETIC NEURONS ((N.V. Marrion)) Vollum Institute, O.H.S.U, Portland, OR 97201.

The M-channel can display both low and high open probability (Po) behavior, with the macroscopic current relaxations arising primarily from high Po activity (Marrion, 1993; Neuron, 11, 77). The putative switch between these behaviors and therefore, the magnitude of the macroscopic M-current is postulated to be influenced by the intracellular calcium concentration. Whole-cell dialysis of bullfrog sympathetic neurons with a preactivated, calcium-independent form of calcineurin (92 nM) reduced the M-current by 59 ± 10% (mean ± s.d, n=11). The effect of calcineurin was blocked by inclusion of the autoinhibitory peptide to calcineurin (75 μM). Dialysis with the peptide inhibitor alone resulted in a larger M-current with a dramatic slowing of M-current 'rundown' over the 20 minutes of dialysis. This data suggests that activation of endogenous calcineurin may underlie 'rundown' of the macroscopic M-current. In approximately 50% of excised patches, M-channel activity was maintained in the 'inside-out' patch configuration. Application of preactivated calcineurin to the inner membrane face of an 'inside-out' patch caused a reduction in patch Po (0.12, control; 0.01 in calcineurin (92 nM), -50 mV), an effect resulting from the selective loss of the long open time constant underlying high Po M-channel behavior. Therefore, calcium-dependent dephosphorylation of the M-channel by calcineurin can promote low Po M-channel activity. Increasing calcium levels bathing an 'inside-out' patch to 200 nM can also result in a loss of high Po activity. This suggests that, in some examples, endogenous calcineurin may remain associated with the patch after excision. Supported by NS29806.

M-Pos97

G PROTEIN-INDEPENDENT REGULATION OF THE ATRIAL MUSCARINIC K^+ CURRENT: MECHANISM OF DESENSITIZATION. ((S-G. Hong, A. Pleumsamran and D. Kim)) Department of Physiology and Biophysics, Chicago Medical School, North Chicago, IL 60064

Acetylcholine (ACh)-activated K^+ (K_{ACh}) current undergoes a rapid desensitization within several seconds. We further investigated the roles of membrane- and cytosol-associated processes in this phenomenon. Rapid desensitization of the K_{ACh} channel current was also observed in cell-attached patches with 10 μM ACh in the pipette. When inside-out patches were formed within ~1 s after formation of such cell-attached patches and GTP applied to the membrane, no desensitization was present. This indicated that certain cytosolic factor(s) mediated the desensitization. Applying atrial cytosol extract to such inside-out patches elicited a rapid desensitization of the K_{ACh} current. These effects of cytosol on the K_{ACh} channel could be reversed (i.e., resensitized) by applying ATP (4 mM) to the desensitized K_{ACh} channel in excised patches, and the cytosol antagonised this effect of ATP. Protein kinase and phosphatase inhibitors did not block the effect of ATP or cytosol. The effects of ATP and cytosol on the K_{ACh} channel could occur in the absence of GTP, indicating that the G protein was not involved. Treatment of the cytosol with proteases or heat-denaturation abolished the effect of the cytosol, indicating that the cytosolic factor was a protein. These results show that the K_{ACh} channel activity observed after exposure to ACh is the net sum of the effects produced by at least three processes: the G protein-mediated opening, the ATP-dependent stimulation, and the cytosolic protein-mediated inhibition of the K_{ACh} channel. The latter two processes appear to mediate the rapid desensitization of the K_{ACh} current via G protein-independent pathways.

M-Pos98

MUSCARINIC M_3 RECEPTOR MEDIATED SUPPRESSION OF K_{ATP} IN ESOPHAGEAL SMOOTH MUSCLE: INVOLVEMENT OF Ca^{2+}, PKC AND TYROSINE KINASE. ((N. Hatakeyama, Q. Wang, R.K. Goyal and H.I. Akbarali)) Program in Smooth Muscle Research, Beth Israel Hospital and Harvard Medical School, Boston, MA 02215

K_{ATP} channels play an important role in smooth muscle membrane hyperpolarization and relaxation. We examined the presence and cholinergic modulation of these channels in the rabbit esophageal muscularis mucosae (TMM). Lemakalim (LMK, 10 μM),an activator of K_{ATP}, increased whole cell currents by 174 ± 15 pA (n=36) in cells dialysed with 0.1 mM ATP and 130 mM K^+, and bathed in 60 mM $[K^+]_o$ (V_H -70 mV; E_K -20 mV). Activation of K_{ATP} was markedly decreased in 5mM [ATP], (-70 ± 11 pA (n=9)) and was abolished by glibenclamide. Carbachol (CCh, 10μM) suppressed LMK currents by 74 ± 4% (internal solution= 0.1 ATP, 10mM EGTA). With no EGTA in the pipette solution, LMK-induced currents were completely abolished by CCh. CCh-suppression was blocked by the M_3 antagonist p-fluro-hexahydrosiladifenidol (pHSD 0.1μM) but not by pirenzipine (M_1) or methoctramine (M_2). K_{ATP} was also suppressed by phorbol ester (PMA) by 63 ± 9% (n=6). The effects of both CCh and PMA were significantly attenuated by protein kinase C inhibitors, calphostin C (1 μM) and H7 (1 μM). The tyrosine kinase inhibitors, tyrphostin (10 μM) and genestein (10 μM) also reduced the CCh suppression to 39 ± 11% and 44 ± 7%, respectively. Supported by NIH DK 46367 and DK 31902

M-Pos99

THE SLOWLY ACTIVATING DELAYED RECTIFIER K^+ CURRENT : A STRETCH SENSITIVE CARDIAC ION CHANNEL. ((W.J. Groh, J.G. Maylie)) Oregon Health Sciences University, Portland, OR 97201.

Myocardial stretch shortens cardiac action potential duration (APD). The modulation of ion channels leading to APD shortening in response to stretch is not well defined. We studied the effect of hypotonic-induced stretch on the slow component (I_{Ks}) of the delayed rectifier K^+ current (I_K) in guinea pig ventricular myocytes using a whole-cell patch clamp technique. Dissociated myocytes were perfused with normal Tyrode soln [300 mOsmol/l] at 23°C and dialyzed with a pipette soln containing (mM):K-Asp 90,KCl 40,NaCl 10,$MgCl_2$ 1,MgATP 5,HEPES 10,EGTA 10,$CaCl_2$ 1, pH 7.1 [310 mOsmol/l]. Addition of 10 μM E-4031 blocked the rapid component (I_{Kr}) of I_K. Perfusion with hypotonic (hT) soln [Tyrode less 35 mM NaCl, 230 mOsmol/l] plus 10 μM E-4031 induced cell swelling with width increasing by 38.7±2.7% (mean±SEM) and length by 6.0±0.7, n=36, P<.001. Nonpatched myocytes showed no change in cell size. APD at 90% repolarization shortened by 38.7±2.7%, n=19, P<.001. I_{Ks} measured during a 5 s depolarization to 60 mV increased by 31.4±8.2% from 8.2±1.1 pA/pF to 10.4±2.5 pA/pF, n=10, P<.001. Addition of 10 μM azimilide (NE-10064), a potent blocker of I_{Ks}, inhibited the time-dependent outward current by 82.0±5.3%, n=10, P=.002. Presoaking myocytes or inclusion in the pipette of the inhibitors of protein kinase A, H-89 or protein kinase C, chelerythrine had no effect on the hT-induced increase in I_{Ks}.
These results show that I_{Ks} is upregulated by myocyte stretch providing one mechanism for APD shortening.

M-Pos100

REGULATION OF 5-HT RECEPTOR-COUPLED K^+ CHANNEL BY ATP and Gβγ IN RAT HIPPOCAMPAL NEURONS. ((U.T. Oh, Y-K. Ho and D. Kim)) Dept. of Physiology and Biophysics., Chicago Med. Sch, N. Chicago, IL 60064 and Dept. of Biochemistry, U. of Illinois, Chicago, IL 60612

The inwardly rectifying K^+ channels activated by 5-HT (K_{5HT} channels) in hippocampal neurons and by ACh (K_{ACh} channels) in atrial cells have identical primary amino acid sequences. It is not known whether the mechanisms of regulation of channel function by second messenger molecules are also identical. The atrial K_{ACh} channel was shown to be activated by $G_{\beta\gamma}$ rather than Gα. When activated by ACh (pipette) and GTP (bath), the K_{ACh} channel activity in inside-out patches can be augmented irreversibly by applying ATP, but not AMPPNP, to inside-out patches. We examined whether such effects were also present for the K_{5HT} channels. Inside-out patches were formed with 10 μM 5-HT in the pipette using hippocampal neurons isolated from adult rat brain. Perfusion of the cytoplasmic side of the membrane with 100 μM GTP caused activation of the K^+ channels with a single channel conductance of ~36 pS (140 mM KCl) and a mean open time of ~0.6 ms. GDPβS (100 μM) inhibited channel opening. When 1 mM ATP and GTP were applied together, the channel activity increased ~4 fold, and the mean open time increased to ~2 ms. These effects of ATP were irreversible and AMPPMP failed to produce the same changes, similar to that observed with the atrial K_{ACh} channel. Application of transducin $\beta\gamma$ ($T_{\beta\gamma}$;50 μg/ml) to inside-out patches activated the K_{5HT} channels and this was reversed by adding $T_{\alpha.GDP}$. These results show that the regulatory properties of the K_{5HT} channels in hippocampal neurons are similar to those of the atrial K_{ACh} channels.

M-Pos101

NEUROPEPTIDE Y, PHENYLEPHRINE, SEROTONIN, AND HISTAMINE INHIBIT ATP-SENSITIVE K^+ CHANNEL CURRENTS BY PROTEIN KINASE C IN SMOOTH MUSCLE CELLS FROM RABBIT MESENTERIC ARTERY.

((Adrian D. Bonev and Mark T. Nelson)) University of Vermont, Dept. of Pharmacology, CMRF, 55A South Park Drive, Colchester VT 05446-2500.

ATP-sensitive K^+ (K_{ATP}) channels are involved in the regulation of vascular tone and appear to be targets of endogenous vasodilators and vasoconstrictors. We explored the possibility that the stimulation of neuropeptide Y (NPY), α-adrenergic, serotonin and histamine receptors can inhibit K_{ATP} channels in vascular smooth muscle. To examine the inhibition of K_{ATP} channels in smooth muscle cells from rabbit mesenteric artery, whole cell currents through K_{ATP} channels were elevated by lowering intracellular ATP to 0.1 mM and by the synthetic K_{ATP} channel opener pinacidil. Pinacidil (5 μM) increased K^+ currents by 135.2 ± 8.4 pA (n = 63) at -70 mV and with 60 mM external K^+. Glibenclamide (10 μM) inhibited the entire pinacidil-activated current as well as 11.7 ± 1.5 pA of steady-state K^+ current. Neuropeptide Y (NPY) (30 nM), phenylephrine (1 μM), serotonin (1 μM) and histamine (1 μM) inhibited the whole-cell glibenclamide sensitive currents in the presence of pinacidil by 41.6 ± 1.9 % (n=6), 48.3 ± 3.9 % (n=8), 39.4 ± 1.9 % (n=10) and 54.6. ± 3.7 % (n=5), respectively. These vasoconstrictors were without effect on the membrane currents when K_{ATP} channels were blocked by glibenclamide. Antagonist of α_1-adrenoceptors prazosin (10 μM) and antagonist of H_1 histamine receptors triprolidine (1 μM) blocked the effect of phenylephrine and histamine, respectively. The blocker of protein kinase C (PKC), GF-109203X (300 nM) greatly reduced the inhibition of K_{ATP} currents by NPY , phenylephrine, serotonin and histamine. The activator of PKC, phorbol 12-myristate 13-acetate (PMA) (100 nM) inhibited K_{ATP} currents by 84.7 ± 4.9 % (n=3). The inactive phorbol ester, 4α-phorbol 12,13-didecanoate (4α-PDD) (100 nM) had little effect. We propose that vasoconstrictors may depolarize arterial smooth muscle in part through inhibition of K_{ATP} channels and that inhibition involves activation of PKC.
Supported by the NIH and NSF.

M-Pos102

THYROID HORMONE ACUTELY ENHANCES INWARD RECTIFIER POTASSIUM CURRENTS THROUGH EXTRANUCLEAR MECHANISMS IN GUINEA-PIG VENTRICULAR MYOCYTES. ((Yoshihide Sakaguchi and Luyi Sen)) UCLA, School of Medicine & VAMC/LA, Los Angeles, CA 90073.

Cardiac myocytes repolarization is shortened in hyperthyroidism, and lengthened in hypothyroidism. However, the mechanism for shortened repolarization by thyroid hormones has not been well defined. To determine the T_3 effect on the inward rectifier potassium current (I_{K1}), the single cell action potential and whole-cell I_{K1} were recorded in guinea-pig ventricular myocytes before and after T_3 superfusion using patch-clamp technique. The action potential duration at 90% repolarization (APD_{90}) was shortened from 647±13 to 391±25 msec at 24°C (n=8, p<0.0001) and from 224±6 to 159±7 msec at 36°C (n=5, p<0.01) by 10^{-6} M T_3. In the same patch, T_3 (10^{-6} M) caused a significant increase in steady-state I_{K1} at -40 mV (33±6% at 24°C, 31±11% at 36°C). A low dose of T_3 (10^{-8} M) shortened APD_{90} from 687±23 to 574±9 msec (24°C, n=5, p<0.05) and slightly increased steady-state I_{K1} at -40 mV (+7±3 % at 24°C, n=5). These effects were initiated at 5-15 min and reached a stable plateau at 25 min. The effect of T_3 on I_{K1} was dose-dependent and temperature-independent. Triiodothyroacetic acid (TAA, 10^{-6} M), an analogue of T_3 that does not stimulate DNA transcription in the nucleus, caused a similar increase in steady-state I_{K1} at -40 mV (34±8% at 24°C, n=7), suggesting that the effects of T_3 on I_{K1} are independent of the cell's nucleus. Reverse-T_3 (rT_3, 10^{-6} M), which has been reported to be inactive and partially compete with T_3 and T_4 on the membrane receptors, did not increase I_{K1}. In contrast, in cells pre-incubated with rT_3 (10^{-6} M) 30 min, the effect of T_3(10^{-6} M) on I_{K1} was blocked by 48% (24°C, n=8). These results suggest that a) T_3 enhances I_{K1} through extra nuclear mechanism; b) the enhanced I_{K1} by T_3 might be one of the causes for shortened APD in hyperthyroidism.

M-Pos103

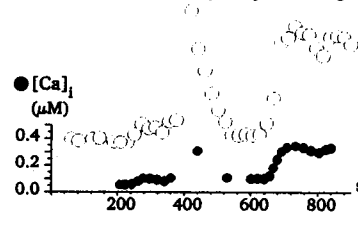
THE DELAYED POTASSIUM CURRENT IN RABBIT VENTRICULAR MYOCYTES IS SENSITIVE TO EXTERNAL BUT NOT TO INTERNAL pH. ((E. Carmeliet))Laboratory of Physiology, University of Leuven, Belgium.

In order to evaluate the role played by the delayed rectifier current in ischemic conditions a study was made of the effect of changes in intracellular and extracellular pH. Whole cell experiments were performed on ventricular myocytes of the rabbit heart. Internal pH was changed in steps between 6.2,7.2 and 8.2, external pH between 6.5, 7.4 and 8.5 by adding appropriate amounts of KOH or NaOH to the solution containing 10 mM Hepes. The results can be summarized as follows. Tail current amplitude or time course were not modified by changes in intracellular pH. External acidification shifted the activation curve to positive potentials, reduced the amplitude of the fully-activated current-voltage relation and caused a shift to positive potentials, shortened the time constant of the tail currents over the whole range of potentials. Prolongation of tail currents was seen in alkaline conditions. It is concluded that the delayed rectifier K current is selectively sensitive to external pH. The charge carried by the current is markedly reduced in acidic conditions.

M-Pos104

POTASSIUM CURRENT IN CILIARY BODY EPITHELIAL CELLS EVOKED BY PHOTOLYSIS OF CAGED IP_3 AND DM-NITROPHEN. ((L.M. Botchkin and G. Matthews)) Dept. of Neurobiology, SUNY, Stony Brook, NY 11794-5230.

Freshly isolated single nonpigmented epithelial cells from rabbit ciliary body have been shown to possess a Ca-activated potassium conductance (Botchkin & Matthews, ARVO Abstract, 1994). Combined whole-cell patch clamp recordings and fura-2 measurements of $[Ca]_i$ show an increase in outward membrane current accompanying increased $[Ca]_i$ elicited by application of ionomycin or the cholinergic agonist carbachol. To further characterize the calcium-dependence of this current, we used photolysis of caged IP_3 to release calcium from internal stores or DM-nitrophen to directly photorelease calcium. Caged compounds were loaded into cells, together with fura-2 to monitor $[Ca]_i$, by dialysis via a whole-cell patch pipette. Photolysis was achieved with the same 340 and 380 nm excitation lights used for fura-2 measurements. Photolysis of IP_3 or DM-nitrophen led to an increase in $[Ca]_i$, accompanied by an increase in outward current (see Figure, which shows response to 4 μM caged IP_3). The magnitude of the current closely tracked the changes in $[Ca]_i$. The effect of photoreleased IP_3 was strongly attenuated when the concentration of EGTA in the pipette solution was increased from 1 mM to 2.5 mM.



(Supported by NIH grant EY08673)

M-Pos105

MODULATION OF A CLONED INWARD RECTIFIER K^+ CHANNEL (HRK1) BY PROTEIN KINASE C. ((P. Henry*, W. Pearson, R. Melton, E.N. Makhina and C.G. Nichols)). Dept. Cell Biol. and Physiol. Washington U. Sch. Med., 660 So. Euclid, St. Louis, MO 63110 and Lab. de Physiol. Cellulaire, U. Paris XI, Orsay, France*

In order to look for modulation of inward rectifier K^+ channels by phosphorylation, we tested the effects of protein kinases A (PKA) and C (PKC) on 3 different cloned inwardly rectifying K channels (ROMK1, HRK1, IRK1) expressed in *Xenopus* oocytes. Stimulation of PKA with a cocktail of forskolin (5 μM), IBMX (500 μM) and cpt-cAMP (50 μM) caused only a small (< 10 %) and rapidly reversible inhibition of the current through each cloned channel. Stimulation of PKC using phorbol esters (PMA 1 μM) had no significant effect on current through ROMK1, or IRK1 channels, but induced a marked (67 ± 2 %), essentially irreversible, reduction of HRK1 channel current. The kinetics and voltage-dependence of HRK1 current were unaffected by PMA. In the presence of staurosporine (10 μM), PMA inhibition of HRK1 current was essentially abolished (<5 % inhibition). PMA did not inhibit current through a chimeric channel between HRK1 (N-terminal through M2) and ROMK1 (C-terminal). These results show that HRK1 channels are sensitive to inhibition by PKC, in contrast to another strong inward rectifier IRK1, and the mild inward rectifier ROMK1. The sensitivity of HRK1 to phosphorylation by PKC seems to involve residues in the C-terminal region.

M-Pos106

INHIBITORS OF TYROSINE KINASE ACTIVITY CAUSE SPIKE BROADENING AND SUPPRESSION OF VOLTAGE-GATED K^+ CURRENTS. ((R.J. Knox and L.K. Kaczmarek)) Dept. of Pharmacology, Yale University, New Haven, CT 06520. (Spon. by L.K. Kaczmarek)

Aplysia bag cell neurons express an insulin receptor tyrosine kinase, which, upon binding of insulin, undergoes tyrosine specific autophosphorylation. We have previously shown that insulin produces an increase in the height of bag cell neuron action potentials by increasing voltage-gated Ca^{2+} currents (Jonas *et al.*, 1994). We have now examined the effects of specific tyrosine kinase inhibitors on action potentials and membrane currents in isolated bag cell neurons.

Action potentials were stimulated by injecting depolarizing current pulses before and after perfusion with a range of concentrations (100 nM-200 μM) of genistein (GEN), a membrane permeant tyrosine kinase inhibitor (Akiyama *et al.*, 1987; 292: 5592-5595, JBC). GEN produced a rapid, dose-dependent, reversible increase in the duration of action potentials. Similar effects were observed with other tyrosine kinase inhibitors, tyrphostin (0.1-100 μM) and herbimycin A (10-1000 nM). Daidzein, an inactive analog of GEN, did not effect the shape of action potentials. Voltage-clamp experiments in isolated neurons revealed that GEN (100 nM- 200 μM) (but not daidzein) produced a rapid, dose-dependent, reversible inhibition of two components of K^+ current. Experiments are in progress to determine whether the effects of the inhibitors are mediated by inhibition of tyrosine kinase activity.

M-Pos107

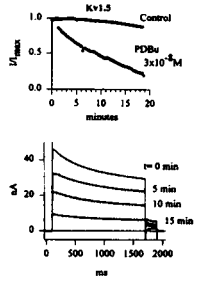
REGULATION OF FAST INACTIVATION IN A CLONED MAMMALIAN I_K(A) CHANNEL BY PROTEIN KINASE C. ((H. Ehmke[1], M. Koenen[1], B. Fakler[2], and J.P. Ruppersberg[2])). [1]Max-Planck-Institut für Medizinische Forschung, 69120 Heidelberg, Germany; [2]Sektion für Sensorische Biophysik der HNO-Klinik, Universität Tübingen, 72076 Tübingen, Germany (Spon. by L. Wollmuth).

Rapidly inactivating (A-type) K^+ channels are important for the integration of synaptic inputs and the determination of firing frequencies. Recent studies have demonstrated that a major principle of rapid inactivation is a reversible block of the inner mouth of the pore by a peptide domain located in the amino-terminal region of the α- or ß-subunits (Jan and Jan, Nature 371:119-122, 1994; Rettig et al., Nature 369:289-296, 1994). Here we report that acceleration of C-type inactivation by protein kinase C may be an independent pathway to confer fast inactivation behaviour. Mouse Kv1.4 channels were expressed in *Xenopus* oocytes and macroscopic currents were obtained in cell-attached and inside-out patches. In the cell-attached configuration, wild-type channels inactivated rapidly, but lost fast inactivation in the excised patch after exposure to an oxidizing solution. Cytoplasmic application of two structurally different, synthetic stimulators of protein kinase C [1-Oleolyl-2-Acetyl-Glycerol (OAG) or SC-10] restored fast inactivation. Similar results were obtained in a mutant channel that lacked the amino-terminal inactivation gate. Fast inactivation induced by stimulation of endogenous protein kinase C was not slowed when the pore blocker TEA was applied to the cytoplasmic side of the excised patch. These results suggest that protein kinase C may alter synaptic transmission by accelerating C-type inactivation of potassium channels.

M-Pos108

REGULATION OF CLONED SMOOTH MUSCLE K^+ CHANNELS $cK_V1.2$ AND $cK_V1.5$ BY PROTEIN KINASE C . ((Fivos Vogalis, Manus W. Ward and Burton Horowitz)) University of Nevada, Department of Physiology (352), Reno, NV 89557.

Acetylcholine (ACh) controls smooth muscle tone and rhythmicity by mechanisms not fully elucidated. In canine colonic smooth muscle, ACh increases the amplitude and duration of the electrical slow waves, consistent with the inhibition of the delayed rectifier current in these myocytes. The consensus amino acid sequence for protein kinase C (PKC) regulation is present on most cloned voltage gated K^+ channels including the $K_V1.2$ and $K_V1.5$ classes, previously cloned from this muscle. Phorbol 12, 13-dibutyrate (PDBu, $3x10^{-9}$ - 10^{-7} M) decreased K^+-channel current (I_{K_V}) in oocytes injected with cRNA encoding $cK_V1.2$, $cK_V1.5$ (see Figure) and those coinjected with both clones. Overnight pretreatment of expressing oocytes with the specific PKC inhibitor staurosporine (500 nM) decreased the PDBu-mediated inhibition of I_{K_V} from 90% to 60 %. Calphostin C (10-7M), however, another putative PKC-inhibitor, alone inhibited I_{K_V} and failed to antagonise the PDBu-mediated suppression of I_{K_V}. The inactive phorbol ester, 4-α phorbol 12,13-didecanoate (10^{-8}M) caused a 20% reduction in I_{K_V} suggesting that the action of PDBu is specific and mediated by activation of PKC. In colonic smooth muscle, ACh stimulates phoshoinositide hydrolysis to generate IP_3 and DAG. Our present data suggest that the excitatory action of ACh in gastrointestinal smooth muscle is partly mediated by suppression of delayed rectifier K^+ channels by PKC-mediated phosphorylation. (Supported by NIDDK)



M-Pos109

NON-INDEPENDENT PHOSPHORYLATION SITES IN THE INACTIVATION GATE OF A CLONED K^+ CHANNEL. ((T.B. Vyas[1], A. Wei[3], S. J. Slater[1], C.D. Stubbs[1], X.-L. Gong[2], R.G. Sorensen[2] and M. Covarrubias[1])) 1) Department of Anatomy, Pathology and Cell Biology, and 2) Department of Medicine, Jefferson Medical College, Philadelphia, PA 19107; and 3) Department of Anatomy and Neurobiology, Washington University School of Medicine, St. Louis, MO 63110.

We have shown that protein kinase C (PKC) specifically eliminates N-type inactivation of a cloned human K^+ channel encoded by *hKv3.4* (Covarrubias et al. *Neuron 11*, in press). In fact, a peptide corresponding to the inactivation gate of hKv3.4 is phosphorylated by PKC in vitro. Four putative phosphate acceptors were identified in this region: S8, S9, S15 and S21. To further study the mechanism of PKC action we studied the following point mutations: S8A/S9A, S15A, S21A, S15A/S21A. We found that all mutations significantly inhibited the effect of PKC: S8A/S9A, 90%; S15A, 60%; S21A, 50%; and S15A/S21A, 70%. Thus, PKC phosphorylation of serine residues in the inactivation gate of hKv3.4 is associated with inhibition of N-type inactivation. However, the effect of the mutations is clearly not additive. This suggests that PKC sites in the inactivation gate of hKv3.4 are not equivalent or independent. For instance, phosphorylation of a particular phosphate acceptor may affect recognition of neighboring PKC sites.

Supported by NIH grant NS32337 (M.C.), and in part by NIH training grant AA07463 (T.B.V.).

M-Pos110

PHOSPHORYLATION BY cAMP-DEPENDENT PROTEIN KINASE ENHANCES 4-AMINOPYRIDINE-SENSITIVE DELAYED RECTIFIER K^+ CURRENT IN RABBIT PORTAL VEIN SMOOTH MUSCLE CELLS. ((E.A. Aiello, M.P. Walsh and W.C. Cole)) Smooth Muscle Research Group, University of Calgary, Calgary, Alberta, Canada.

The effect of modulating cAMP-dependent protein kinase (PKA) activity on 4-aminopyridine (4AP)-sensitive delayed rectifier current (I_{4K}) in isolated rabbit portal vein smooth muscle cells was studied using whole-cell voltage clamp. Run-up of 4AP-sensitive I_{4K} after gaining access was assessed in myocytes dialysed with 5 mM EGTA and 5 mM ATP (20-22 °C). A three-fold increase in peak time-dependent and tail current was observed which was completely suppressed by 4AP (5 mM). Run-up was reduced by 50 and 70 % in myocytes dialysed with the non-hydrolysable ATP analogue, AMP-PNP (5 mM), or PKI (10 μM), the specific peptide inhibitor of PKA, respectively. Delayed dialysis with PKI reversed run-up and inhibited I_{4K} to below the level recorded upon gaining access. Activation of adenylate cyclase with forskolin (1 μM) caused a reversible 24% increase in I_{4K} at 0 mV which was completely inhibited by 4AP (5 mM). Forskolin also induced a negative shift (6.6±1.5 mV) in the reversal potential of the net whole-cell current-voltage relation consistent with a hyperpolarization of resting membrane potential. Isoproterenol (1 μM) reversibly enhanced I_{4K} by 21% at +10 mV. The increase due to isoproterenol was sensitive to propranolol (2 μM) and 4AP (5 mM), and prevented by dialysis with PKI (10 μM). Analysis of activation and deactivation kinetics before and after isoproterenol revealed a decrease in activation time constant (from 19.4±1.6 to 16.4±1.4 ms) but no change in deactivation kinetics. Isoproterenol also prolonged the slow inactivation of I_{4K} at positive potentials. The decay in current was best fitted with a bi-exponential function and only the fast time constant was affected by isoproterenol, doubling in magnitude from 0.20±0.03 to 0.38±0.3 s at +20mV. The data suggest that I_{4K} is regulated by intracellular ATP via a phosphotransferase reaction mediated by PKA during isoproterenol activation of ß-adrenoceptors coupled to adenylate cyclase. Supported by MRC (DG-N025) to WCC and HSFA to MPW.

M-Pos111

MODULATION OF THE VOLTAGE-GATED K^+ CURRENT BY ARACHIDONIC ACID IN RAT PULMONARY ARTERIAL MYOCYTES. ((S.V. Smirnov and P.I. Aaronson)) UMDS, St Thomas's Hospital, London SE1 7EH (Spon. by S.J. Smith)

The voltage-gated delayed rectifier K^+ current (I_K) was studied in single smooth muscle cells isolated from rat small pulmonary artery using whole cell patch clamp technique. The cell membrane was stepped for 300 ms to +60 mV at 0.1 Hz to elicit I_K. Bath application of 10 or 50 μM arachidonic acid (AA) caused a dual effect on I_K. Firstly, the activation of I_K was substantially accelerated and the initial amplitude of the current measured increased. Secondly, the rate of decay of I_K increased very markedly. The amplitude of I_K measured at 300 ms decreased by 64±3%, n=8, (mean ± S.D.) and 78±6%, n=7 for 10 and 50μM, respectively. Linoleic acid (50 μM) had similar but smaller effects, while myristic acid (50 μM) caused little inhibition of I_K. Bath perfusion with 20 μM indomethacin (an inhibitor of cyclooxygenase), or 20 μM nordihydroguaiaretic acid (an inhibitor of lipoxygenases) did not prevent the effects of AA. Conversely, the effect of AA was significantly suppressed (46±2%, n=8, $p<0.03$, and 41±7%, n=4, $p<0.003$, for 10 and 50 μM AA, respectively) in the presence of 10 μM ethoxyresorufin (an inhibitor of monooxygenases), suggesting a possible involvement of cytochrome P450-mediated metabolism in the inhibition of I_K. A direct effect of AA on channel gating may also occur.

SVS supported by the British Heart Foundation.

M-Pos112

PHASIC STIMULATION AND USE DEPENDENT INHIBITION: EXPLICIT SOLUTION FOR A 3-STATE ION CHANNEL SYSTEM. **((V. Uteshev and P. Pennefather)) Fac. Pharmacy, U. Toronto, Toronto ON., M5S 2S2, Canada.**

We have derived generalized, recurrent and explicit formula describing the interaction between phasic activation and use-dependent inhibition within the framework of a cyclic three-state kinetic model [closed (C), open (O) and inactivated (I)]. The formula make use of matrix solutions that can be dealt with by symbolic calculator software such as *Mathamatica* and allow prediction of the degree of use dependent inhibition at any point during a train of repeated stimuli. Each state is defined by two functions of time (y or z) that define the fraction of channels in that state during the stimulation and recovery phases, respectively. For a train of repeated stimuli we defined vector $\vec{Z}_{2n}$ that has coordinates z^O_{2n} and z^I_{2n} representing the values for O and I states at the end of the n-th recovery phase. We then defined a recurrent relationship, $\vec{Z}_{2n} = F\vec{Z}_{2n-2} + \vec{G}$. Therefore, $\lim_{n\to\infty} \vec{Z}_{2n} = \lim_{n\to\infty} \vec{Z}_{2n\pm2} = \vec{Z}_{ss}$, where $\vec{Z}_{ss} = (E-F)^{-1}\vec{G}$, $F = \begin{pmatrix} c_{11}c_{12} \\ c_{21}c_{22} \end{pmatrix}$, $\vec{G} = \begin{pmatrix} c_{13} \\ c_{23} \end{pmatrix}$ and E - (2x2) identity matrix. Matrix and vector elements, c_{ij}, can be defined in terms of duration of the repeated stimulation and recovery phases and the two sets of 6 rate constants that describe the three state model during those two phases. We will show that for all steady state solutions the matrix F is such that $\lim_{n\to\infty} \prod^n F = \prod^\infty F = 0$. By factoring out one coordinate of the vector $\vec{Z}_{2n}$, a recurrent expression for the other can be obtained. For the state (I) the solution is $z^I_{2n+4} = \xi_1 z^I_{2n+2} + \xi_2 z^I_{2n} + \xi_3$, where $\xi_1 = c_{11} + c_{22}$, $\xi_2 = c_{12}c_{21} - c_{11}c_{22}$ and $\xi_3 = c_{13}c_{21} - c_{23}c_{11} + c_{23}$. Use of this approach for investigating modulation of $GABA_a$ receptor desensitization by the anesthetic propofol will be presented.

M-Pos113

PERIODIC FORCING OF A K^+ ION CHANNEL AT SEVERAL STIMULATION FREQUENCIES

((M. C. Menconi°, M. Pellegrini°, M. Pellegrino°, D. Petracchi*)) (spons. by F. Lenci)

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In a recent paper [1] we forced K^+ channels by varying the holding potential in a periodic way at frequencies at which the system cannot reach the stationary condition associated with the time dependent holding potential. The results show that periodic forcing is an useful tool which allows to give a quite direct evaluation of the rate constants in a non steady situation and that in the studied channel the closed-open rate constant leads in phase respect to the applied potential. Here a bistable model, partially described by differential equations, is used to mimic the channel open-closed transitions. The same technique of analysis used on experimental data is applied in analysing the model behaviour searching, both qualitatively and by computer simulations, for the expected phase shift. It is shown that only a phase lag can be expected in such models, and only at frequencies at which the internal dynamics becomes relevant. On the experimental side the dependence of the measured rate constants on the stimulation frequency is studied and in particular the variations of their phase respect to the periodic potential are measured.

[1]D. Petracchi, M. Pellegrini, M. Pellegrino, M. Barbi, F. Moss - Periodic forcing of a K+ channel at several temperatures. Biophys. J.,66, 1844-1852, 1994

M-Pos114

ELECTRIC FIELDS IN A TAPERED PORE WITH WATER AND CHARGES: CALCULATION VIA MONTE CARLO SIMULATION ((Jianjun Lu and Michael E. Green)) Dept. of Chemistry, City College of CUNY, New York NY 10031

The electric fields and potentials which are present in a tapered pore which is a model for an ion channel have been calculated in the course of doing a Monte Carlo simulation of the behavior of the water. The pore contains water, with or without an ion. The electric fields show maxima which can be attributed to the presence of charges in the boundaries of the pore. Simulations were also carried out with an ion in the pore. Fields and potentials were calculated separately for the contributions of the water, and for the total field and potentials. In the model, the walls of the pore are assigned a dielectric constant of 4, comparable to that of a protein, and the upper boundary is given a dielectric constant of 80. The dielectric in the pore is determined by the presence of the water molecules, which are explicitly represented. The fields are large compared with those produced by the membrane potential, and are comparable to those in electrical double layers. Results will be shown for pores with charge configurations which produce increased density of water in the pore, and for configurations which produce reduced density.

M-Pos115

MODULATION OF OOCYTE-EXPRESSED SKELETAL MUSCLE SODIUM CHANNELS BY PROTEIN KINASE A AND PROTEIN KINASE C. ((J. Paul Mounsey, J. Edward John, J. Randall Moorman)) University of Virginia, Charlottesville, VA 22908

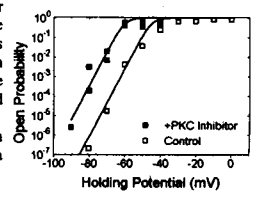
Phosphorylation of Na channels by cyclic AMP-dependent protein kinase (PKA) and protein kinase C (PKC) reduces Na current amplitude (I_{Na}). Unlike brain Na channels, which have multiple phosphorylation sites, skeletal muscle Na channels have only a few potential sites of which the most likely is in the highly conserved cytoplasmic linker between homologous domains III and IV (S1321). To test the idea that this site is a substrate for phosphorylation, we measured I_{Na} through *Xenopus* oocyte-expressed skeletal muscle Na channel α-subunits alone, and in the presence of mRNA encoding either the catalytic subunit of PKA or a constituitively active PKC. Coexpression of PKA reduced I_{Na} by an average of 53% ($p<0.001$; 8-15 frogs, 65-139 oocytes) whilst coexpression of PKC reduced I_{Na} by 60% ($p<0.001$; 6-15 frogs, 40-139 oocytes). If the protein kinase effect was the result of phosphorylation at S1321, disabling this site by Ser to Ala mutation (S1321A) should abolish the effect. However, although S1321A induced normal Na currents when expressed in oocytes, I_{Na} amplitude was reduced following coexpression of PKA or PKC by the same amount as the reduction of currents through wild type channels (average reduction 56% for PKA ($p<0.001$; 3-9 frogs, 26-85 oocytes) and 51% for PKC ($p<0.0001$; 4-9 frogs, 28-85 oocytes) (p=NS for the comparison between wild type and S1321A effects). Thus, although skeletal muscle I_{Na} is modulated by protein kinases, the effect does not appear to be mediated solely by phosphorylation at site S1321 in the III-IV linker.

M-Pos116

MODULATION OF WILDTYPE AND NON-INACTIVATING SKELETAL MUSCLE SODIUM CHANNELS EXPRESSED IN XENOPUS OOCYTES BY PROTEIN KINASE C. ((B. Hirschberg, M. Lieberman, A. Rovner, and J. Patlak)) Dept. of Molecular Physiology, Univ. of Vermont, Burlington, VT 05405.

Voltage-dependent sodium channels have been shown to be phosphorylated *in vitro* and *in situ* by protein kinase C and cAMP-dependent protein kinase in a number of preparations. However, the physiological role, and thus the possible implications for regulation of sodium channel activity are unclear. One topic of debate is whether the reduction in peak sodium current seen with protein kinase C and cAMP-dependent protein kinase is due to a shift in the channel's voltage-dependence. Two processes contribute to the current-voltage relationship: activation and inactivation. An effect of protein kinase C on inactivation might be expected, since a conserved protein kinase C consensus site exists on the inactivation gate. Modulation was examined in the sodium channel mutant IFM1303QQQ expressed in *Xenopus* oocytes. This mutant lacks fast inactivation allowing in detail analysis of effects on activation.

The protein kinase C inhibitor, Chelerythrine chloride, left-shifted the activation curve by 10-20mV. This finding suggested that a protein kinase C phosphorylation site exists, that it affects the voltage-dependence of activation and that it was endogenously phosphorylated in our preparation. Further phosphorylation of the channel either by the protein kinase C activator, ß-phorbol 12-myristate 13-acetate, or through inhibiting endogenous phosphatases by okadaic acid, lead to a reduction in peak sodium current without further affecting the current-voltage relationship. This reduction seemed to be due to an altered availability of channels to open.



In this preparation neither the cAMP-dependent protein kinase activator, forskolin, nor the inhibitor H-89 had a significant effect on channel behavior.

M-Pos117

MODULATION OF BRAIN Na^+ CHANNELS BY A G PROTEIN-COUPLED PATHWAY. ((J.Y. Ma, M. Li, W.A. Catterall and T. Scheuer)) Department of Pharmacology, SJ-30, U. of Washington, Seattle, WA 98195.

Na^+ channels in rat hippocampal neurons and in Chinese hamster ovary (CHO) cells expressing the α subunit of rat brain type IIA Na^+ channel (CNaIIA-1 cells) are modulated by G protein-coupled pathways under whole cell voltage clamp. Activation of G proteins by 0.2 to 0.5 mM guanosine 5'-O-(3-thiotriphosphate) (GTPγS) increased Na^+ currents in both cell types. The increase in current was caused by an 8 to 10 mV negative shift in the voltage-dependence of activation. Inactivation was also shifted 8 to 10 mV negative. The effects of G protein activators were blocked by treatment with pertussis toxin (PTX) or guanosine 5'-O-2-thiodiphosphate (GDPβS), a nonhydrolyzable GDP analog, but not by cholera toxin. GDPβS (2 mM) alone had opposite effects to GTPγS, causing an 8 to 10 mV positive shift in Na^+ channel gating and suggesting that basal activation of G proteins is sufficient to modulate Na^+ channels. In CNaIIA-1 cells, thrombin, which activates PTX-sensitive G proteins in CHO cells, caused a negative shift in the voltage dependence of Na^+ channel activation and inactivation which was blocked by PTX. The results in CNaIIA-1 cells indicate that the Na^+ channel α subunit alone is sufficient to respond to G proteins. Na^+ channel modulation by G-proteins may regulate electrical excitability through integration of different G protein-coupled synaptic inputs.

M-Pos118

MODULATION OF INACTIVATING AND NON-INACTIVATING Na CURRENTS BY DIACYLGLYCEROLS (DAGs). ((M. Renganathan, and S. Cukierman)). Dept. Physiology, Loyola Medical Center, Maywood, IL 60153.

DAGs are commonly used as potent activators of protein kinase C. These compounds can however, modulate Na currents in N1E-115 neuroblastoma cells independently of protein kinase C activation. DAGs attenuated normally inactivating Na currents in a weakly voltage dependent manner, shifted their steady-state inactivation curve to more negative voltages, and did not affect activation or deactivation of currents. These effects were fully reversible upon DAG washout. To examine the possible influence of fast inactivation on DAGs' effects, we have eliminated fast inactivation irreversibly with proteolytic enzymes. With short duration voltage clamp pulses (≈4 ms), where slow inactivation of Na currents was not significantly present, DAGs were still able to attenuate currents without noticeable effects on the kinetics of activation and deactivation. With longer pulse durations (≥100 ms), DAGs increased the rate of decay of slowly inactivating macroscopic currents. The time course of development of slow inactivation was accelerated by DAGs (from τ=75 ms to 20 ms at -40 mV). In addition, the steady-state inactivation curve, present in normally inactivating Na currents, was partially "restored" by DAGs. Contrary to normal fast inactivation of Na channels however, this slow inactivation process does not end into an absorbing state: channels can reopen from the inactivated state in response to voltage clamp pulses ≥20 mV. In this sense, DAGs resemble the effects of some local anesthetics on different voltage-dependent Na channels without inactivation. Our results clearly demonstrate that Na channels can be modulated by DAGs independently of metabolic pathways. An important question to be addressed concerns the molecular basis of DAG-induced inactivation on Na channels.

M-Pos119

THE MODULATION OF THE CARDIAC SkM2 SODIUM CHANNEL BY PKA DOES NOT DEPEND ON PHOSPHORYLATION OF SERINE(1504) IN THE SP19 LOOP ((B. Frohnwieser[1], L. Weigl[1], B. Spreitzer[1], R.G. Kallen[2] and W. Schreibmayer[1])) [1]: Institute for Medical Physics and Biophysics, Harrachgasse 21, University of Graz, A-8010 Graz, Austria. [2]: Department of Biochemistry and Biophysics, University of Philadelphia, Philadelphia PA 19104-6059, U.S.A.. (Spon. by H. Tritthart)

Stimulation of cyclic AMP dependent protein kinase (PKA) leads to an increase in activity of the cardiac SkM2 sodium channel isoform, expressed in *Xenopus laevis* oocytes (Schreibmayer et al., 1994). On the contrary, calcium dependent protein kinase (PKC) downregulates this channel (Weigl et al. 1994). Another sodium channel isoform from rat brain (IIA) becomes attenuated by both PKA and PKC. A serine (S1504) in the cytosolic SP19 loop has been identified to be responsible for the effects of PKC. In addition phosphorylation of this serine is required for the effects of PKA (Li et al., 1993). As this SP19 loop is highly conserved among sodium channel isoforms, we have constructed a set of mutant channels where the corresponding serine in SkM2 has been replaced by alanine and permanently negatively charged aspartate or glutamate, respectively. No differences in the stimulatory action of cAMP injection into the oocytes could be observed between wild type and mutant channels.

The data show that PKA acts not only differentially on neuronal and cardiac sodium channels, but also that PKC phosphorylation of serine 1504 is not required for PKA modulation of the cardiac isoform.

Supported by the Austrian Research Foundation.

Li et al., Science 261, 1439-1442 (1993)
Schreibmayer et al., Receptors and Channels, in press
Weigl et al. Biophys. J. 66, A202 (1994)

M-Pos120

MODULATION OF SODIUM CHANNELS IN N1E-115 CELLS USING CAGED COMPONDS.
((M. Egger and N.G. Greeff)) Physiologisches Institut, Universität Zürich, Winterthurerstr. 190, 8057 Zürich, Switzerland.

Under whole cell patch clamp conditions the activation of PKA (separation of regulatory and catalytic subunit) via intracellular photorelease of caged cAMP induced a decrease of peak I_{Na} uniformly over the voltage range of -40 mV to +70 mV of about 11% without any shifts of the I-V relation or the reversal potential. These observations are in agreement with phosphorylation studies of Na^+-channel function in inside-out and excised patches. After photolysis of cAMP activation (τ_m) and inactivation (τ_h) of I_{Na} both showed slower time courses compared with the control values. Peak I_{Na} in N1E-115 cells was also influenced by intracellular treatment with diazo-2, a photosensitive Ca^{2+} scavenger. The $[Ca^{2+}]_i$ shift from pCa 8 to pCa 9 induced two functional effects: slowing of inactivation and up- regulation of peak I_{Na} by about 18%. Control measurements with diazo-3 (similar photochemical properties to diazo-2 but no Ca^{2+} binding) showed no effect. The diazo-2 results suggest that the Na^+-channel function may be modulated by the Ca^{2+}/diacylglycerol signalling pathway resulting in a protein kinase C phosphorylation of the α-subunit of the Na^+-channels. (Supported by SNF 31-37987.93.)

RYANODINE RECEPTOR: MOLECULAR BIOLOGY AND BIOCHEMISTRY

M-Pos121

PRIMARY STRUCTURE AND PROPERTIES OF HELOTHERMINE, A PEPTIDE TOXIN THAT BLOCKS RYANODINE RECEPTORS. ((J. Morrissette[1], J. Kratzschmar[2], B. Haendler[2], R. El-Hayek[1], J. Mochca-Morales[3], B. M. Martin[4], J.R. Patel[1], R.L. Moss[1], W.-D. Schleuning[2], R. Coronado[1] and L. D. Possani[3])) [1]Department of Physiology, University of Wisconsin; [2]Schering AG, Berlin, Germany; [3] Institute of Biotechnology, UNAM, Mexico; [4]NIH, Bethesda, MD.

Helothermine, a protein from the venom of the Mexican beaded lizard (Heloderma horridum horridum) was found to inhibit [^{3}H]ryanodine binding to cardiac and skeletal sarcoplasmic reticulum, to block cardiac and skeletal ryanodine receptor channels incorporated into planar bilayers, and to block Ca^{2+}-induced Ca^{2+} release triggered by photolysis of nitr-5 in saponin-permeabilized trabeculae from rat ventricle. Cloning of the helothermine cDNA revealed that the protein is composed of 223 amino acids with a molecular mass of 25,376 daltons, and is apparently stabilized by eight disulfide bridges. The peptide sequence showed significant homology with a family of cysteine-rich secretory proteins found in the male genital tract and in salivary glands. The interaction of helothermine with ryanodine receptors should serve to define functional domains within the channel structure involved in the control of Ca^{2+} release from sarcoplasmic reticulum.(Supported by NIH, MDA, AHA, HHMI, and Schering AG).

M-Pos122

EXCHANGE OF BOUND FKBP-12 FROM THE SKELETAL MUSCLE RYANODINE RECEPTOR COMPLEX BY WILD-TYPE OR PPIASE DEFICIENT FKBP-12 MUTANTS ((Anthony P. Timerman, Greg Wiederrecht*, Alice Marcy*, and Sidney Fleischer)) Dept. of Mol. Biol., Vanderbilt University Nashville, TN 37235 and *Merck Research Laboratories, Rahway NJ 07065

FKBP-12 (FKBP), the soluble receptor for the immunosuppresant drug FK-506, is tightly bound to the calcium release channel/ryanodine receptor (RyRec) of skeletal muscle (SKM) terminal cisternae (TC) of sarcoplasmic reticulum; the stoichiometry is 4 moles FKBP per tetrameric RyRec complex. FKBP displays peptidyl-prolyl isomerase (PPIase) activity that is inhibited by FK-590 or rapamycin. In SKM TC, these ligands bind to and dissociate FKBP from the RyRec in a temperature dependent manner which increases the channel's open probability. The net energized Ca^{2+} uptake rate of TC vesicles devoid of FKBP is decreased due to an increased leak rate specifically via the RyRec which is reversed upon rebinding FKBP. Thus, the RyRec is modulated by FKBP. We now find that bound [^{35}S] FKBP is displaced from the FKBP•RyRec complex by exchange with unlabeled FKBP in solution. The EC_{50} for exchange is 0.30 uM for wild type FKBP vs. 0.6 to 2.4 uM for three different site-directed mutants that are practically devoid of any measurable PPIase activity. Substitution of wild-type FKBP on the RyRec complex with the PPIase deficient mutants did not alter the Ca^{2+} flux of TC vesicles, whereas dissociation of FKBP from TC with FK-590 increased the Ca^{2+} leak rate. Our studies show that, in-vivo, the FKBP•RyRec complex is in equilibrium with the cytosolic pool of FKBP (~3 uM) and suggest that modulation of the channel by FKBP is independent of PPIase activity. (Supported by NIH HL 32711 & Muscular Dystrophy Association).

M-Pos123

CO-LOCALIZATION OF FKBP WITH RYANODINE RECEPTOR IN RABBIT SKELETAL MUSCLE ((Hitoshi Onoue, David Samuelson, Tom Jetton**, Loice Jeyakumar, Eunice Ogunbunmi, Akitsugu Saito and Sidney Fleischer)) Dept. of Molecular Biology, ** Dept. of Molecular Physiology and Biophysics, Vanderbilt University, Nashville TN 37235 (Spon by P. Flicker)

The calcium release channel/ryanodine receptor of skeletal muscle terminal cisternae (TC) is tightly associated with FKBP (Jayaraman et al., J. Biol. Chem., 267 1992). We have developed methodology to dissociate and rebind FKBP from and to TC (Timerman et al., J. Biol. Chem, 268, 1993). Using this reconstitution approach, we have shown that FKBP modulates the function of the calcium release channel. In order to assess association, *in situ,* immunolocalization of the calcium release channel and FKBP in skeletal muscle sections has been performed. The calcium release channel and FKBP were detected in rat skeletal muscle using specific antibodies raised against each protein. Both calcium release channel and FKBP were colocalized in a banding pattern normal to and throughout the length of the fiber. The location is at the junction of the A and I bands at each sarcomere where the triad junction is located in mammalian skeletal muscle. Our observation confirms that FKBP and the ryanodine receptor are associated *in vivo,* and supports the concept that FKBP modulates the calcium release channel. (Supported by NIH HL 32711 and Muscular Dystrophy Association)

M-Pos124

AFFINITY PURIFICATION OF THE RYANODINE RECEPTOR/CALCIUM RELEASE CHANNEL FROM SKELETAL MUSCLE BASED ON ITS TIGHT ASSOCIATION WITH FKBP-12. ((Hong-Bo Xin, Anthony P. Timerman, Hitoshi Onoue, Gregory J. Wiederrecht*, Sidney Fleischer)) Dept. of Mol. Biol., Vanderbilt University, Nashville TN 37235; *Department of Immunology, Merck Research Laboratories, Rahway, NJ 07065-0090 (Spon. by C. Cobb)

The ryanodine receptor (RyRec)/calcium release channel (CRC) isolated from skeletal muscle terminal cisternae (TC) of sarcoplasmic reticulum (SR) is tightly associated with FK-506 binding protein of 12.0 kDa (FKBP-12.0) (Jayaraman et. al. (1992), J. Biol. Chem. 267: 9474-9477). In this study, we describe a new method for purifying RyRec from skeletal muscle SR based on; 1) its tight association with FKBP-12; and 2) the finding that soluble FKBP exchanges with bound FKBP-12 on the ryanodine receptor (Timerman et. al., 1995, Biophys J., in press). The principle of affinity chromatography was applied, using a fusion protein of Glutathione-S-Transferase/FKBP-12.0 (GST/FKBP 12.0), which was expressed in E. coli. GST/FKBP 12.0 was first exchanged with bound FKBP-12 on the ryanodine receptor. The GST/FKBP 12.0 was then purified by affinity chromatography on glutathione-Sepharose 4B. After selective binding of GST/FKBP•RyRec, the complex was selectively eluted with glutathione. The RyRec, purified by this method, possesses similar characteristics in radioligand binding and immunoreactivity as the RyRec purified by heparin-agarose chromatography. (Supported by NIH HL32711 and Muscular Dystrophy Assoc.)

M-Pos125

EVIDENCE FOR THE PRESENCE OF A NOVEL SKELETAL-LIKE RYANODINE RECEPTOR (RyR) ISOFORM IN STRIATED MUSCLES OF FISH. ((Jens P.C. Franck, John E. Keen, Richard L. Londraville, Mark B. Beamsley, and Barbara A. Block)) Hopkins Marine Station, Stanford University, Pacific Grove, CA, 93950.

The skeletal muscles of non-mammalian vertebrates are known to co-express two RyR isoforms, α and β, whereas mammals express a single skeletal isoform. Phylogenetic and biochemical analyses have determined that the α isoform is more closely related to the mammalian skeletal isoform while the β isoform appears homologous to the brain isoform of mammals. The frog α and β isoforms are approximately 70% identical when compared at the amino acid level (Oyamada *et al.*, J. Biol. Chem. 269: 17206 (1994)). We have characterized the cDNA sequence for the α skeletal RyR isoform from fish extraocular muscle (only α RyR expressed). To date we have cloned and sequenced 13 kbp of contiguous cDNA clones. In addition to the major α isoform cDNA we have identified a second isoform that shares extensive sequence homology with the mammalian skeletal isoform. Screening of a cDNA library from the superior rectus muscle of marlin with a PCR product generated from conserved primer pairs yielded two clones designated λBM3 and λBM4 with 1032 bp of overlapping sequence.The overlapping sequence corresponds to nucleotides 10500 -11500 in the rabbit skeletal RyR open reading frame. Re-screening of the cDNA library has yielded approximately 2 kbp of overlapping sequence for the two isoforms. The two isoforms are 80% identical when the derived amino acid sequences are aligned and compared. A phylogenetic analysis of the two fish isoforms was performed from a multiple alignment with the corresponding regions of the three rabbit RYR isoforms (skeletal, cardiac, brain), human skeletal, and frog α and β isoforms. The resulting phylogeny based on parsimony analysis with the *Drosophila* RyR sequence as a designated outgroup, clustered both fish RyR isoform sequences in the same clade as the frog α, rabbit and human skeletal sequences, distinct from the clade clustering the frog β and mammalian brain isoforms, and also distinct from the rabbit cardiac isoform sequence. Reverse transcriptase and ratio PCR assays have revealed that the two skeletal isoforms are differentially distributed. Primer pairs specific for the novel skeletal isoform generated products from RNA of superior rectus, cardiac, and skeletal muscles, whereas primer pairs specific for the designated α isoform sequence only generated products from superior rectus and skeletal muscle. Neither isoform was detected in total RNA derived from smooth muscle. The novel RyR isoform therefore appears to represent a second γ-like isoform ubiquitously expressed in the striated muscles of fish. (Supported by NIH AR40245.

M-Pos126

DISTRIBUTION AND QUANTITATION OF RYANODINE RECEPTOR EXPRESSION IN MAMMALIAN TISSUES. ((James R. Mickelson, Jacquelyn K. Priener, and Charles F. Louis)) Department of Veterinary PathoBiology, University of Minnesota, St. Paul, MN 55108

Ryanodine receptor (ryr) expression in various porcine tissues was determined using reverse transcription-PCR (RT-PCR). cDNA was synthesized from total tissue RNA with reverse transcriptase and random hexamer primers. PCR primer sets were selected to specifically amplify a 500 bp segment from homologous regions near the 5' end of the skeletal (ryr1), cardiac (ryr2), or brain (ryr3) ryr cDNA sequences. Specific amplification was confirmed by restriction enzyme mapping and DNA sequencing. A ryr1 RT-PCR product was identified in skeletal muscle and esophagus, a ryr2 RT-PCR product was identified in cardiac muscle, aorta and esophagus, and a ryr3 RT-PCR product was identified in skeletal and cardiac muscle, aorta, esophagus, adrenal gland, small intestine, and lung. All three ryr isoforms were identified throughout the brain, including the parietal, frontal, and temporal lobes of the cerebrum, thalamus /hypothalamus, cerebellum, and brain stem. Quantitation of mRNA for the three ryr isoforms in striated muscle and brain sections using competitive PCR and ryr cDNA standards is in progress. These results thus demonstrate expression of two ryr isoforms in both skeletal and cardiac muscle, and all ryr isoforms in a number of regions of the central nervous system. Determination of the relative level of expression of each ryr isoform in various tissues will ultimately allow a better understanding of the Ca^{2+} signaling pathways which function in different cell types. Supported by NIH GM-31382.

M-Pos127

COMPARATIVE MOLECULAR FIELD ANALYSIS OF RYANODINE BINDING TO CARDIAC AND SKELETAL MUSCLE RYANODINE RECEPTORS ((K.E. Mitchell, J. J. Bottorff, S. D. Sperry, J. A. Airey, J. L. Sutko, K. Gerzon, H.R. Besch, Jr., L. Ruest, and W. Welch)) Univ. Nevada, Reno, NV 89557.

Ryanodine is a specific effector of the ryanodine receptor (RyR) family of intracellular calcium channels. A comparative molecular field analysis (CoMFA) was developed in which structural elements of ryanodine analogs were correlated with their binding to the skeletal muscle RyR. The pyrrole and isopropyl loci were found to have the strongest steric correlations with binding whereas the dominant electrostatic correlation is localized to a single hydroxyl group in the molecule. The CoMFA predictive function was used to elucidate the binding orientation of two structural isomers of ryanodine, 10-ryanodine and 3-epiryanodine. The CoMFA predicts that 10-ryanodine assumes a ryanodine-like orientation with the pyrrole, now at the 10-position, buried in theRyR binding pocket. Thus, 10-ryanodine binds in an orientation with the body of the molecule rotated ~ 180° with respect to ryanodine. 3-epiryanodine is predicted to bind in a ryanodine-like orientation with the body of the molecule shifted to accommodate proper positioning of the pyrrole in the binding pocket. To study binding interactions at the 10-position, a series of ryanodine and ryanodol derivatives with various aromatic and charged substituents at this locus were synthesized. Our data suggest that these derivatives bind to the receptor in one of two modes; with the 10-substituent buried in the binding cleft or with the 10-substituent positioned near the mouth of the binding pocket. The insights gained from the CoMFA of ryanoid binding to skeletal RyR are being used to develop a CoMFA for ryanoid binding to cardiac RyR. Although we find some exceptions, in general ryanoids bind with 2-3X greater affinity to the cardiac than to the skeletal RyR.

M-Pos128

THE αRYR INFLUENCES THE TYPE OF E-C COUPLING USED IN EMBRYONIC CHICKEN SKELETAL MUSCLE. ((D. McKemy, A. Ivanenko, J.L. Kenyon, J.A. Airey & J.L. Sutko)) Univ. Nevada, Reno, NV 89557.

Cultured embryonic chick skeletal muscle cells initially exhibit a Ca_o-dependent E-C coupling and then switch to a Ca_o-independent coupling as they mature. The time course of this switch is influenced by the density at which the cells are plated. The initial Ca_o-dependent Ca transients exhibit long time courses and slow on and off kinetics, whereas the Ca_o-independent transients are more rapid and twitch-like. Cultured Crooked Neck Dwarf (cn) mutant skeletal muscle cells do not express normal α ryanodine receptor (RyR), but have normal levels of ßRyR. These cells initially exhibit Ca_o-dependent E-C coupling, but do not switch to the Ca_o-independent coupling. In addition, with further maturation cn/cn cells develop another defect in E-C coupling and do not respond to electrical stimuli. The latter is not due to a defective ßRyR or a failure to maintain releasable SR Ca stores as caffeine elicits ryanodine-sensitive Ca transients. These results suggest the αRyR is required for development of Ca_o-independent E-C coupling, as well as for maintaining the ability to activate ßRyR-mediated Ca transients.

M-Pos129

THE MOLECULAR BASIS OF THE CROOKED NECK DWARF (CN) MUTATION IN CHICKENS. ((J.F. Gera, M.C. Bohlman, J.A. Hamerman, J.L. Sutko & J.A. Airey)) Univ. Nevada, Reno, NV 89557.

The cn mutation causes a failure to make normal α ryanodine receptor (RyR) in skeletal muscle and results in a muscle dysgenesis, the degenerative loss of skeletal muscle and death at the time of hatching (Airey et al. Dev. Dynam. 197:169, 1994). The following results suggest the defect underlying this mutation occurs in the αRyR gene. (i) A normal αRyR is missing in both skeletal muscle and brain. (ii) The levels of other E-C coupling components (mRNAs and proteins: ßRyR, α_1 dihydropyridine subunit, Ca^{2+}-ATPase, calsequestrin) are normal. (iii) Abnormally localized αRyR immunoreactivity co-localizes with the rough endoplasmic reticulum protein, BiP; (iv) The level of skeletal muscle αRyR mRNA is reduced to 25% of normal. These observations suggest a truncated and defective αRyR protein is rapidly degraded. Using a RNase protection assay we have identified a difference in the sequence of normal and cn/cn αRyR mRNAs. We are currently determining the nature of this difference to ascertain if it causes an inappropriate termination of translation of cn/cn αRyR mRNA.

M-Pos130

CALMODULIN AFFINITY LABELING OF THE TURKEY SR Ca^{2+}-CHANNEL: EFFECTS OF IONIC STRENGTH AND Ca^{2+}-RELEASE MODULATORS. ((L.-J. Wang, J. Korlach, and G.M. Strasburg)) Dept. of Food Science and Human Nutrition, Michigan State University, E. Lansing, MI 48824.

Calmodulin (CaM) is an inhibitor of Ca^{2+}-release by the SR Ca^{2+}-channel protein. We have previously shown that CaM affinity labeling of the porcine skeletal muscle channel protein is Ca^{2+}-independent [Yang et al., Biochemistry (1994) 33:518]. In contrast, azido-CaM binding to the chicken skeletal muscle channel protein which consists of 2 isoforms (α and ß) is Ca^{2+}-dependent [Airey et al., Biochemistry (1993) 32:5739]. To determine whether observed differences in affinity labeling patterns are the result of species differences or of crosslinking conditions, turkey skeletal muscle SR channel protein was affinity labeled with ^{125}I- and benzophenone-4-maleimide-labeled wheat CaM (^{125}I-Bz-CaM) under various Ca^{2+} and Mg^{2+} concentrations and buffer conditions. In the presence of either EGTA or Ca^{2+} and no added NaCl, both isoforms bound ^{125}I-Bz-CaM, although Ca^{2+} enhanced affinity labeling. The α isoform showed much greater affinity labeling than ß. Affinity labeling of both isoforms was substantially increased with increasing [Mg^{2+}]. Addition of 0.15 M NaCl resulted in Ca^{2+}-insensitive affinity labeling of both isoforms as well as little Mg^{2+}-dependence of labeling. Furthermore, labeling of the α isoform was now comparable to that of ß. These results suggest that discrepancies in CaM affinity labeling patterns between avian and mammalian channel proteins are affected by crosslinking conditions and perhaps by the CaM affinity label.

M-Pos131

THIMEROSAL INDUCES CALCIUM RELEASE AND INHIBITS RYANODINE BINDING TO SKELETAL MUSCLE SARCOPLASMIC RETICULUM. ((T.G. Favero, A.C. Zable *, N. Perumal #, G. Salama # and J.J. Abramson *)) Biol. Dept., Univ. of Portland, * Physics Dept., Portland State Univ, and # Physiology Dept., Univ. of Pittsburgh.

The thiol reagent, thimerosal, has been shown to increase the intracellular Ca^{2+} concentration in several cell types and to induce repetitive Ca^{2+} spikes. Here we report that micromolar concentrations of thimerosal stimulate Ca^{2+} release from SR vesicles actively loaded with Ca^{2+}, inhibit high affinity ^{3}H-ryanodine binding, and modify channel activity of the reconstituted Ca^{2+} release protein. Thimerosal inhibits ryanodine binding by decreasing the binding capacity (B_{max}), but does not effect the binding affinity or the dissociation rate of bound ryanodine. Single channel reconstitution experiments show that thimerosal (10 μM to 20 μM) modifies single channel behavior in a time dependent manner. Within 1 min, the Po increases. After 5-10 min, the channel closes. Activation of the channel at early times is apparently responsible for thimerosal induced Ca^{2+} release, while time dependent inactivation causes a decrease in ryanodine binding. Once inactivated, the channel can not be reactivated by Ca^{2+} or ATP.

M-Pos132

CALMODULIN BINDING LOCATION ON THE THREE-DIMENSIONAL ARCHITECTURE OF THE SKELETAL MUSCLE CALCIUM RELEASE CHANNEL/RYANODINE RECEPTOR. ((T. Wagenknecht, R. Grassucci, J. Berkowitz, A.P. Timerman, and S. Fleischer)) Wadsworth Center for Laboratories and Research, New York State Dept. of Health, Albany, NY 12201 and Department of Molecular Biology, Vanderbilt University, Nashville, TN 37235.

Calmodulin (CaM) regulates the calcium release channel/ryanodine receptor (RyR) in a Ca^{2+}-dependent manner by direct binding. Previously, we mapped the location of a CaM binding site on the RyR by cryo-electron microscopy of RyR:CaM complexes in which a 1.4-nm diameter gold cluster was covalently attached to the CaM. In that study the gold cluster served as a high-contrast marker of the CaM site, but the accuracy of the localization was limited to 4 nm. To improve the accuracy of the localization we have attempted to directly detect CaM bound to the RyR, i.e. without a gold cluster marker. Images of frozen-hydrated RyR:CaM and of RyR complexes were independently averaged from electron micrographs, and the difference of the two computed. The difference image showed a major peak corresponding to additional mass in the RyR:CaM complexes which is attributed to bound CaM. This peak is displaced ≈3 nm from the position of the gold cluster determined previously. A three-dimensional reconstruction of the RyR (Radermacher et al. (1994) J. Cell Biol., in press) shows a deep cleft on its cytoplasmic-facing surface at the location of the CaM binding site. Supported by NIH AR40615 (TW) and NIH HL32711 (SF).

M-Pos133

ONTOGENY OF CALCIUM RELEASE CHANNEL AND CALCIUM PUMP IN SARCOPLASMIC RETICULUM OF RAT MYOCARDIUM. V. Ramesh, M.J. Kresch, and D.H. Kim. Divs. of Neonatology & Cardiology, Univ. of Connecticut Health Center, Farmington. CT 06032, USA; K- JIST (DHK), Kwangju, Korea.

During fetal and neonatal development, various changes take place in myocardial contractile properties. The objective of this study was to define the ontogeny of Ca release channel (CRC) and Ca-pump (CaATPase) which play a major role in contraction and relaxation. We used whole ventricular homogenates of fetal (F)(19 and 22 days in gestation), postnatal (N)(1 and 7days postnatal) and adult (A)(5 weeks postnatal) Sprague-Dawley rat hearts to study ^{3}[H]ryanodine binding and oxalate-supported ^{45}Ca uptake. For CRC, the major findings were: 1) CRC density as determined by maximal ^{3}[H]ryanodine binding (Bmax) increased 3 fold between the fetal (F22) and adult (A) periods, (0.21± 0.1 vs. 0.73 ± 0.7 pmoles/g protein; $p<0.01$), whereas there was no significant change during the fetal and early postnatal development phases (N1: 0.34 ± 0.15). 2) Affinity of CRC to ryanodine (Kd) decreased significantly as development progressed from fetal (F22) to adult (A) myocardium; (1.5 ± 0.2 vs. 3.9 ± 1.0; $p<0.05$). For Ca-pump, changes started early with increased rate of Ca uptake in the fetal periods (F19: 8.1 ± 1.2 vs. F22: 19.3 ± 2.1 nmoles/g protein/min; $p<0.05$) and peaked by 7 days (N7) of postnatal age (34.8 ± 2.1). Thus our study suggests that both quantitative and qualitative changes occur in CRC during myocardial development and maturation of Ca uptake appears to start earlier than that of Ca release. (This study was supported by NIH Grant (HL-07420) and Established Investigatorship (DHK) from AHA).

M-Pos134

MOLECULAR INTERACTION BETWEEN RYANODINE RECEPTOR AND GLYCOPROTEIN TRIADIN INVOLVES REDOX CYCLING OF FUNCTIONALLY IMPORTANT HYPERREACTIVE SULFHYDRYLS. ((Isaac N. Pessah and Guohua Liu)) Department of Molecular Biosciences, University of California, Davis, CA 95616.

The fluorogenic maleimide 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin (CPM) has been shown to selectively form Michael adducts with hyperreactive sulfhydryl goups on the skeletal SR ryanodine receptor protomer (RyR1) and glycoprotein triadin which are essential for normal Ca^{2+} channel function (Liu *et al., Molec. Pharmacol.* 45:189-200, 1994). The present report demonstrates a functionally important interaction between RyR1 and triadin which involves, in part, redox cycling of hyperreactive sulfhydryls in response to channel activation and inactivation. Nanomolar CPM is shown to selectively label RyR1 and triadin, only in the presence of Ca2+ channel inhibitors (millimolar Mg2+, micromolar neomycin or ruthenium red, or anti-triadin antibody). Treatment of SR with channel activators (micromolar Ca2+, nanomolar ryanodine, or millimolar caffeine), 1) slows CPM labeling kinetics >10-fold, 2) negates CPM labeling of channel-associated sulfhydryls, and 4) stabilizes a high molecular weight complex (HMWC) which appears on non-reducing SDS-PAGE gels. The HMWC is positively identified as RyR1 and triadin by Western blot and immunoprecipitation analyses. High-affinity [3H]ryanodine binding sites are immunoprecipitated by either anti-RyR1 or anti-triadin antibody in a dose-dependent manner. 1,4-Naphthoquinone (≤40 pmol/μg protein) directly and selectively oxidizes hyperreactive sulfhydryls on RyR1 and triadin, induces Ca2+ efflux from SR, and stabilizes the HMWC. The HMWC is reduced by ß-mercaptoethanol or dithiothreitol into its component RyR1 and triadin protomers. Tyrptic digestion of CPM-labeled SR reveals that ~32% of the hyperreactive sulfhydryls are cytoplasmic, whereas the remainder are localized within the SR lumen. The results provide direct evidence for the existence of a functionally important complex between RyR1 and triadin whose stability is determined by the redox state of hyperreactive sulfhydryl moieties which are allosterically regulated by physiological and pharmacological channel ligands. The present results suggest a possible molecular mechanism by which localized transient changes in the redox state within the RyR1/triadin complex can signal information across the SR membrane.

M-Pos135

MODIFICATION OF RYANODINE RECEPTOR CHANNELS BY SULFHYDRYL-REACTING COMPOUNDS. ((K.E. Quinn and B.E. Ehrlich)) University of Connecticut, Farmington, CT 06030

Sulfhydryl-reacting compounds iodoacetamide (IAM) and iodoacetic acid (IAA) were used to study structure-function relationships in ionic channels; specifically to modify cysteine residues of the ryanodine receptor (RyR) calcium channel in what is believed to be the pore-forming region. The relationship of the structural configuration and functional properties of the RyR was studied in SR vesicles from rabbit skeletal muscle incorporated into planar lipid bilayers. Addition of IAM and IAA (5 mM) increased the channel open probability (Po) which was reversed following washout. IAM, but not IAA, reduced channel Ca conductance by 55% (108 vs 49 pS) but this was not reversed by perfusion with IAM-free buffer. These results show that IAM is partially reducing the Ca current through the pore of the RyR channel, probably by binding to a thiol group (ie, a cysteine residue). The addition of IAM can therefore be used as a tool for probing the structure of the RyR calcium channel. Supported by NIH HL-33026 and The Donaghue Foundation.

M-Pos136

EXPRESSION STUDIES WITH cDNAs ENCODING THE HUMAN CARDIAC RYANODINE RECEPTOR-CALCIUM RELEASE CHANNEL. ((Richard E.A. Tunwell, Colin Wickenden, Benedicte M.A. Bertrand, Martina Walsh, Valery Shevchenko, Paul D. Allen* and F. Anthony Lai)) MRC National Institute for Medical Research, Mill Hill, London NW7 1AA, UK. and *Brigham and Women's Hospital, Boston, MA 02115, USA.

Calcium induced calcium release via the ryanodine-sensitive calcium release channel (RyR) is a fundamental event in excitation-contraction coupling in cardiac muscle. The cardiac muscle RyR has been identified as a tetrameric complex comprising subunits of M_r 565K, and displays a large cation conductance in single channel recordings. We have isolated aand sequenced nine overlapping cDNA clones that encode the human cardiac muscle isoform of the RyR. Two in-frame insertions were observed in the overlap region of certain clones. The deduced amino acid sequence of this RyR isoform is very similar to that of the rabbit cardiac RyR isoform. Alignment of all RyR amino acid sequences and hydropathy analysis suggest that a conserved C-terminal region may constitute the channel-forming transmembrane domain. Areas of non-conserved residues within and upstream of this region may encompass regulatory sites that could account for the functional differences observed between the three known RyR isoforms. Expression of human cardiac RyR cDNA fragments as fusion proteins in bacteria has indicated the C-terminal region to be relatively labile. To investigate the putative channel-forming domain, we have transfected cells with various human cardiac RyR cDNA constructs. Antibodies prepared to regions specifically conserved in rabbit and human cardiac RyRs were used to demonstrate the presence of expressed proteins, and to identify the native protein in human cardiac muscle.

M-Pos137

RYANODINE RECEPTORS: ISOFORMS EXPRESSION IN MAMMALS AND CHICKEN.
((A. Conti, P. Tarroni, D. Rossi and V. Sorrentino)) DIBIT, Istituto Scientifico H. S. Raffaele Milano, Italy. (Spon. by M. Mazzanti)

Ryanodine receptors (RyRs), are a family of intracellular calcium release channels. They have been known for many years as the calcium channels of the sarcoplasmic reticulum of both skeletal and cardiac muscle. Recently, both pharmacological and molecular studies have shown that in many tissues, cells are present with intracellular calcium stores regulated by channels with properties similar to those of RyRs of muscle cells. We have studied, both at level of gene expression and of protein localization, the presence of the three members of the RyR family in several tissues and cell lines and we have observed a widespread pattern of expression for all of them. Evidences of coexpression of more than one isoform in several cell types have also been found. A further level of diversification of these channels is apparently mediated by a mechanism of alternative splicing.
In chicken we have cloned the homologs of mammalian RyR1 and RyR3 and demonstrated that they correspond to the two isoforms expressed in chicken skeletal muscle, formerly identified as α and β. In order to understand the role of RyR3 in muscle cells we have started the analysis of RyR expression in chicken and mammalian muscles to verify the extend of a differential regulation/utilization of RyR3 in skeletal muscles.

M-Pos138

PROBING THE STRUCTURE OF THE SKELETAL MUSCLE CA^{2+} RELEASE CHANNEL WITH PROTEOLYSIS AND SULFHYDRYL LABELING. ((Dolores H. Needleman and Susan L. Hamilton)) Department of Molecular Physiology and Biophysics, Baylor College of Medicine, Houston TX.

Proteolysis of the Ca^{2+} Release channel in sarcoplasmic reticulum membranes has been used to determine the location of ryanodine binding sites on this channel (Callaway *et al., J.Biol. Chem.* 269:15876-15884, 1994). We have examined the proteolytic fragments generated in high and low Ca^{2+} and in the presence and absence of bound ryanodine. We have also examined the labeling of sulfhydryl groups on the protein under these conditions. Peptide fragments (including those labeled with sulfhydryl reagents) were identified by sequencing and using sequence specific antibodies. We have detected differences in both the proteolytic patterns and in the labeling of sulhydryls under the different experimental conditions. Our results indicate that this is a useful approach for examining the structure of this protein in different functional states.

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M-Pos139

CALMODULIN BINDING SITES OF THE SKELETAL, CARDIAC AND BRAIN RYANODINE RECEPTOR Ca^{2+} CHANNELS: MODULATION BY THE CATALYTIC SUBUNIT OF cAMP-DEPENDENT PROTEIN KINASE?
Remo Guerrini$, Paola Menegazzi, Roberto Anacardio$, Mauro Marastoni$, Roberto Tomatis$, Francesco Zorzato*, Susan Treves*· $ Dipartimento di Scienze Farmaceutiche, Universita' degli Studi di Ferrara, Ferrara, Italy, $ Research Group Dompè, L'Aquila, Italy, *Istituto di Patologia Generale, Universita' degli Studi di Ferrara, Ferrara, Italy.* (Spons. by M.C. Sorgato) *Cardiac and brain RYR peptides corresponding to the calmodulin binding sites present in the skeletal RYR (Biochemistry 33: 9078-9084) were synthesized and their interaction with calmodulin was monitored by fluorescent techniques. The central portions of the skeletal, cardiac and brain RYR protomers displayed one high (CaM#1) and one low (CaM#2) affinity calmodulin binding site. A third calmodulin binding site (CaM#3) was identified a few residues upstream the putative transmembrane segment M5. Its affinity for calmodulin varied between the RYR isoforms: the cardiac RYR CaM#3 has high affinity, while the skeletal and brain RYR CaM#3 have low affinity. The RYRs calmodulin binding site CaM#1 was phosphorylated in vitro by the catalytic subunit of the cAMP-dependent protein kinase. Phosphorylation of binding site CaM#1 was inhibited by calmodulin binding. These data indicate that the interaction of calmodulin with the CaM#1 site may be regulated by the phosphorylation state of the Ser residue localized within the binding site.*

M-Pos140

CHARACTERIZATION OF TERATOMA PRODUCED SKELETAL MUSCLE CELLS LACKING A FUNCTIONAL RYR-1. **((P. D. Allen^, H. Nguyen^, E. D. Buck^ and I. N. Pessah^)) *Dept. of Cardiology, Children's Hospital, Boston MA, 02115 and ^Dept of Molecular Biosciences, School of Veterinary Medicine, University of California, Davis, CA 95616**

The skeletal ryanodine receptor (RyR-1) gene was interrupted in the middle of an exon which codes for amino acids 268-320 by transfection of J1 ES cells with a replacement vector in which a PGK-Neo resistance gene was inserted into the middle of the exon. A PGK-TK gene was attached to the end of the genomic fragment. Cell selection with G418 and gangcyclovir resulted in 24 of 190 cells which contained the mutant allele by southern blot analysis. We used Mortensen's technique of increased G418 to produce an ES cell line which was homozygous for the mutant allele. Both heterozygous (+/-) and homozygous (-/-) ES cells were injected subcutaneously in SCID mice and the resultant teratocarcinomas analyzed by immunohistochemistry for reactivity to skeletal myosin and RyR-1 antibodies. Other aliquots of the same tumors were assayed for the binding of [3H]ryanodine and stimulation of radioligand occupancy by bastadin 5 which is selective for RyR-1. The tumors were variable in their expression of skeletal muscle ranging from scarce to 30% of tumor mass. The +/- tumors showed several regions which were reactive to both RyR-1 and skeletal-specific myosin antibodies, whereas the -/- tumors showed reactivity only to myosin antibody. Both the +/- and -/- tumors possessed high-affinity [3H]ryanodine binding sites. The +/- tumors exhibited a 1.5 to 2.5-fold enhancement of specific [3H]ryanodine occupancy in the presence of bastadin 5. [3H]ryanodine occupancy in the -/- tumors was consistently insensitive to bastadin 5 indicating the absence of RyR-1. Myogenic cells lacking a functional RyR-1 will provide an excellent model to study RyR-1 structure/function.

M-Pos141

REGULATION OF THE SKELETAL MUSCLE RYANODINE RECEPTOR BY METABOLIC FACTORS ASSOCIATED WITH MUSCLE ACTIVITY. ((B.R. Fruen, P.K. Kane, J.R. Mickelson, and C.F. Louis)) Department of Veterinary PathoBiology, University of Minnesota, St. Paul, MN USA 55108.

We have previously reported that the *in vitro* activity of the skeletal muscle sarcoplasmic reticulum (SR) ryanodine receptor (RYR) Ca^{2+} channel is stimulated by concentrations of inorganic phosphate (3-30 mM) that occur *in vivo* during exercise (*J.Biol.Chem.* **269:** 192-198, 1994). Here we examine the influence of other metabolites that either accumulate or are depleted during exercise on [^{3}H]ryanodine binding to porcine skeletal muscle SR vesicles. In the presence of optimal Ca^{2+}, pH 7.1, phosphocreatine inhibited [^{3}H]ryanodine binding to skeletal muscle SR vesicles (50% inhibition at 40 mM phosphocreatine), whereas creatine (up to 40 mM) had no significant effect. [^{3}H]Ryanodine binding to skeletal muscle SR vesicles was also inhibited by lactate (40% inhibition at 40 mM lactate). However, like phosphate, neither phosphocreatine nor lactate altered [^{3}H]ryanodine binding to cardiac muscle SR, demonstrating the specificity of these metabolite's effects on the skeletal muscle RYR isoform. Inhibition of skeletal muscle SR [^{3}H]ryanodine binding by phosphocreatine, lactate, or decreased pH did not significantly alter the half-maximally effective concentration of P_i (approximately 5 mM) for stimulation of [^{3}H]ryanodine binding. These results support the hypothesis that changing levels of certain metabolites during muscle activity may influence SR Ca^{2+} release via effects at distinct sites on the skeletal muscle RYR Ca^{2+} channel. Supported by NIH grant GM31382.

M-Pos142

INACCESSIBILITY OF THE C-TERMINUS OF THE SKELETAL MUSCLE RYANODINE RECEPTOR TO SITE-SPECIFIC ANTIBODIES. ((Ron Grunwald and Gerhard Meissner)) Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, NC 27599-7260.

In a previous report (*Biophys. J. A152, 1993*) we found that antibodies against the C-terminus of the ryanodine receptor (RyR) showed increased binding after permeabilization of SR vesicles, suggesting the possibility that the C-terminus may be luminally oriented. In this work we report that a C-terminal directed antibody (Ab[5006-5037]) bound efficiently to SR vesicles which were pre-treated by brief exposure at pH 8.5, conditions where few of the vesicles were permeabilized. Permeabilization was monitored by: (1) the release of calsequestrin, (2) accessibility of calsequestrin to trypsin, (3) binding of antibody Ab[4581-4640] to a luminal epitope of the RyR , and (4) binding of antibody A20 (gift of D.H. MacLennan) to a luminal epitope of the SR CaATPase. This result is consistent with a cytoplasmic localization of the RyR C-terminus. However, Ab[5006-5037] was not able to bind SR vesicles under some conditions where the vesicles were permeabilized ([CHAPS] $\leq$ 0.1%). Extensive treatment with higher concentrations of detergent resulted in exposure of the C-terminus with a slow time course. Conditions which increased accessibility also decreased [^{3}H]ryanodine binding. The exposure of the C-terminus to antibodies may therefore be associated with either partial denaturation of the RyR or the removal of protein-protein interactions.

M-Pos143

MOLECULAR CHARACTERIZATION OF CALCIUM RELEASE CHANNELS IN HYPERTROPHIED RAT HEART. Katerina M. Michaels, Nikolaus A. Spoerel and Do Han Kim. Division of Cardiology and Department of Biochemistry, University of Connecticut Health Center, Farmington, CT 06032, USA; K-JIST(DHK), Kwangju, Korea.

Previous studies have shown that the density of the calcium release channel (CRC) in the left ventricle is significantly lower (68%) in severely hypertrophied rat hearts than in sham hearts (Kim et al., J. Mol. Cell. Cardiol., in press). The present study was undertaken to assess and confirm this difference at the level of gene expression using competitive reverse transcription (cRT) and competitive polymerase chain reaction (cPCR). CRC mRNA templates of sham and hypertrophied rat heart left ventricles and a known amount of partially deleted CRC RNA (competitor) were amplified by cRT and cPCR in the same tube. The reaction also included CRC primer sets and ^{32}P-labeled dNTP. Glyceraldehyde phosphate dehydrogenase (GAPDH) wild-type and deleted templates were used as controls. Our preliminary results show that whereas GAPDH copy numbers were similar in sham and hypertrophied animals, CRC copy numbers were $13.8 \pm 2.3 \times 10^9$ and $5.0 \pm 1.2 \times 10^9$ per 1 µg mRNA for the sham and the hypertrophy, respectively ($p<0.05$). This decrease in the copy number of CRC mRNA in hypertrophied hearts (64%) is consistent with the lower CRC density in hypertrophied hearts, suggesting that CRC expression during the development of left ventricular hypertrophy is regulated at the transcriptional level.

This study was supported by NIH Grants and Established Investigatorship from American Heart Association (D.H.K.)

M-Pos144

IDENTIFICATION OF CALMODULIN-BINDING DOMAINS IN THE SKELETAL MUSCLE SARCOPLASMIC RETICULUM (SR) Ca^{2+}-RELEASE CHANNEL. ((H. Yang, Delores Needleman, S.L. Hamilton and G.M. Strasburg)) Dept. of Food Science and Human Nutrition, Michigan State University, E. Lansing, MI 48823 and Baylor College of Medicine, Houston, TX 77030.

The Ca^{2+}-release channel of skeletal muscle SR is partially inhibited by the direct binding of calmodulin (CaM). Our previous data (Yang et al., Biochemistry (1994) 33:518] have indicated that there may be several CaM binding sites per channel subunit.. This study was conducted to localize the CaM binding domains in the channel protein primary structure. Rabbit skeletal muscle SR membranes were briefly trypsinized in the presence of 0.1 mM $CaCl_2$ and affinity-labeled with wheat germ CaM derivatized with ^{125}I and benzophenone-4-maleimide (^{125}I-Bz-CaM). Four major CaM binding fragments of the channel protein have been indentified by autoradiography of SDS-PAGE. The apparent molecular masses of the crosslinked products obtained in the presence of 1 mM EGTA were 142,000, 115,000, 68,000, and 48,000. Assuming 1 CaM per fragment, these would correspond to channel protein fragments of 125,000, 98,000, 51,000 and 31,000. When affinity labeling was performed in the presence of 0.1 mM $CaCl_2$, only the two smallest complexes were observed. Sequence specific antibodies are being used to identify the labeled fragments.

Ca-DEPLETION AND Ca-ENTRY

M-Pos145

REGULATION OF DEPLETION-ACTIVATED Ca^{2+} CURRENT BY PROTEIN KINASE IN RBL CELLS ((Anant B. Parekh & Reinhold Penner)) *Max-Planck Institut für biophysikalische Chemie, Am Faßberg, 37077 Göttingen, Germany.*)

Depletion of intracellular Ca^{2+} stores activates a voltage-independent, highly selective Ca^{2+} current I_{CRAC} (Ca^{2+} release activated Ca^{2+} current) in many non-excitable cells. I_{CRAC} was activated in rat basophilic leukaemia cells by dialysing the cells with $InsP_3$ via a patch pipette. In the absence of ATP and using a high buffering capacity, little inactivation of the current was observed over a 5 minute recording period. However, inclusion of ATP in the pipette accelerated the inactivation of I_{CRAC}. The ATP effect was mimicked by ATPγS, an ATP analogue that is readily used by kinases but only weakly dephosphorylated by phosphatases. Application of various activators and inhibitors of protein kinases revealed that a serine/threonine kinase was responsible for this inactivation. Exposure to antigen in sensitised RBL cells resulted in large I_{CRAC} currents and a sustained plateau of Ca^{2+} influx in fura-AM loaded cells, demonstrating that I_{CRAC} is the major source of Ca^{2+} for secretion in these non-excitable cells. Hence the kinase regulation of I_{CRAC} is likely to have widespread implications for key cellular processes like secretion.

M-Pos146

THE ROLE OF $[Ca^{2+}]_i$ IN THE "Ca-DEPLETION-Ca-ENTRY COUPLING". ((Ph. Gailly, E.Hermans and J.M.Gillis)) Physiology and Neurochemistry. Faculty of Medicine, Univ. of Louvain. Belgium.

Internal Ca stores depletion induces a Ca influx to replenish them. The role of Ca in this coupling is controversial. We used transfected CHO fibroblasts expressing the neurotensin (NT) receptor, coupled to the PLC-IP3 cascade, in two series of experiments. (1) Cells were maintained in a 0.2 mM EGTA solution and complete depletion of the Ca stores was obtained either by stimulation with NT (1nM) wich produces large $[Ca^{2+}]_i$ transients, up to 2.5 µM (from 35-50 nM, at rest), or by thapsigargin (15 nM) producing slight $[Ca^{2+}]_i$ increases up to 100 nM. These Ca-depleted cells were extracted following the procedure of Randriamampita & Tsien, 1993 (Nature, 364,809) and the extracts were pressure-injected into normal fibroblasts. This produced $[Ca^{2+}]_i$ transients, amounting to 103 nM (from cell treated with thapsigargin), and three times larger (305 nM) from cells treated with NT, extracts from controls had no effect (n=5, for each condition). We thus confirm the existence of an extractable "*Calcium influx factor*", the efficiency of which seems to depend on the amplitude of the Ca^{2+} rise which accompanied store depletion. (2) Store depletion was also obtained (fibroblasts maintained in a 0.2 mM EGTA solution) with various concentrations (0.01 to 1.0 µM) of thapsigargin to obtain peak $[Ca^{2+}]_i$ transients (1rst peaks) of various amplitudes. Return to normal external Ca^{2+}, produced a second $[Ca^{2+}]_i$ transient. We found that the amplitudes of these second peaks depended on the amplitudes of the first peaks, in a range covering pCa from 7.5 to 5.5. Thus the rise of $[Ca^{2+}]_i$ promotes re-entry of Ca^{2+}.

M-Pos147

OLIGOMYCIN INHIBITS STORE-DEPENDENT CALCIUM INFLUX IN THAPSIGARGIN-TREATED CHO AND JURKAT CELLS. ((J. H. Cho, M. Balasubramanyam, A. Aviv, J. P. Gardner and J. P. Reeves)) Department of Physiology and Hypertension Research Center, UMDNJ-NJ Medical School, Newark, NJ 07103

Thapsigargin (Tg) inhibits SERCA-type ATPases and leads to a permanent depletion of $InsP_3$-sensitive Ca stores, an effect which activates Ca influx pathways in the plasma membrane (store-dependent Ca influx or SDCI). When suspensions of either Chinese hamster ovary (CHO) cells or Jurkat cells where pretreated with 200 nM Tg, oligomycin inhibited the increase in $[Ca]_i$ observed upon addition of extracellular Ca. Half-maximal effects were observed at 0.4, 0.7 and 3.2 μg/ml of oligomycin B, C and A respectively. These effects were not mimicked by NaN_3, rotenone, KCN or ouabain, indicating that oligomycin's effects were independent of its inhibition of mitochondrial energy production or the Na,K-ATPase. Oligomycin also blocked the acceleration of Mn entry elicited by Tg in these cells, as indicated by the rate of quenching of intracellular fura 2 by extracellular Mn. In addition, oligomycin inhibited the Tg-induced increase in ^{45}Ca influx in CHO cells. The effects of oligomycin were greatly reduced in confluent CHO cells grown on a solid substrate compared to non-confluent cells, or cells in suspension. The results indicate that oligomycin inhibits SDCI in these cells; it is not known whether oligomycin inhibits the influx pathway itself or the process by which this pathway is activated.

M-Pos148

Ca^{2+} CHANNEL MODULATION BY 'CALCIUM INFLUX FACTORS' (CIF'S) PRODUCED FOLLOWING THE DEPLETION OF INTRACELLULAR Ca^{2+} STORES. ((B. A. Premack, D. W. Thomas*, M. R. Hanley*, and P. Gardner)) Molecular Pharmacology, Stanford University Medical School and *Biological Chemistry, University of California Davis Medical School. (Spon. P. Gardner).

In many non-excitable cells the depletion of stored intracellular Ca^{2+} initiates Ca^{2+} influx across the plasma membrane. So far, the biochemical events signalling stores depletion and leading to Ca^{2+} channel activation remain obscure. Randriamampita and Tsien (Nature 364: 809, 1993) provided the first evidence for a novel small messenger, termed CIF (calcium influx factor), which was shown to cause Ca^{2+} influx when applied extracellularly to several cell types. We have further examined the diffusible messenger hypothesis using crude and partially purified extracts from Jurkat T cells treated with the Ca^{2+}-ATPase inhibitor thapsigargin (Tg). The extracts at several stages of purification were evaluated for both extracellular and intracellular activity using whole-cell patch-clamp and the Ca^{2+} indicator indo-1. Crude, neutralized, Ba^{2+} and MeOH precipitated, HCl extracts from Tg-treated cells could activate Ca^{2+} influx when applied extracellularly to unstimulated Jurkat cells. However, patch-clamp analysis revealed that this influx was *not* due to stimulation of the stores-depletion Ca^{2+} current, which has been well characterised in these cells. Rather, extracellular application of crude extract appeared to activate a non-selective cation conductance not normally observed in Jurkat cells. Intracellular perfusion with crude extract did not activate non-selective conductances but did significantly increase Ca^{2+} current recorded in response to depletion of the Ca^{2+} stores. To date, the crude extracts appear to modulate, but not to directly activate, Ca^{2+} influx when applied intracellularly in Jurkat cells.

M-Pos149

FAST INACTIVATION OF CA^{2+} RELEASE-ACTIVATED CA^{2+} (CRAC) CHANNELS RESULTS FROM COOPERATIVE CA^{2+} BINDING TO SITES ON THE CHANNEL PROTEIN. ((Adam Zweifach and Richard S. Lewis)) Dept. Mol. Cell. Physiol., Stanford U. Sch. Med., Stanford, Ca 94305.

CRAC channels inactivate within tens of msec during hyperpolarizing pulses to potentials below -40 mV. We have shown previously that inactivation is not voltage-dependent but occurs through the action of Ca^{2+} in private microdomains (A. Zweifach and R.S. Lewis, *Biophys. J.* 66:A153, 1994). The extent of I_{CRAC} inactivation was measured in Jurkat T cells during 200-ms pulses to -80 to -160 mV in the presence of 2 or 22 mM $Ca^{2+}{}_o$. 12 mM intracellular BAPTA reduced inactivation, while 12 mM EGTA did not, implying that the site of Ca^{2+} action is close to the pore. A simple model of Ca^{2+} diffusion in the presence of a highly mobile, nonsaturable chelator (E. Neher, *Exp. Brain Res.* 14:80-96, 1986) predicts that the steady-state $[Ca^{2+}]_i$ gradient near the pore is steeper in the presence of $BAPTA_i$ relative to $EGTA_i$ due to BAPTA's ~400-fold faster Ca^{2+} binding rate. Estimates of single-channel current under the different conditions listed above were used to compute $[Ca^{2+}]_i$ profiles, allowing us to determine the location and affinity of the Ca^{2+} binding site(s). Data obtained with $BAPTA_i$ and $EGTA_i$ are predicted by the model to exhibit identical Ca^{2+} dependence only if the binding site(s) for Ca^{2+} are assumed to be 3-4 nm from the mouth of the pore, consistent with a location on the channel protein itself. The predicted Ca^{2+} sensitivity of inactivation is best described by the Hill equation with $n_H = 2.0$ and $K_{1/2} = 3.6$ μM, suggesting that at least two Ca^{2+} ions must bind cooperatively to induce inactivation.

M-Pos150

PURINERGIC RECEPTOR STIMULATION IN RAT MEGAKARYOCYTES ACTIVATES RECEPTOR-OPERATED, SECOND MESENGER-OPERATED AND CALCIUM-RELEASE-ACTIVATED CURRENTS. ((B.Somasundaram and Martyn, P.Mahaut-Smith)) The Physiological Laboratory, Cambridge UK. (Spon.by J.Requena)

Simultaneous patch clamp and fura-2 fluoresence measurements were used to study ATP-evoked membrane currents and intracellular ($[Ca^{2+}]_i$) changes in rat megakaryocytes. At negative potentials, under conditions that blocked K^+currents, 20μM ATP activated a biphasic inward current and a concurrent biphasic increase in $[Ca^{2+}]_i$. The initial increase in $[Ca^{2+}]_i$ was due to influx whereas the delayed increase was at least partly due to the release of internal Ca^{2+} stores.The initial current had the characteristics of a receptor operated channel and was carried by both Na^+ and Ca^{2+}. The delayed current was also transient and carried by monovalent cations; this conductance did not require an increase in $[Ca^{2+}]_i$ for activation but was triggered by 10μM IP_3 in the pipette. Buffering internal Ca^{2+} with 20mM BAPTA revealed a third current activated by Ca^{2+} release from internal stores. This channel was highly selective for Ca^{2+}. ATPγS, like ATP, activated all three currents wheras ADP only stimulated the calcium release and the two currents associated with it.Increasing the external divalent cation concentration abolished the ATP-evoked Ca^{2+} release and delayed currents but not the initial transient current. We conclude that rat megakaryocytes express two types of purinergic receptor. One is activated by ATP,and closely coupled to a non selective cation channel; the other recognises ATP^{4-} and ADP^{3-} and activates Ca^{2+} release and two types of membrane conductance, one selective for monovalent cations, another selective for Ca^{2+}.

M-Pos151

ACTIVATION OF STORE-DEPENDENT CALCIUM CURRENTS IN RAT MEGAKARYOCYTES IS BLOCKED BY PRIMAQUINE, AN INHIBITOR OF VESICULAR TRANSPORT. ((B.Somasundaram, J.C.Norman & M.P.Mahaut-Smith)) Physiological Laboratory, Cambridge, U.K. (Spon. by R.D.Keynes)

In many non-excitable cell types, release of calcium from intracellular stores stimulates a calcium-selective current (I_{crac}) in the plasma membrane. We are studying the mechanism coupling store depletion to opening of membrane calcium channels in rat megakaryocytes using whole-cell patch clamp. The pipette contained a Csgluconate saline and internal Ca^{2+} was strongly buffered with 10mM Cs_4BAPTA. Emptying of internal Ca^{2+} stores with 3μM ionomycin activated a Ca^{2+}-selective current indistinguishable from I_{crac} previously reported in other cells. Dialysis of the cytoplasm by the pipette saline removed a factor required for I_{crac} activation, therefore to compare I_{crac} in different cells, ionomycin was applied within a standard time range, normalised for series resistance, of 10-15 s $M\Omega^{-1}$. Addition of primaquine (PQ), a blocker of vesicle transport, prior to emptying of internal Ca^{2+} stores, inhibited I_{crac} with a half-maximal concentration of about 100μM. This is similar to the concentration of PQ required to block vesicular transport and receptor recycling in a number of cell lines. A maximal dose of PQ (1mM) caused a 90±5% inhibition. 1mM PQ added after activation of I_{crac} caused only a small (approx. 10%), slow (2-5 min) block of the current indicating a minimal effect of PQ on the channels underlying I_{crac}. A related compound, chloroquine, which is less effective at blocking vesicular secretion, had no effect on I_{crac}. GTPγS (1mM in the pipette), also an established inhibitor of vesicular transport, reduced I_{crac} by 56±20%. The recognised role of GTPases in regulation of vesicular trafficking, together with block of I_{crac} activation by PQ provide evidence that CRAC channels may be stored in a vesicular membrane compartment and transferred to the plasma membrane following store depletion.

Funded by the BHF & BBSRC

M-Pos152

A NOVEL Ca^{2+} INFLUX PATHWAY ACTIVATED IN THAPSIGARGIN-TREATED DDT_1MF-2 SMOOTH MUSCLE CELLS ((A.D. Short, C.A. Ufret-Vincenty, and D.L. Gill) Department of Biological Chemistry, University of Maryland School of Medicine, Baltimore, MD 21201.

Ca^{2+} influx activated by Ca^{2+} pool depletion represents an important component of Ca^{2+} signals generated in cells. This capacitative entry process varies widely between different cell types. In DDT_1MF-2 smooth muscle cells, the influx component after emptying pools with either thapsigargin (TG) or agonists (e.g. bradykinin) is small and short-lived, even though Ca^{2+} released from pools Ca^{2+} represents a substantial Ca^{2+} component of signals. After treatment of DDT_1MF-2 cells for 16 hr with 3 μM TG, capacitative Ca^{2+} entry is considerably enhanced as revealed by transient $Ca^{2+}{}_o$ removal (the Ca^{2+} "overshoot" response) with fura-2-loaded cells. Interestingly, 10 mM caffeine also induced a large transient influx of Ca^{2+} (i.e. dependent on $Ca^{2+}{}_o$), but not dependent on transient $Ca^{2+}{}_o$ removal. When added immediately following a transient $Ca^{2+}{}_o$ removal, caffeine induced a very large transient influx of Ca^{2+}; this additive effect suggested caffeine was inducing a distinct influx mechanism from capacitative Ca^{2+} entry. Normal DDT_1MF-2 cells (i.e. not TG-treated) had no effect of caffeine or other methylxanthines. Mn^{2+}-quench experiments revealed that caffeine induced a 3.5-fold increase in the rate of Mn^{2+} entry; 10 mM theophylline induced a larger and more rapid Ca^{2+} transient than caffeine, and a 5.2-fold increase in Mn^{2+} entry rate. In contrast, capacitative entry in these cells did not allow passage of Mn^{2+}, providing more evidence of a distinct methylxanthine-activated channel mechanism. Dibutyryl cAMP or cGMP, isobutylmethylxanthine, and forskolin had no effects, indicating no direct action of cyclic nucleotides. 3,7-dimethyl-1-propargylxanthine, a good methylxanthine activator of ryanodine receptors, was almost without effect indicating the noninvolvement of ryanodine receptors. The Ca^{2+} entry inhibitor, SKF 96365 did not block methylxanthine-induced Ca^{2+} entry. Although 0.5 μM nitroprusside and 15 mM $NaNO_2$ induced substantial Ca^{2+} influx, unlike the actions of methylxanthines the effects were seen in normal as well as TG-treated cells. The results indicate that in TG-treated cells, a novel methylxanthine-sensitive Ca^{2+} influx pathway exists which appears distinct from capacitative Ca^{2+} entry. (NIH grant NS19304; NSF grant DCB 9307746).

M-Pos153

SWELLING- AND DEPLETION-ACTIVATED Ca^{2+} INFLUX PATHWAYS IN MOUSE THYMOCYTES.

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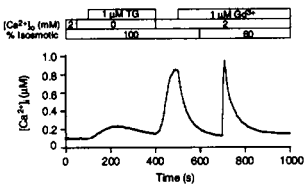
Using fura-2 imaging, we characterized two Ca^{2+} influx pathways in thymocytes. 1) Superfusion with 60% isosmotic media elicited a sharp rise in $[Ca^{2+}]_i$ in 77% of thymocytes. $[Ca^{2+}]_i$ rose steeply after a delay of ≈ 70 s to ≈ 650 nM from resting levels of ≈ 100 nM. Cell phenotyping demonstrated that $[Ca^{2+}]_i$ transients were primarily associated with immature $CD4^-CD8^-$ and $CD4^+CD8^+$ thymocytes. Mature mouse or human T-cells did not display $[Ca^{2+}]_i$ transients. Thymocytes swollen in Ca^{2+}-free media failed to respond, indicating that Ca^{2+} influx underlies $[Ca^{2+}]_i$ transients. Furthermore, the lanthanides Gd^{3+} and La^{3+} inhibited swelling-induced Ca^{2+} influx with K_d's of 3.8 and 2.4 μM, respectively. 2) Thymocytes treated with 1 μM thapsigargin (TG), a Ca^{2+}-ATPase inhibitor which depletes intracellular Ca^{2+} stores, displayed a rise in $[Ca^{2+}]_i$ that peaked at ≈ 900 nM. TG stimulated Ca^{2+} influx in all four classes of CD4/CD8 thymocytes and in mature T-cells. Gd^{3+} block of depletion-activated Ca^{2+} influx was 136-fold more potent (K_d = 28 nM) than block of swelling-induced transients. Swelling-induced Ca^{2+} influx could be observed after the complete inhibition of depletion-activated Ca^{2+} influx with 1 μM Gd^{3+} (*see Figure*). Therefore, we conclude that swelling- and depletion-activated Ca^{2+} influx represent distinct Ca^{2+} entry pathways through the plasma membrane of thymocytes.



Supported by NIH grant NS14609.

MUSCLE REGULATORY PROTEINS I

M-Pos154

CHARACTERIZATION OF STRIATED MUSCLE ISOFORMS OF TROPOMYOSIN FROM SALMONID FISH.

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Tropomyosin (TM) was isolated from the cardiac muscle, and the fast and slow trunk muscles of mature salmonid fish. The TMs consisted of the following, distinct and exclusively distributed, isoforms: one each for fast and cardiac, and two for slow. On a variety of gel systems the cardiac and slow isoforms migrated in close proximity to rabbit α TM while the fast isoform migrated in close proximity to rabbit β TM. All of the purified fish TMs were predominantly unphosphorylated and were blocked. Upon carboxypeptidase-A treatment, histidine was released from slow TM and cardiac TM but not fast TM (which contained two asparagines). All other amino acids were either similar or identical. In viscometry experiments, carried out at temperatures close to physiological (T = 5°C, pH = 7.00), the strength of the end to end interaction increased in the order: slow < cardiac < fast. The coding sequence of fast TM was determined and found, overall, to more closely resemble α-type TMs than β-type TMs. The sequence was corroborated by a number of lines of evidence, including mass spectral analysis of fragments spanning over 90% of the molecule. Thus, electrophoretic mobility is not in all cases a suitable criterion to classify striated muscle isoforms of TM.

M-Pos155

A MOLECULAR MECHANICS APPROACH TO ACTIN-TROPOMYOSIN INTERACTIONS. I. TROPOMYOSIN.

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A molecular mechanics model for rabbit tropomyosin was constructed from low-resolution Cα structure (Phillips et al, 1986; Brookhaven pdb2tma.ent, 1988), using SYBYL. Main-chain and side-chain atoms were added, followed by geometric minimization and energy minimization using the Kollman-unified atom force-field, non-bonded cutoff 10Å, and distant-dependent dielectric constant, with iterations continued until convergence at ΔE < 0.01 kcal/mole. Tropomyosin is known to have periodicities along its heptad sequences (McLachlan & Stewart, 1976; Hitchcock & associates, 1990). The molecular mechanics model shows comparable features. The charged groups at heptad positions (b, c, f) > (e, g) are of special interest. The external charges which may interact with actin are taken as those with distance ρ > 6Å between charged group and local center-of-mass. The external charge density of H^+-groups and net charge show peaks along the tropomyosin axis, with period ~60Å. The azimuthal region of tropomyosin which may interact with actin varies with axial distance. Allowing for this shift, external charge density of tropomyosin shows striking variation with azimuthal angle, with period ~90°. The lysine-arginine residues in the repeat regions which may interact with actin include pairs of adjacent residues in 5 of 7 cycles. The charge-repeat regions are also characterized by strong van der Waals interactions between (a,d) residues of the 2 chains, which would tend to stabilize coiled-coil structure in these regions.

M-Pos156

A MOLECULAR MECHANICS APPROACH TO ACTIN-TROPOMYOSIN INTERACTIONS. II. ACTIN-TROPOMYOSIN IN DIFFERENT Ca^{2+}-STATES.

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Recent x-ray and electron microscopic studies localize tropomyosin (TM) along the actin filament, and Lorenz (1994) has identified TM binding sites near actin 203-216 and 300-328 in Ca^{2+}-ON state. A molecular mechanics model was developed for interaction of TM with actin in different Ca^{2+}-states. Actin filament coordinates were kindly provided by M. Lorenz (JMB 1993). Charged groups of actin which may interact with TM form 3 groups as to azimuthal position and net charge: OFF, NEAR-ON, and FAR-ON regions. TM contains charged groups with 60Å axial repeat and 90° azimuthal period. This suggests a simple electrostatic model for ON-OFF transition. In OFF-state, negatively-charged residues (asp25, glu334) may interact with positively-charged TM residues. A 90° clockwise rotation of TM brings negatively-charged TM residues near positively-charged actin residues in NEAR-ON region, and further 90° rotation brings positively-charged TM groups (opposite chain from OFF-state) near negatively-charged actin groups in FAR-ON region. This scheme was elaborated into a molecular model for each state by energy minimization of TM(1-106) and 3 actin monomers in sequence, using the Kollman-unified atom force-field, with constraint-free iterations to convergence ΔE<0.01 kcal/mol. All 3 actin-TM models show more favorable energies than actin + TM, reflecting acto-TM interactions and induced structural changes in TM. Periodic interactions and local changes in each state are further detailed by energy calculations along actin-TM complex.

M-Pos157

USE OF A TRANSGENIC (TG) MOUSE MODEL TO INVESTIGATE THE ROLE OF TROPOMYOSIN (Tm) ISOFORMS IN THE REGULATION OF CARDIAC MYOFILAMENT ACTIVATION.

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We examined the functional significance of Tm isoforms in cardiac myofilament activation by the use of TG mice which overexpress striated muscle specific ß-Tm in the heart. Although ß-Tm message is abundant, preliminary results suggest that the total amount of Tm in the thin filament does not change thus indicating that the relative amount of α-Tm has decreased in the myofilament. Force generated by skinned fiber bundles was measured as a function of pCa and pMgATP, both alone, and in the presence of MCI-154, a pharmacological agent that alters the myofilament response to Ca^{2+}. In myofilaments demonstrating a ß/α-Tm ratio of 0.7, there was a small but significant leftward shift of the pCa-force and the pMgATP-force relation (as measured at pCa 9.0) when compared to the nontransgenic (NTG) controls (100% α-Tm expression). In myofilaments with a ß/α-Tm ratio of ≥ 1.9 there was a significant rightward shift of the pCa-force relation. MCI-154 significantly increased the maximal force in these TG fibers but the effect was not as pronounced as that demonstrated in the NTG controls. Our results indicate that the higher the proportion of ß-Tm in the population, the lower the sensitivity to Ca^{2+}, the lower the cooperative activation of the thin filaments by strong cross-bridges, and the greater the attenuation of the effects of MCI-154 on the myofilament response to Ca^{2+}.

M-Pos158

REGULATED ACTOMYOSIN ATPASE AND THE SIX STATE MODEL

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There has been a great deal of interest in the regulation of muscle contraction. Prior biochemical studies have demonstrated that the binding of regulated actin to S-1-ATP is unchanged at low [Ca^{2+}], even though the ATPase activity of regulated actomyosin is inhibited under these conditions. Prior structural studies using Xray diffraction techniques have suggested that the tropomyosin-troponin complex may move and inhibit the actomyosin interaction at low [Ca^{2+}] (i.e., steric blocking). In physiologic fiber experiments, 'weak-binding' crossbridges have been found to bind to the actin filament at low [Ca^{2+}], especially at low ionic strength, and other experiments have suggested that Pi release is not directly regulated by calcium. In biochemical studies in the absence of ATP, inhibition of the binding of strong binding states have been reported in both equilibrium and transient kinetic studies. The current work suggests that all of these observations can be explained in terms of a six state model in which regulation affects one particular weak binding state that contains strongly bound ADP and Pi: AM-ADP-Pi_2, and this implies that regulation affects a kinetic transition as well as a weak binding isotherm.

M-Pos159

CALCIUM REGULATION OF CARDIAC NATIVE THIN FILAMENT MOVEMENT IN MOTILITY ASSAYS. ((E. Homsher, J. Chang, B. Kim, and L. Tobacman)) Physiol. Dept. UCLA, Los Angeles, CA 90024 and *Dept. of Internal Med., University of Iowa Coll. of Medicine, Iowa City, IA 52242.

In motility assays of reconstituted thin filaments, Honda and Asakura (1989) found a very steep relationship between filament sliding velocity and pCa in what appears to be almost an "on or off" type of regulation. We examined calcium regulation in motility assays with "native" thin filaments isolated from bovine cardiac muscle (Tobacman and Sawyer, 1990) using a rabbit skeletal HMM motor assay and analysis system similar to previously described (Sellers, et al 1993). [Ca^{+2}] was varied from pCa 9 to 4.5. At pCa >7, fewer than 25% as many filaments bound to the surface as at pCa 4.5, and as the pCa concentration rose, the number of filaments bound to the surface increased. Further between pCa 7 to 9 more than 65% of the filaments bound to the surface did not move at all and a small fraction (<2%) moved uniformly at a velocity that was 90% maximal. As the pCa declined from 7 to 4.5 the number of filaments moving at a uniform velocity progressively increased with the [Ca^{+2}] and the number of non-moving filaments decreased markedly until at pCa 4.5 more than 80% of the filaments were moving and >15% of the filaments were moving at a uniform velocity. At each pCa those filaments moving at a uniform velocity moved at the maximal rate (2-5 μm/s, depending on the HMM prep) which was itself independent of pCa. Unregulated f-actin filaments exhibited no dependence on pCa. Thin filament movement at pCa 9 might be a result of dissociation of the regulatory proteins (troponin, Tn, and tropomyosin, Tm) from the native filaments. However, addition of 1μM Tm/Tn to motility buffers did not significantly reduce the fraction of filaments moving at pCa 9. These results are most consistent with a regulatory mechanism in which calcium controls the number of attached and cycling crossbridges. Supported by NIH grants AR-30988 [EH] and HL-38834 [LT]).

M-Pos160

CERAMIDE STIMULATES DEPHOSPHORYLATION OF CARDIAC MYOFILAMENT PROTEINS. ((XuPei Huang and Jeffery W. Walker)) Department of Physiology, University of Wisconsin, Madison, WI 53706

Ceramide, a naturally occurring lipid formed by hydrolysis of sphingomyelin, has been hypothesized to be a second messenger that controls cell growth and differentiation (Hannun & Bell, Adv. Lipid Res. 1993,25:27). While its principal molecular targets are unknown, ceramide has been shown to activate certain protein kinases and protein phosphatases. We tested the effects of a cell-permeant ceramide, C_2-ceramide, on the level of phosphorylation of cardiac myofilament proteins. Rat ventricular myocytes were isolated by collagenase digestion, then stimulated with the β-agonist isoproterenol (Iso), the protein kinase C activator phorbol myristate acetate (PMA), or the phosphatase inhibitor okadaic acid (OA). Phosphorylation was assessed by pre-labelling cells with ^{32}P-P_i, or by back phosphorylation using protein kinase A/protein kinase C and γ-^{32}P-ATP after skinning with Triton X-100. Iso (0.1-1 μM) increased phosphorylation of troponin I (TnI) and C-protein by 3-6 fold, whereas PMA (100 nM) and OA (10 μM) increased phosphorylation of TnI, C-protein and myosin light chain 2 (LC_2) by 1.5-3 fold. Treatment of intact myocytes with C_2-ceramide (20-100 μM) decreased the level of phosphorylation of TnI and LC_2 by 10-50%, regardless of how phosphorylation had been stimulated. C_2-Ceramide treatment of Triton X-100 skinned myocytes also promoted protein dephosphorylation. The results suggest that ceramide can activate protein phosphatases in cardiac myocytes, thereby reversing the actions of specific kinases on the regulatory proteins TnI and LC_2.

M-Pos161

EFFECT OF MUTATIONS IN HELIX C OF TROPONIN C ON THE Ca^{2+} REGULATION OF MUSCLE CONTRACTION. ((B. Parsons, A. Mandveno, R. Zhang, J. Zhao, J.D. Potter)) Dept. of Mol. & Cell. Pharm., Univ. of Miami Sch. of Med., Miami, FL 33136

In order to investigate the role of helix-C in the regulation of contraction we have prepared a mutant (SUB5262) in which we have changed negatively charged helix-C amino acid residues (T53,E55,E56,Q58,E59,D62,E63) in mouse cardiac TnC (MCTnC) into neutrally charged alanine residues using site directed mutagenesis. We also prepared a mutant (5262) with amino acids 52-62 deleted. Endogenous CTnC was extracted from a cardiac skinned muscle preparation (CSM)and the two mutants dissolved in pCa 8 relaxing solution were tested for their ability to rebind to the CTnC depleted CSM. Both mutants rebound to the fiber but neither was able to activate force in the pCa 4, contraction solution. Evidence that the two proteins rebound to the fiber comes from the fact that addition of wildtype (WT) mouse CTnC to the mutant substituted fibers failed to generate any additional force. An energy minimized (CVFF force field) model of the crystal structure of TnC mutated to SUB5262 indicates that the WT tertiary structure in helix C is not altered from its original α-helical structure. It is possible that an interaction may occur between this negatively charged region of TnC and the positively charged region of 96-115 in TnI. We are currently investigating the interaction of these mutants with CTnC. (Supported by NIH HL42325, AR37701 & AR40727).

M-Pos162

EFFECTS OF GENETICALLY ENGINEERED TnC ON FORCE PRODUCTION IN SKINNED MYOFIBRILLAR BUNDLES FROM THE BARNACLE BALANUS NUBILUS. ((*CC Ashley, *T Miller, *S Lipscomb, *JD Potter)) *Univ. Lab Phys., Oxford UK, **Friday Harbor Labs, WA, *Dept. of Mol. & Cell. Pharm., Univ. of Miami Sch. of Med., Miami, FL 33101.

Recombinant $BTnC_2$ ($rBTnC_2$) containing mutations of either Ca^{2+} binding site II or Ca^{2+} binding site IV were studied in native TnC depleted muscle fibers. Previous studies (Collins *et al.*,Biochem. 30:702, 1991; Ashley *et al.*, J. Mus. Res. 12:532 1991) suggested that only sites II and IV bind Ca^{2+} in $BTnC_2$. To test the function of sites II & IV, mutants were prepared where either site II or site IV was rendered unable to bind Ca^{2+} by insertion of an ALA for an ASP residue in the X coordinating position of each site. Myofibrillar bundles (100-150 μm diam) were isolated from single depressor muscle fibers. These bundles lost their Ca^{2+} sensitivity as judged by the loss of the force response when challenged at pCa 3.0 after exposure to 10 mM orthovanadate in relaxing solution (pCa 7.0, pH 6.8, I=0.15M for 5 min. @ 20 C). Subsequent exposure (30 min at 2 mg/ml) to native $BTnC_2$, $rBTnC_2$ or $rBTnC_2$ lacking either Ca^{2+} binding to site II or site IV dissolved in relaxing solution, were all able to bind in the absence of Ca^{2+} and restored force responses subsequently at pCa 3. These findings are in contrast to our earlier findings where $rBTnC_2$ lacking the last eleven amino acids at the C terminal end and hence most of site IV (TRUNC), only regulated force in the presence of Ca^{2+}. (Supported by MDA, NIH HL42325, AR37701, & AR40727).

M-Pos163

PREDICTED SOLUTION STRUCTURES OF TROPONIN C. ((Alan Mandveno, Bruce Parsons, Ren Zhang, and James D. Potter)) Dept. of Mol. & Cell. Pharm., Univ. of Miami Sch. of Med., Miami, FL 33136

Solution studies of skeletal troponin C (STnC) at physiological pH indicate that amino acid distances are significantly different from values obtained from the pH 5.1 derived crystal structure. Heidorn and Trewhella's (*Biochem.* 1988, 27:909) data suggest that TnC folds at glycine-92 while maintaining the fundamental dimensions of each domain. We computer modeled (Biosym Inc.) a modified crystal structure of chicken STnC based on published distances of $2Mg^{2+}$-TnC in solution: glycine 92 angle=65.4°, M28 to C101=15Å±15, F13 to F112=15Å±5, D36 to Q85=23Å±2. We also modeled a modified crystal structure of chicken STnC based on solution measurements of $4Ca^{2+}$-TnC: M28 to C101=22Å±4, F13 to F112=15Å±5, D36 to Q85=23Å±2. Energy minimization of the above STnC's led to conformations with distances well within half width distributions of the solution measured mean distances. Interestingly, the minimized models predicted that methionine-28 is exposed in the $2Mg^{2+}$-TnC but buried in the $4Ca^{2+}$-TnC state which is in agreement with experimentally measured DANZ labeled TnC fluorescence. These results demonstrate that empirically derived template forcing results in energetically feasible models upon which biophysical predictions can be made. (Supported by NIH HL42325, AR37701 & AR40727).

M-Pos164

N- & C-TERMINAL DOMAIN RECOMBINANT FRAGMENTS OF SKELETAL TnC AND TENSION REGULATION IN SKELETAL MYOFIBERS. ((*J. Gulati, A. Babu Akella and Hong Su*)) The Molecular Physiology Lab, Albert Einstein College of Medicine, Bronx, NY 10461

Skeletal TnC is a dumbbell shaped protein, with Ca-specific sites I and II in the N-terminal lobe joined by a long central helix to Ca-Mg sites III and IV in the C-terminal lobe. Sites I and II form the trigger domain for the Ca-switch and sites III and IV the structural domain for anchoring TnC to the regulatory complex. But whether or not these functions depend on the dumbbell structure of TnC is unclear. Presently, we synthesized two fragments of rabbit sTnC to test this: (1) N-terminal fragment, comprised residues 1-102 and contained sites I and II as well as the central helix. (2) C-terminal fragment, comprised residues 73-159 and sites III and IV as well as the central helix. The regulation of force development was investigated in skinned skeletal myofibers (rabbit muscle) devoid of endogenous sTnC. The N-fragment fully restored the force regulation, but required continuous presence of the fragment in the activating medium. The C-fragment was unable to restore Ca-regulation under any of the conditions examined so far. The findings indicate that sites I and II retain the triggering function in the fragment and can regulate the contraction switch independently of the putative structural sites in the C-terminal domain of TnC. Furthermore, the presence of the central helix may be essential to maintain active configuration of the N-terminal regulatory sites. This is currently under study with recombinant proteins deleting a portion of the central helix.

M-Pos165

NMR STUDIES OF CALCIUM BINDING TO C-DOMAIN OF CHICKEN TROPONIN C. ((Monica X. Li, Larry A. Calhoun, Lawrence B. Smillie and Brian D. Sykes)) MRC Group in Protein Structure and Function, Department of Biochemistry, University of Alberta, Edmonton, Canada T6G 2H7.

This work was directed toward elucidating the mechanism for calcium binding to the C-terminal domain of troponin C (TnC). The mechanism has been investigated with the use of heteronuclear multidimensional NMR spectroscopy. Recombinant C-domain (residues 88-162) of chicken TnC has been cloned in pET3a vector and expressed in minimal media to allow uniform ^{15}N and ^{13}C labeling. The NMR spectra have been resolved and assigned. Calcium titration monitored by ^{1}H, ^{15}N HMQC spectral changes revealed that two equivalents of calcium are required to fold the entire C-terminal fragment completely and that calcium binding to sites III and IV of TnC undergoes a cooperative transition with dissociation constants in the range of 0.1-1μM. This mechanism is consistent with that obtained from our previous flourescence and far UV CD studies on C-domain fragment (88-162) (Li *et al.*, (1994) *Biochemistry* 33, 917-925). (Supported by MRC of Canada and the Alberta Heritage Foundation for Medical Research.)

M-Pos166

CHARACTERIZATION OF TNC BINDING TO EXTRACTED MYOFIBRILS. ((Darl R. Swartz)) Anatomy Department, Indiana University School of Medicine, Indianapolis, IN 46202

Troponin C (TnC) is the calcium-binding subunit of troponin that acts as the primary switch for activation of contraction. The interactions between the troponin subunits have been characterized in solution studies, but they have not been studied in native (myofibrillar) thin filaments. Native TnC was removed from myofibrils by extraction with Tris-EDTA buffer, and the binding of TnC to thin filaments was investigated using fluorescent conjugates of TnC and fluorescence microscopy. Conjugates of TnC were prepared using thiol-specific probes (rhodamine-X-iodoacetamide and fluorescein-5-iodoacetamide). Functional studies (in a solid-phase assay) showed that the conjugates bound TnI in a calcium-sensitive manner. In highly extracted myofibrils, the pattern of fluorescent TnC binding was dependent upon concentration of TnC, pCa, and crossbridge status (rigor or relaxed). At pCa 9.0, fluorescent TnC bound in the overlap region; when saturating levels of S1 were added at pCa 9.0, fluorescent TnC bound in both the overlap regoin and I-band. At pCa 4.5 and under rigor conditions, binding was observed in the overlap region at low [TnC] without S1 and in the overlap region and I-band with S1. Higher levels of TnC at pCa 4.5 without S1 resulted in TnC binding in both the overlap and I-band region. When fluorescent TnC was incubated with myofibrils under relaxing conditions (ATP, pCa 9.0), the binding of TnC was much less than that observed under rigor conditions. When partially extracted myofibrils were incubated with fluorescent TnC under conditions which give maximal incorporation, most of the fluorescence was observed in the I-band rather than the overlap region suggesting that extraction occurred preferentially in the I-band region. These observations suggest that both calcium and strong-binding crossbridges influence the interaction between TnC and TnI/TnT; calcium via binding to TnC and crossbridges via conformational changes in tropomyosin/TnT/TnI.

M-Pos167

MODIFICATIONS IN THE N-AND D-HELICES ALTER TROPONIN C STABILITY AND CONFORMATION ((L. SMITH)) Dept. of Neuroscience and Cell Biology, Robert Wood Johnson Medical School, Piscataway, NJ 08854.

Deletion of the N-helix of chicken skeletal TnC (Δ14-TnC) affected the stability, regulation of the actomyosin ATPase, Ca^{2+}affinity and increase in the α-helix upon Ca^{2+} binding to the high affinity sites. (Smith et al.,1994, *JBC* 269:9857-9863). To determine the minimum length of the N-helix required for TnC function, residues 1-7 (Δ7-TnC) were deleted. CD studies showed that the deletion had no affect on the stability or the increase in α-helix upon Ca^{2+} binding. Thus, residues 8-14 of the N-helix are sufficient. The X-ray structure of TnC shows there is a salt bridge between Arg11 in the N-helix and Glu76 in the D-helix. To learn if these residues and / or their interaction are important for TnC function, they were replaced with cysteines. Cys 101 was substituted with Leu. The consequences of the mutation (red-11/76 TnC) and its crosslinked (ox-11/76 TnC) form were investigated using CD. The thermal denaturation curves of red-11/76 TnC show that it is as stable as wildtype TnC in the presence of Mg/EGTA and Ca^{2+}(T_m=70°C and 90°C, respectively). In contrast, in the apo-state, the major transition occurs at 42°C compared to 65°C for wildtype TnC. In the absence of calcium, only the N-terminal domain is believed to be folded. Therefore, the mutation has destabilized the N-terminal domain of TnC. Disulfide crosslinking (ox-11/76TnC) showed no improvement. Ca^{2+} binding monitored by CD, demonstrated that binding of Ca^{2+} to the high affinity sites resulted in only 60% of the maximum change for red-11/76 and ox-11/76 TnC compared to wildtype TnC. Moreover, the Ca^{2+}affinity is altered; increased for the high affinity sites and reduced for the low affinity sites. These results show that Arg 11 and Glu 76 may be essential for the calcium-induced conformational change and the overall stability of TnC. (Sup. NIH and MDA).

M-Pos168

CALCIUM-INDUCED STRUCTURAL CHANGES IN TROPONIN-C. ((Stéphane M. Gagné, Sakae Tsuda, Monica X. Li, Lawrence B. Smillie, and Brian D. Sykes)) MRC Group in Protein Structure and Function, Department of Biochemistry, University of Alberta, Edmonton, Alberta, T6G 2H7.

In the thin filament of skeletal muscle, conformational changes are induced in the protein troponin-C (TnC) by the binding of two calcium ions to the N-domain of the protein; this is the first step in a process that ultimately leads to muscle contraction. We have solved the three-dimensional solution structures of the apo and Ca^{++}-saturated states of the N-domain of TnC, based on a series of multinuclear 2D and 3D nuclear magnetic resonance (NMR) experiments. The study has been done on the $^{15}N/^{13}C$-labelled recombinant regulatory domain (1-90) of chicken skeletal TnC. The high quality solution structures in the apo and Ca^{++} state clearly indicate an opening of the regulatory domain of TnC upon Ca^{++}-binding, due to the reorientation of two helices. This opening of the structure, along with some detailed structural changes, provides information about the mechanism of the muscle contraction trigger, TnC.

M-Pos169

CALCIUM-INDUCED FLUORESCENCE CHANGES OF PROBES ATTACHED TO CYS-84 OF CARDIAC TROPONIN C. ((W.-J. Dong and H.C. Cheung)) Dept. Biochem. & Mol. Genetics, Univ. Alabama at Birmingham, Birmingham, AL 35294.

Cys-84 of central helix D in the N-domain of cardiac troponin C (cTnC) was selectively labeled with the fluorescent probes IAANS and acrylodan in the presence of bound Ca^{2+} at site II. The fluorescence of the attached probes was studied to gain an insight about activator Ca^{2+}-induced conformational changes in the regulatory region of cTnC. The experimental emission spectra, quantum yields and lifetimes do show Ca^{2+}-induced conformational changes of cTnC in the region of Cys-84. The accessibility of the attached probes was determined from quenched lifetime data. A two-fold increase of the bimolecular collisional quenching constant was observed with both probes in the presence of calcium. This increased exposure of residue 84 to the solvent is consistent with the H-M-J model of calcium activation. Calcium also induced blue shifts of the emission spectra, increase in emission intensity and the excited state lifetimes. Interpretation of these spectral changes is not straight forward because the Ca^{2+}-induced spectral changes are not consistent with increased exposure to the solvent. The fluorescence results obtained from the probes dissolved in various solvents provide an insight into possible changes in the interactions between the attached probes and neighboring residues in both apo and 3Ca states. In the absence of bound Ca^{2+}, the observed spectral properties suggest interactions between the attached probes and neighboring charged side chains. These interactions are disrupted when site II is filled with Ca^{2+}, resulting in the observed blue shifts of the emission spectra and increases in lifetimes. The effect of increased exposure apparently is overcompensated by the effect of elimination of the interactions. The origin of these specific interactions is not yet clear. Supported in part by NIH and by MDA (WJD).

M-Pos170

INTRAMOLECULAR DISTANCES IN SKELETAL TROPONIN C MUTANTS.
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The binding of activator calcium to troponin C from skeletal muscle is believed to induce reorientations of several helices in the N-domain, thus creating a more "open" conformation favorable for interaction with troponin I. Certain inter-site distances are expected to be longer in the "open" conformation than in the "close" conformation. We have studied this by using four engineered chicken fast skeletal troponin C mutants generated by site-directed mutagenesis. Each mutant contained a Trp-Cys pair suitable for determination of inter-site distance by fluorescence resonance energy transfer, with Trp serving as energy donor and Cys for attachment of an energy acceptor. The mutants are: (1) F22W/N52C/C101L, (2) F13C/N52W/C101L, (3) N52C/A90W/C101L, and (4) F13C/A90W/C101L. Mutant (1) has W on helix A and C on the helices B-C linker; mutant (2) has W on the linker and C on helix A; mutant (3) has W on the central helix and C on the B-C linker; and mutant (4) has W on the central helix and C on helix A. The single C residue in each mutant was separately modified with the following acceptor probes: IAEDANS, IAANS, and MIANS. Donor-acceptor distances were determined by the steady-state method at 20 °C in 0.1 M KCl, 25 mM MOPS, pH 7.2. In the presence of activator Ca^{2+}, an increase in distance was observed in all 4 mutants. The magnitude of the increase was probe-dependent and ranged from less than 1 Å to over 6 Å (mutant 3 with IAEDANS as acceptor). In general, the observed increases were smaller than have been anticipated from the modeled $4Ca^{2+}$ structure of troponin C.

M-Pos171

THE N-HELIX OF cTnC IS SPECIFIC FOR Ca^{2+}-SWITCHING MECHANISM OF CARDIAC CONTRACTION AND CAN NOT BE REPLACED BY THE N-HELIX OF sTnC.
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Troponin C (TnC) has a 10 residue α helix (Nt-helix) at the extreme N-terminus that is highly variable between cardiac and skeletal TnC isoforms. We previously indicated that the N-helix arm of sTnC has a specific role in the switching mechanism for contractile activation of skeletal muscle (Ding et al, *Protein Science*, 1994). The maximal contractile force in myofiber was diminished with a modified sTnC in which either the Nt-helix was deleted (ΔNt•sTnC4) or was replaced with the cardiac arm (cNt•sTnC4). Because site I in cTnC is Ca-deficient, uncertainty remained regarding the specificity of cNt in cardiac TnC. To investigate this, we made mutants of bovine cTnC, by either deleting the Nt-helix (ΔNt•cTnC3) or replacing it with a skeletal Nt-helix (sNt•cTnC3). Maximal force regulation was studied by pCa4 activation of the rat skinned cardiac trabeculae with TnC-exchange. The force responses normalized to cTnC3-exchange were: 46%P_o with ΔNt•cTnC3 and 53%P_o with sNt•cTnC3. The results provide evidence that cardiac N-terminal helix is essential for the regulatory function of cardiac TnC. Moreover, the skeletal Nt helix was unable to substitute for the endogenous cardiac N-helix for force development in maximally activated trabeculae, indicating that the structural requirements for the regulatory sites of cTnC are also specific in the mechanism of tension generation.
Supported by NIH & NY Heart Association

M-Pos172

STRUCTURAL CHANGES IN THE Ca-DEFICIENT EF-HAND OF CARDIAC TnC DURING ACTIVATION: *STEADY-STATE FLUORESCENCE AND POLARIZATION*
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The cardiac troponin C (cTnC) comprises four EF-hands, of which two are in the N-terminal trigger domain (sites I & II), but site I is unable to bind Ca^{2+}. Despite the Ca^{2+} deficiency, by using a cardiac-skeletal chimera, we have recently indicated that cardiac site is active in myocardial contractility (Biochemistry,33,1994). To gain further insights into the switching mechanism, we have genetically engineered a variant of cTnC3 by inserting a tryptophan-26 (cTnC3.F26W). Comparisons of the tryptophan fluorescence spectra in EGTA (apo relaxed state) and pCa4 (Ca-saturated active state) showed a 44% increment during activation, in agreement with findings on the chimera. This indicated that structural alterations during transition between the relaxed and activated states registered by the tryptophan reflected the intrinsic property of the cardiac site. To further explore the activation mechanism, we measured steady-state fluorescence polarizations in cTnC3.F26W and sTnC4.F26W (tryptophan derivative of recombinant skeletal TnC). The polarization parameters in EGTA (relaxed state, P_{EGTA}) were highly variable between cTnC3.F26W (0.137) and sTnC4.F26W (0.166). In saturating Ca^{2+}, the activated values (P_{Ca}) were: cTnC3.F26W, 0.106 and sTnC4.F26W (0.103).The polarization results suggest that the cardiac switch in the *OFF*-state is already predisposed towards the activated state; however, the final *ON*-state is similar in cardiac and skeletal TnCs.
Supported by NIH and New York Heart Association

M-Pos173

N-CAP MUTANTS IN THE REGULATORY DOMAIN OF CHICKEN TROPONIN-C. ((Louise LeBlanc and Thor Borgford)) Department of Chemistry and Institute of Molecular Biology and Biochemistry, Simon Fraser University, Burnaby BC, CANADA V5A 1S6 (spon. Dr. G. Tibbits)

In a previous study (Trigo-Gonzalez *et al, Biochem.* 37, 9826-9831 (1993)) the calcium affinity of the Ca^{2+}/Mg^{2+} sites, in the carboxyl-terminal domain of recombinant chicken troponin-C, was "tailored" by mutagenesis. Amino acid substitutions were made at position 130, the N-cap residue of helix G. Substitutions either stabilized or destabilized the G-helix and in doing so shifted the affinity of associated binding sites. To determine if this relationship between helix stability and ion affinity is a general property of paired calcium binding sites, we created mutants with substitutions at position 54, the N-cap residue of helix-C. Position 54 (Thr) is in the regulatory domain of troponin-C and topologically equivalent to position 130. Variant proteins were made that contain the substitutions Ser,Gly,Ala,Val,Ile,Asp or Asn. The effect of mutation on calcium affinity was monitored with the aid of a single tryptophan at position 29 (Phe29->Trp). The results are discussed in relationship to findings involving C-domain mutants. (Supported by a grant from the BC Health Research Foundation)

M-Pos174

EFFECT OF CALTROPIN ON CALDESMON-ACTIN INTERACTION.
((R.S. Mani and C.M. Kay)) MRC Group in Protein Structure and Function, Dept. of Biochemistry, Univ. of Alberta, Edmonton, Canada, T6G 2H7.

The binding of caldesmon (CaD) to actin was studied both in the presence and absence of caltropin (CaT) using airfuge centrifugation, disulfide crosslinking and the fluorescent probe acrylodan. In co-sedimentation studies most of the caldesmon pelleted along with actin. However, when caldesmon in the presence of caltropin was mixed with actin, caldesmon did not pellet with actin. Treatment of a solution of CaD and actin with 5,5'-dithiobis (2-nitrobenzoic acid) resulted in the formation of a disulfide cross-linked product with a molecular mass of 180,000. Incubation of CaD with actin in the presence of CaT resulted in reduced cross-linking between CaD and actin. Titration of acrylodan labelled CaD with actin indicated a strong affinity ($K_a \approx 6 \times 10^7 M^{-1}$). However, in the presence of CaT, actin exhibited a lower affinity ($K_a \sim 2 \times 10^7 M^{-1}$) for CaD. CaT also affected the CaD-G-actin interaction. CaD induced polymerization of G-actin into filaments in the absence of salt and this was accompanied by an increase in relative viscosity. This effect of CaD was essentially abolished by CaT in the presence of Ca^{2+}. Acrylodan labelled G-actin when excited at 375_{nm} exhibited emission maximum of 482_{nm}. Polymerization of G-actin resulted in shifting the emission maximum to 465_{nm}. The kinetics of G-actin polymerization in the presence of CaD was followed by observing the increase in fluorescence intensity at 465_{nm} as a function of time. When CaD was added to G-actin containing CaT, the rate of G-actin polymerization was reduced considerably suggesting that CaT influences CaD-actin interaction. CaT which is very effective in reversing caldesmon's inhibition of the actin-activated myosin ATPase also exhibits a pronounced effect on CaD binding to actin.

M-Pos175

TRYPTOPHAN RESIDUES ARE CRITICAL FOR CALDESMON TO INTERACT WITH CALMODULIN. ((Enzhong Wang, Shaobin Zhuang, and C.-L. Albert Wang)) Muscle Research Group, Boston Biomedical Research Institute, Boston, MA 02114.

Smooth muscle caldesmon (CaD) contains five tryptophan residues; three of them appear in the C-terminal region of the molecule: W659, W692 and W722. There is a sizable tryptophan fluorescence enhancement accompanied with binding of CaD to calmodulin (CaM), indicating that at least one of these Trp residues is at or near the interacting regions between CaD and CaM. In fact, W659 and W692 are indeed within the peptide stretches of putative CaM-binding sites (referred to as Sites A and B). One possibility is that CaM recognizes Trp residues, in addition to nearby positive charges, as the sites for interaction. To test this hypothesis, we have carried out mutagenesis on CaD, at both levels of recombinant fragments and synthetic peptides, replacing the Trp residue with Gly, and examined the effect of such changes on its ability to bind CaM. We have previously shown that GS17C (Site A; from G651 to S667, containing W659) binds CaM with an affinity similar to that of CaD ($1.9 \times 10^6 M^{-1}$; measured at 50 mM KCl); when W659 was changed to Gly, the binding affinity for CaM was decreased by at least 10-fold. Another peptide, VG29C (from V685 to G713, thus containing W692), was designed to cover a second CaM-binding site (Site B). VG29C binds CaM with an affinity of $3.8 \times 10^5 M^{-1}$; but lost binding almost completely when W692 was changed to Gly. We have also expressed a 22K CaD fragment (from K579 to Pro756) and its mutant 22K/W692G in E. coli. By fluorescence titration we have determined the binding constants of 22K and 22K/W692G for CaM to be, respectively, $4.2 \times 10^6 M^{-1}$ and $8.5 \times 10^5 M^{-1}$. Thus Trp residues appear indeed important for CaD to interact with CaM. Supported by grants from NIH.

M-Pos176

FUNCTIONALLY RELEVANT CALMODULIN-BINDING SITE ON SMOOTH MUSCLE CALDESMON. ((Shaobin Zhuang, Enzhong Wang, and C.-L. Albert Wang)) Muscle Research Group, Boston Biomedical Research Institute, Boston, MA 02114.

The C-terminal region of smooth muscle caldesmon (CaD) contains most of the functional properties of the intact protein, among which is the ability to interact with calmodulin (CaM) in a Ca^{2+}-dependent manner and thereby to reverse the inhibitory effect of CaD on the acto-myosin ATPase activity. We have previously shown that the major CaM-binding site (Site A) in this region is localized within the segment from Met-658 to Ser-666 (Zhan, Q., Wong, S.S. and Wang, C.-L.A. (1991) *J. Biol. Chem.* **266**, 21810-21814). Recently, another peptide segment, from Asn-675 to Lys-695 (Site B), was suggested to also interact with CaM (Mezgueldi, M., Derancourt, J., Calas, B., Kassab, R. and Fattoum, A. (1994) *J. Biol. Chem.* **269**, 12824-12832). In order to determine which of these two putative CaM-binding sites is relevant to the regulatory properties of CaD, we have examined three synthetic peptides in terms of their effects on the ability of CaM to reverse the CaD-induced inhibition of acto-myosin ATPase activity. These peptides are: GS17C (from Gly-651 to Ser-667), VG29C (from Val-685 to Gly-713), each containing one CaM-binding site, and MG56C (from Met-658 to Gly-713) which contains both sites. We found that although VG29C was indeed able to bind CaM with an affinity somewhat lower than that of GS17C, it failed to compete with CaD for CaM, whereas GS17C effectively displaced CaD from CaM and resulted in re-inhibition; MG56C also achieved similar effect as GS17C. These experiments demonstrated that Site A is the CaM-binding site responsible for regulating the inhibitory function of CaD. Supported by grants from NIH.

M-Pos177

TWO SITED INTERACTION OF CALDESMON WITH CALMODULIN ((P.A.J. Huber, Z. ¶Grabarek *D.A. Slatter, *B.A. Levine and S.B. Marston)) Dept. Cardiac Medicine, NHLI, Dovehouse St. London SW3 6LY, UK, *University of Birmingham, Edgbaston, Birmingham B15 2TT, UK ¶ Dept. Muscle Res., Boston Biomed. Res. Inst., Staniford St. Boston, MA, USA

We have tested the proposal that there are 3 calmodulin contact sites on caldesmon A, B, and B', centred on trps 659, 692 and 722, respectively. Only binding at B and B' is coupled to reversal of caldesmon inhibition (Marston et al., JBC, 269: 8134-9). Ca^{2+}-calmodulin enhances the intrinsic trp fluorescence of the recombinant caldesmon fragments H2 (aa 626-710, containing site A and B) and H9 (aa 669-737, containing site B and B'). This fluorescence enhancement is accompanied by a blueshift and is reversed in EGTA. We have used two calmodulin mutants to assess the interaction site(s) within calmodulin. Calmodulin mutant F92A has a C-terminal phe to ala mutation and calmodulin cys41/75 has N-terminal mutations, where two cys replace positions 41 and 75 and are additionally zero crosslinked. Both calmodulin mutants were not able to reverse the ATPase inhibition of smooth muscle caldesmon. We did however observe a decrease of the trp fluorescence within H2 and H9 when titrated with the calmodulin mutants, suggesting some interaction took place. Ca^{2+}-F92A caused additionally a small blueshift of the trp fluoresence maximum, which was reversed by EGTA. The intrinsic trp fluorescence of two peptides, V12T (^{683}VGVSSRINEWLTKT^{696}), representing site B and G12S (^{713}GSVSGK-RNLWEKQS^{726}), representing site B' was also observed to decrease when titrated with calmodulin. Thus caldesmon sites B and B' and calmodulin species with two functional sites are required to give full regulatory function.

M-Pos178

LOCALIZATION OF CALDESMON IN CARDIAC MYOCYTES. ((G.C. Scott-Woo, M.P. Walsh and G.J. Kargacin)) Depts. of Medical Physiology and Medical Biochemistry, University of Calgary, Calgary, Alberta, Canada.

Caldesmon is a heat stable, actin- and myosin-binding protein that has been studied primarily in smooth muscle and non-muscle cells. Although it has been hypothesized that caldesmon regulates the interaction of actin and myosin, its cellular function is not entirely clear and it has also been suggested that caldesmon is involved in organizing contractile filaments or in the assembly of microfilaments. Caldesmon has been reported to be present in cardiac muscle; however, its function and localization in the heart have not been determined. Western blot analysis revealed the presence of caldesmon in both atrial and ventricular myocytes isolated from rabbit heart. The ratio of caldesmon to tropomyosin in cardiac muscle was found to be markedly less than that in gizzard smooth muscle. Immunofluorescence microscopy revealed a striated pattern for the distribution of caldesmon in fixed and un-fixed relaxed cardiac myocytes from a variety of mammalian sources. In dual-label experiments, caldesmon co-localized with α-actinin at the center of the I-band but not with myosin in the A-band of cardiac cells. This distribution was unexpected and argues against the involvement of caldesmon in regulating the interaction of actin and myosin in relaxed cardiac cells. Our results are consistent with a structural role for caldesmon in the heart. Supported by NIH AR39678, MRC (Canada), AHFMR and the Heart and Stroke Foundation of Alberta.

M-Pos179

INTERACTION OF THE NH_2 AND COOH-TERMINAL DOMAINS OF CALDESMON ((T.S. Tsuruda, D.B. Foster and A.S. Mak)) Department of Biochemistry, Queen's University, Kingston, Ontario, Canada, K7L 3N6

Phosphorylation of chicken gizzard caldesmon by mitogen activated protein (MAP) kinase at the COOH-terminal domain inhibits its phosphorylation by casein kinase II at the amino-terminal domain. This observation suggests that the NH_2- and COOH-terminal domains of caldesmon may communicate directly. To test this hypothesis, we have studied the interaction between the two domains using two recombinant fragments of human fibroblast caldesmon, CaD40 (residues 1-160) and CaD39 (residues 234-538). Fluorescence studies show that anilno(napthalene)maleimide (ANM)-labelled CaD39 interacts specifically with CaD40. This observation is corroborated by changes in CaD39's intrinsic tryptophan fluorescence induced by titration with CaD40. CaD40 also interacts with a synthetic peptide of chicken gizzard caldesmon (residues 655-709) which contains binding sites for calmodulin, actin and is able to inhibit actin-activated myosin ATPase activity. The CaD39 fragment also binds to a CaD40-affigel affinity column. It is not clear at present whether the interaction between the domains is intra- or intermolecular although there is evidence that the cysteine residues in the two domain can be close to each other to generate pyrene excimer fluorescence. (Supported by MRC of Canada and Ontario Heart and Stroke Foundation).

M-Pos180

KINETIC STUDIES OF COMPETITIVE BINDING OF CALDESMON AND MYOSIN SUBFRAGMENT-1 TO ACTIN ((Yi-der Chen, J. M. Chalovich, and Hai Luo)) NIDDK, NIH, Bethesda, MD 20892- and Dept. of Biochemistry, East Carolina University School of Medicine, Greenville, NC 27858-4354

Caldesmon can bind to actin filament and inhibit the actin-activated ATP hydrolysis of myosin subfragment 1 (S-1) by reducing the degree of binding of S-1 to actin. Binding studies of enzyme-digested caldesmon subfragments directed at mapping the actin binding site of caldesmon have shown that a small 8-kD fragment around the COOH-terminal can compete directly with the S-1 binding to actin; at least one other fragment that binds to actin does not inhibit the actin-activated ATPase activity of S-1 (1). This prompted us to suggest that the binding of caldesmon and S-1 to actin might not be pure competitive, but of the "mosaic multiple-binding" type (2). In this study, we explored the possibility of differentiating these two modes of binding by investigating the kinetics of exchange between free S-1 and bound caldesmon molecules (or vice versa). Kinetics of these exchange reactions for the two models were first evaluated theoretically using Monte Carlo simulations and then compared with measured kinetic data obtained recently (3). Conditions for differentiating between pure competitive binding and mosaic multiple-binding models based on kinetic data were obtained and will be discussed.

(1). Velaz L, et al., Biophys. J. 65, 892 (1993).
(2). Chen Y. and Chalovich J.M., Biophys. J. 63, 11063 (1992).
(3). Chalovich J.M. and Luo H., Biophys. J. 66, A198 (1994).

M-Pos181

EFFECT OF CALDESMON FLEXIBILITY ON ITS BIOCHEMICAL PROPERTIES ((R.H. Crosbie[1], F.W.M. Lu[2], J. M. Chalovich[2], and E. Reisler[1])) [1]Dept. of Chemistry and Biochemistry and the Molecular Biology Institute, University of California, Los Angeles, CA 90024 [2]Dept. of Biochemistry, East Carolina University School of Medicine, Greenville, NC 27858

The contribution of the extended and bent forms of caldesmon to this protein's function was investigated by examining chemically modified forms of the protein. First, we examined the influence of cellular factors on the excimer fluorescence of pyrene labeled caldesmon. The folded, 'hairpin' form of caldesmon was enhanced at physiological pH and at low ionic strengths, as detected by an increase in excimer fluorescence under these conditions. The presence of nucleotides also produced significant conformational changes in caldesmon, as detected by fluorescence measurements and protease digestions. Titrations of pyrene caldesmon with actin, HMM, and calmodulin resulted in a decrease in excimer fluorescence. The function of the folded form of caldesmon was investigated by using intramolecular EDC-crosslinked caldesmon. The inhibition of acto-S-1 ATPase activity by crosslinked caldesmon was less efficient compared with pyrene modified and control caldesmons. Caldesmon's ability to switch from an activator to an inhibitor of actin activated ATPase of myosin was also affected by the folding. Cosedimentation experiments revealed normal binding of crosslinked caldesmon to smooth muscle myosin. These results indicate the importance of caldesmon's transition from extended to folded forms and suggest possible functional roles for these different forms of caldesmon.

M-Pos182

TROPOMYOSIN-ACTIN COOPERATIVE EFFECTS DURING MYOSIN S1 AND HEAVY MEROMYOSIN TITRATIONS. ((N.L. Golitsina*, S.E.Hitchcock-DeGregori# & S.S. Lehrer*)), *Boston Biomedical Research Institute, Boston, MA 02114; #UMDMJ-R.W.Johnson Medical School, Piscataway, NJ 08854.

The excimer fluorescence of pyrene-labeled Tm (Tm*) has been used to monitor the myosin S1-induced "off-on" transition of rabbit skeletal Tm*.actin (Ishii & Lehrer,1990; Geeves & Lehrer,1994) and gave n = 5-6 for the number of actin subunits "turned-on" per bound S1. Now we show that n = 9 for the gizzard $\alpha\alpha$Tm*-actin complex, indicating that smooth Tm*.actin should be more readily "turned-on" than skeletal Tm*.actin, in agreement with ATPase studies. Titrations of gizzard Tm*-actin with heavy meromyosin (HMM) under conditions where all heads of HMM are bound gave n=4, indicating that the second head does not appreciably increase the number of actin subunits "turned-on"; i.e., the two heads of HMM bind within the same cooperative unit.

S1 induces Tm to bind to actin under unfavorable binding conditions (Eaton, 1976). We studied the S1-induced binding of recombinant striated $\alpha\alpha$Tm* to actin in 5mM Mg^{2+}, 30mM NaCl, buffer pH 7.5 (where lack of binding to actin is due to lack of N-terminal acetylation of Tm*) and native skeletal Tm* in 1mM Mg^{2+}, 10mM NaCl, buffer pH7.0 (conditions unfavorable for binding) and found that 2 or more S1's bound to 7 actins are required to bind these Tms to actin. Addition of TnT1 (N-terminal 2/3 of TnT) to recombinant $\alpha\alpha$Tm*, facilitates S1-induced Tm* binding to actin, with a similar cooperativity as for the S1-induced "off-on" transition of native rabbit skeletal Tm*.actin.

S1 titrations of recombinant smooth $\alpha\alpha$Tm*, which binds well to F-actin, gave n=7 compared to n=9 for native gizzard $\alpha\alpha$Tm*, suggesting decreased communication between neighboring Tms due to lack of N-terminal acetylation.

M-Pos183

ADENOSINE A_1 RECEPTOR AND PROTEIN KINASE C ACTIVATION DECREASE VELOCITY OF UNLOADED SHORTENING IN RAT VENTRICULAR MYOCYTES.
((J.W. Lester, K.F. Gannaway, and P.A. Hofmann)) Department of Physiology, University of Tennessee, Memphis, TN 38163.

Brief periods of myocardial ischemia precondition the heart to limit subsequent ischemia-induced necrosis. Adenosine A_1 receptor activation has been implicated as the mediator of this cardioprotective effect, but its mechanism of action is unknown. The purpose of the present study was to determine if the adenosine A_1 agonist R-phenylisopropyladenosine (R-PIA) influences Ca^{2+} sensitivity of isometric tension, maximum tension generation, and unloaded shortening velocity in skinned ventricular myocytes. In addition we examined the effects of dioctanoyl glycerol (DOG), a diacylglycerol analog, on cardiac contractile function to determine if protein kinase C (PKC) activation has effects similar to that of R-PIA. Ventricular myocytes were enzymatically isolated, agonist treated, skinned with Triton X-100, and attached to a force transducer and piezoelectric translator. Cardiac myocytes treated for 5 minutes with 100 μM R-PIA had a significantly lower ($p<0.03$) velocity of unloaded shortening (2.91±0.22 ML/s; n=13) as compared to control myocytes (3.70±0.31 ML/s; n=8). Velocity of unloaded shortening of DOG treated myocytes (2.45±0.71 ML/s; n=5) was also lower ($p<0.05$) than control myocytes (3.07±0.24 ML/s; n=5). No significant difference in myofilament sensitivity to Ca^{2+} or maximum tension generation was observed between control and R-PIA treated cardiac myocytes. These studies support the hypothesis that the effects of adenosine may be mediated by PKC and raise the possibility that decrease in velocity of myofilament shortening contributes to the cardioprotective ability of adenosine. (Supported by HL48839).

M-Pos184

TUNING CALCIUM BINDING TO AN EF-HAND-LIKE SITE: MUTATIONAL STUDIES OF EF-LOOP POSITION NINE.
((Steven K. Drake, Keith L. Lee, Loree J. Kim, Joseph J. Falke)) Department of Chemistry and Biochemistry, University of Colorado, Boulder, CO 80309-0215

The EF-hand calcium binding motif is widely used in the activation of calcium signaling pathways: over 1000 examples of this motif have been identified in protein sequences. The heart of the motif is the twelve residue EF-loop, which exhibits both conserved and variable positions. The calcium binding parameters of different EF-hand proteins are tuned to yield optimized control of their respective pathways. Part of this tuning stems from the variable positions of the EF-loop, such as position nine, which we have previously shown to control ion binding specificity and kinetics. Using the EF-hand-like site of the *E. coli* galactose binding protein as a model isolated site, we have further investigated the tuning capabilities of the ninth position. This position has been engineered to incorporate each of the side chains observed at position nine in natural sites, as well as several side chains not observed at position nine. The resulting engineered sites exhibit significantly altered ion binding properties, including altered charge selectivity, size selectivity, and ion dissociation kinetics. Details of these studies will be presented.

M-Pos185

ENGINEERING CARDIAC TROPONIN C FOR STUDIES OF EF-HAND ACTIVATION TUNING. ((A.L. Hazard, N. Stricker and J. J. Falke)) Department of Chemistry and Biochemistry, University of Colorado, Boulder, CO 80309.

Calcium signaling proteins of the calmodulin superfamily typically possess four EF-hand Ca^{2+} binding sites: sites I and II in the N-terminal domain, and sites III and IV in the C-terminal domain. The multi-site, cooperative nature of these complex Ca^{2+} binding systems make quantitative studies of the ion binding parameters of individual sites quite problematic. In order to carefully probe the tuning of an isolated EF-hand site, cardiac troponin C (cTnC) has been chosen as a model protein since the N-terminal domain of cTnC contains only one functional EF-hand site (site II). The cTnC binding system can be further simplified by eliminating sites III and IV via appropriate mutations in their Ca^{2+} binding loops to yield cTnCΔIII,IV (J. Putkey *et al.*). We have further engineered cTnCΔIII,IV by substituting cysteine 35 with serine, yielding a protein containing a single cysteine at position 84 on the interdomain helix. This remaining cysteine can then be labeled with a suitable environment-sensitive fluorescent probe. Ca^{2+} binding to the modified protein yields up to a two-fold increase in fluorescence intensity, suggesting enhanced hydrophobicity in the environment of the fluor. The modified protein enables quantitation of ion binding to an independent EF-hand site. The altered ion binding features of sites containing engineered substitutions at key positions of the EF-hand loop will be discussed.

M-Pos186

TUNING THE CA^{2+} ACTIVATION OF CALMODULIN VIA PROTEIN–PROTEIN INTERACTIONS WITH TARGET HELICES.
((Olve B. Peersen and Joseph J. Falke)) Department of Chemistry & Biochemistry, University of Colorado, Boulder, CO 80309-0215

Calmoldulin (CaM) plays a central role in Ca^{2+} mediated signaling, where it activates a wide range of cellular pathways. CaM binds four Ca^{2+} ions using two pairs of 'EF-hand' type binding sites, and upon doing so it undergoes a conformational change that results in the exposure of hydrophobic patches on its two domains. These patches interact with amphipathic helices on specific target proteins to form tight CaM•Target complexes with nanomolar dissociation constants. The resulting protein-protein interactions yield significantly increased Ca^{2+} binding affinity and cooperativity for CaM in the presence of either whole target proteins or small peptides corresponding to their CaM binding helices. We are systematically studying the effects of different target helices on Ca^{2+} binding to CaM. We suggest that the CaM–target helix interactions may be used to tune and optimize the activation parameters of CaM in a cellular pathway specific manner. The results of these studies will be discussed.

M-Pos187

MYOSIN CROSSBRIDGE ARRANGEMENT IN FREEZE-ETCH REPLICAS OF UNFIXED, RAPIDLY FROZEN PLAICE FIN MUSCLE. ((M. E. Cantino*, M. W. K. Chew, P. K. Luther and J. M. Squire)) Biophysics Section, The Blackett Laboratory, Imperial College, London, SW7 2BZ and *Department of Physiology and Neurobiology, University of Connecticut, Storrs, CT 06269

Previous electron microscopy studies of myosin filament structure in the fish have utilised either chemically fixed muscle (Luther et al., 1981, J. Mol. Biol. *151:703*; Varriano-Marston et al., 1984, J. Mus. Res. Cell Motil. *5:363*) or isolated myofilaments (Kensler and Stewart, 1989, J. Cell Sci. *94:391*). We are investigating crossbridge arrangement *in situ* in unfixed skinned fish muscle fibers using freeze-fracture deep-etch rotary shadowing methods. Fibers from the plaice fin muscle were chemically skinned, placed in relaxing or rigor solutions, and rapidly frozen by immersion in liquid ethane. Samples were then fractured, etched and replicated in a JEOL JFD-9010C Freeze Etch Unit. Myosin heads in relaxed fibers were closely packed around the thick filament backbone along helical tracks. Myosin heads under rigor conditions were generally extended away from the backbone in the H-zone, and bound to actin in the overlap region. Analysis is underway to further characterise crossbridge arrangement under these conditions and to determine locations of accessory proteins such as C protein.

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M-Pos188

MOLECULAR PACKING IN THE VERTEBRATE MYOSIN FILAMENT BACKBONE. ((M.W.K. Chew and J.M. Squire)) Biophysics Section, The Blackett Laboratory, Imperial College, London SW7 2BZ, UK.

Vertebrate muscle thick filaments are made from myosin molecules that pack to form a cylindrical backbone with the S1 portion or heads in a helical array on the filament surface. Radial projections of the helical arrays of myosin heads from different muscle sources reveal patterns that are remarkably similar. With the myosin head ends of the molecules placed on the surface lattice positions, the rod parts can be packed in a number of ways to form the filament backbone. Assuming that myosin rods would tend to pack in exactly equivalent ways, at least in the bulk of the myosin filaments where end effects would not be involved, a number of possible models may be obtained. Models considered include those in which the α-helical coiled-coil rod parts of myosin are packed into various kinds of subfilaments or in a uniform cylindrical layer. Computed Fourier transforms of these models for the vertebrate striated muscle myosin filament backbone have been compared with observed high-angle X-ray diffraction patterns. The relevant parts of the transforms are the high angle equatorial and near equatorial regions related to d-spacings between 2.0 nm and 1.0 nm which arise from the molecular packing and the meridional reflections at 0.51 nm from the α-helical motif of all myosin rods. From a qualitative comparison of the calculated Fourier transforms and observed X-ray patterns, and taken together with electron micrograph information, it is concluded that the uniform layer model appears to explain the observations better than the alternatives.

(Supported by a grant from the MRC, UK.)

M-Pos189

RABBIT THICK FILAMENT STRUCTURE REFLECTS THE STATE OF MYOSIN LIGHT CHAIN PHOSPHORYLATION. ((R. Levine, H.L. Sweeney, R. Kensler and Z. Yang)) Med. Coll. PA, Philadelphia, PA, Univ. of Puerto Rico, San Juan, PR and University of PA, Philadelphia, PA.

Previously[1], we showed that incubation of rabbit psoas muscle thick filaments with skeletal muscle myosin light chain kinase (MLCK)[2] perturbs the ordered arrangment of relaxed myosin heads at ≥23° C.[3] We proposed that the disordered structure of filaments with phosphorylated myosin regulatory light chains (MRLCs) reflects increased mobility of myosin heads, possibly due to incorporation of negative charge, and further, that mobile heads spend more time near thin filaments, thus producing the observed potentiation of tension development[4]. We have now exposed filaments with phosphorylated MRLCs to skeletal muscle phosphatase[2]. Ordered, relaxed filaments become disordered after 30 sec incubation with MLCK and calmodulin at a pCa of 5.0. Such filaments regain their ordered appearance after 2 min exposure to phosphatase. This result supports the conclusion that disorder of the surface myosin array is a specific result of MRLC phosphorylation. We also removed the native MRLCs from skinned fibers, in preparation for replacing them with recombinant LCs with different charges. Preliminary results show extreme disorder of surface structure of filaments lacking MRLCs (and probably essential LCs). We are examining the effects of re-incubation of LC-depleted skinned fibers with native and recombinant LCs on thick filament structure and tension development.

[1]Levine et al. Biophys. J. **64**:A142 '93; [2] Donated by Dr. J. Stull; [3] Kensler et al. J. Mus. Res. Cell Motil. **15**:69 '94; [4] Yang et al., Biophys. J. **61**:A267.

M-Pos190

DIRECTIONAL MOVEMENT OF ACTIN FILAMENTS ON ORIENTED RABBIT SKELETAL MUSCLE MYOSIN MOLECULES. ((Akira Yamada and Haruto Nakayama)) Kansai Advanced Research Center, Communications Research Laboratory, Iwaoka, Nishi-ku, Kobe 651-24, Japan.

We purified a novel protein from the byssus retractor muscles and the posterior adductor muscle of *Mytilus edulis*, as a mixture of about equal quantity of 125kD and 115kD peptides revealed with the SDS-PAGE. It is a component of thick filaments and forms filaments under the low ionic strength condition *in vitro*. By lowering ionic strength slowly, we could get spindle-shaped filaments of more than 50μm in length. After mixed with rabbit skeletal muscle myosin, the filaments were shown to adsorb actin filaments complexed with rhodamine-phalloidin in the absence of MgATP, indicating that myosin molecules attached to these long filaments. In the presence of 1mM MgATP, actin filaments moved along these filaments at 4.7 ± 0.6μm/s toward the center of the filaments, and at 1.9 ± 0.2μm/s in the opposite direction (28 ℃). These values are comparable to the speeds of actin filaments on synthetic rabbit skeletal muscle myosin filaments (Yamada & Wakabayashi, 1993, *Biophys. J.*, **64**, 565-569.), and thus it was suggested that the myosin molecules on the half of the filaments were aligned in the same orientation like those in myosin filaments. Since the filaments in the present system are much longer than both native and synthetic myosin filaments, they are suitable for studying the movement by oriented myosin molecules.

M-Pos191

LINEAR MOVEMENT OF ACTIN FILAMENTS ON MYOSIN HEADS ALIGNED ON POLY(TETRAFLUOROETHYLENE) THIN FILM ((H. Suzuki, K. Oiwa, A. Yamada, H. Sakakibara, S. Mashiko & H. Nakayama)) Kansai Adv. Res. Center, C.R.L., Kobe 651-24, Japan (Spon. by K. Oiwa)

The *in vitro* motility assay in which actin filaments can be seen moving on a myosin-coated glass surface has become a popular method for studying the actin-myosin interaction. Random distribution of myosin heads on the surface, however, leads to an erratic movement of actin filaments, which hampered the precise measurements of force and velocity in the *in vitro* system. Hence, we have developed a new *in vitro* system in which myosin heads adsorbed by a poly-(tetrafluoroethylene) (PTFE) thin film formed linear tracks and showed linear movement of actin filaments over the distance of 100 μm. A solid PTFE bar was moved against a glass surface while applying pressure (7×10^4 Pa) and high temperature (ca. 220°C). The procedure left a thin film on the surface with 10-20 nm height ridges running parallel to the direction of the deposition over 100 μm. Myosin and its subfragments (S1 and HMM) were adsorbed by the ridges and formed linear tracks, on which actin filaments moved smoothly over a distance of 100μm with a constant velocity of ca. 1 μm/sec (for HMM at 23°C). No difference of the velocities of actin filaments between moving in one direction along a track and in the opposite direction was observed. These results suggest that adsorption of myosin heads onto this PTFE films shows a remarkable degree of alignment, while they are randomly oriented relative to the polarity of an actin filament.

M-Pos192

ANALYSIS OF BOUND NUCLEOTIDE IN THE *IN VITRO* MOTILITY ASSAY USING RADIOLABELLED ATP. ((M.S.Z. Kellermayer)) Central Laboratory, Medical University of Pécs, Szigeti ut 12., Pécs, H-7624 Hungary

We have previously shown that *in vitro* actin motility persists at nanomolar ATP concentrations, if the actomyosin is pretreated with millimolar levels of the nucleotide (Kellermayer, M.S.Z., *et al. Biophys. J.* **64**, A232, 1993.). In the above experiments, ATP concentration was reduced from the millimolar by successive washes of the sample chamber with rigor solution (devoid of ATP). A possible source of artifact is that ATP binds to myosin and remains bound during the washout procedure. The bound ATP might then later be released and utilized for fueling motility. To test for this possibility, we carried out the *in vitro* motility assay using radiolabelled ATP, and directly measured the amount of bound ATP. To exclude large variations caused by the generally used, non-standard sample chamber, the motility assay was carried out in a standard, 3-μl capillary, using gravity-fed constant rates of solution flow. We used either alfa- or gamma-phosphate labelled ATP. After the addition of HMM and actin, the capillary was filled with radioactive ATP and the total radioactivity measured in a scintillation counter. This initial measurement was used for normalizing data from the subsequent steps of the experiment. The capillary was then washed with rigor solution, and both the total remaining ATP and the washed-out ATP measured. According to our results, 1-2% of the ATP remained bound. This fraction, however, could not be further reduced by extremely large volumes of washes (500 times that of the capillary volume). Further, we could not detect phosphate release following the washouts. Control experiments indicate that the bound fraction might be localized on nitrocellulose, since 1-2% bound ATP was detected when using nitrocellulose alone. Thus, the bound ATP seems to be stable and not available for maintaining the persisting *in vitro* actin motility.

M-Pos193

NON-NUCLEOTIDE AND SEMI-NUCLEOTIDE TRIPHOSPHATE ANALOGUES FOR SKELETAL ACTOMYOSIN MOTILITY. ((D. Gopal[†], M. Ikebe[‡] and M. Burke[†]))
Department of Biology[†], College of Arts and Sciences and Department of Physiology and Biophysics[‡], School of Medicine, Case Western Reserve University, Cleveland, OH 44106

Eight triphosphate analogues were synthesized and examined for their ability to support sliding of fluorescently labeled F-actin over adsorbed myosin by the standard *in vitro* motility assay. These were based on the non-nucleotide NANTP, (2-[(4-azido-2-nitrophenyl)amino]ethyl triphosphate), class of analogues with substitutions at either (i) the 2 (Y) or 4 (X) positions of the aryl ring, (ii) or the N of the aryl amino, or (iii) in the length of the spacer between the aryl ring and the triphosphate group. The fastest sliding velocities were found with 2-NPhAETP (4.1 μ/s) and DNPhAETP (2.1 μ/s) corresponding to 91 and 47% respectively of the velocity observed in the presence of ATP, in reasonable agreement with the order of maximum shortening velocities observed in skinned fibers with these two analogues (Wang et al., J. Muscle Res. & Cell Motil. 14, 484-497 (1993)). No sliding was observed with 4-NPhAETP suggesting that the presence of the NO_2 group at the 2 position is a requirement for chemo-mechanical coupling for this class of analogues as suggested by Wang et al., (1993). DNPMAETP, which has a methylated aryl amino group, did not produce any sliding even though the length of the spacer was the same as those of the force producing analogues. Substitution at the 4-position by a CF_3 lowered the sliding velocity to 0.91 μ/s while substitution with a bulkier benzoyl group (Bz-NPhAETP) abolished sliding all together. In contrast to the reported loss of chemo-mechanical coupling observed on extending the length of the spacer by a single methylene to a propyl linker, an analogue with a much larger extension, 2-[2-[(2-nitrophenyl)amino] ethoxy] ethyl triphosphate produced a sliding velocity of 0.88 μ/s. The semi-nucleotide analogue, [1-(2,3-O-isopropylidene-5-triphospho-β-D ribofuranosyl)] 2,4 dinitrophenylamine, produced sliding velocities of 0.92 μ/s. (supported by NIH NS 15319)

M-Pos194

ACTIN-MYOSIN MOTILITY ASSAY USING AN ANTIBODY CAPTURE TECHNIQUE. ((A. Ott, L. Bourdieu (#), D. A. Winkelman (!) and A. Libchaber (#)))Institut Curie, Paris, France; (#) Princeton University and NEC Research Institute, Princeton, NJ; (!) Rutgers University, Rutgers, NJ.

We studied the motion of fluorescently labeled actin on a myosin covered substrate by means of fluorescence microscopy. Myosin was deposited on the substrate using antibodies against the myosin heavy chain and this was found to improve the accuracy and the reproducibility of the experiment significantly. Observations were made for three different antibodies, each sensitive to a different portion of the myosin heavy chain, and at different temperatures for various concentrations of ATP, Mg and KCl. The motion of actin was found to be roughly independent of the length of the free myosin heavy chain. It is furthermore suggested that friction and adhesion play a major role in defining the speed and the size of actin filaments and it is shown that the motor does not follow a simple one step Arrhenius law.

M-Pos195

PHOSPHORYLATION INDEPENDENTLY ACTIVATES THE TWO HEADS OF SMOOTH MUSCLE MYOSIN. ((D. Harris & D. Warshaw)) Mol. Physiol. & Biophys., U. of Vermont, Burlington, VT 05405. (Spon. by B. Hamrell)

Smooth muscle myosin is activated by phosphorylation of the regulatory light chain (LC20). To determine if myosin's two heads are activated independently or cooperatively by LC20 phosphorylation, smooth muscle myosin was randomly phosphorylated to < 100% so that none, one or both of myosin's two heads were phosphorylated. Myosin's activation was then tested by actin activated ATPase measurements and *in vitro* motility experiments in which fluorescently labeled actin filaments move over a monomeric myosin-coated surface. ATPase was directly proportional to phosphorylation level. *In vitro* actin filament velocity increased monotonically at a rate indistinguishable from that of unphosphorylated and fully phosphorylated smooth muscle myosin mixed in known ratios (Harris et al., 1994). Thus, smooth muscle myosin heads are activated by LC20 phosphorylation independently of the phosphorylation state of the other head in the pair. To confirm this, myosin phosphorylated to < 100% was subjected to conditions (100 mM KCl, 8 mM MgCl2, 1 mM ATP, pH=7.4) under which all myosins with both heads phosphorylated form filaments and can be removed by centrifugation (confirmed by nondenaturing and glycerol gels). All remaining phosphorylated myosin must now be paired with unphosphorylated partners. These phosphorylated heads were 70% activated. Thus, phosphorylated smooth muscle myosin heads retain substantial activation even when paired with unphosphorylated partners.

M-Pos196

VELOCITY AND FORCE OF SINGLE SMOOTH MUSCLE CELLS CONTAINING MUTANT REGULATORY LIGHT CHAINS. ((K.M. Trybus[1], J. Carmichael[3], S. Yagi[3], H.L. Sweeney[2], F.S. Fay[3]))
[1]Brandeis University, Waltham, MA; [2]U. Pennsylvania School of Med., Philadelphia, PA; [3]U. Mass. Med. Center, Worcester, MA.

Phosphorylation of S19 of the regulatory light chain (RLC) triggers smooth muscle contraction. Here we assess if replacement of T18 and S19 of the RLC with negatively charged amino acids mimics the effect of phosphorylation to activate shortening and force production within the cell. Endogenous RLCs (> 50%) were extracted from permeabilized single smooth muscle cells isolated from the toad stomach. The RLCs were replaced with thiophosphorylated WT RLC (P-WT), or mutant RLCs in which both T18 and S19 were mutated to Ala (A), Glu (E), or Asp (D). Immunofluorescent images are consistent with incorporation of the RLCs into filamentous structures within the cell. Unloaded shortening was initiated by photolyzing caged ATP in the absence of calcium. Maximum unloaded shortening velocities 100 x ($\Delta L/L_i$)/sec increased in the following order: no added RLC, 2.0 ± 0.5 (n = 7; where n refers to individual experiments with 10 cells each); T18A/S19A, 4.5 (n = 2); T18E/S19E, 6.9 (n = 2); T18D/S19D, 8.1 ± 0.5 (n = 3); P-WT RLC, 9.0 ± 1.5 (n = 6). Cells reconstituted with P-WT RLC also generated levels of force at least as great as that of activated intact cells. These results suggest that under low load, two negatively charged amino acids can partially mimic the ability of phosphorylation to activate cellular shortening.

M-Pos197

STRUCTURAL ELEMENTS DEFINING THE FUNCTIONAL DIFFERENCE BETWEEN SKELETAL AND SMOOTH MUSCLE REGULATORY LIGHT CHAINS. ((Zhaohui Yang and H. Lee Sweeney))
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Regulation of the ATPase activity of smooth and non-muscle myosin II involves reversible phosphorylation of the regulatory light chain (RLC). The RLC from skeletal muscle myosin (skRLC) is unable to confer regulation (i. e. no inhibition of ATPase activity) to smooth muscle myosin when substituted for the endogenous smooth RLC (sm RLC). Studies of chimeric light chains comprised of the N- or C-terminal half of each skRLC and smRLC (Trybus, K M et al, J.B.C, vol 268, 4412-4419, 1993) suggest that the structural basis for the loss of this regulation is a difference in the C-terminal half of regulatory light chain. The purpose of this study is to delineate the structural elements within the C-terminal half of the smRLC that are absent in the skRLC and are necessary for regulation. By sequence comparison, six residues Arg103, Arg123, Met129, Gly130, Arg143 and Arg160 were identified in smRLC which are conserved in regulated myosin RLCs but missing in non-regulated myosin RLCs. Thus these residues are potential structural elements necessary for confering phosphorylation-mediated regulation to the smRLC. To test this hypothesis, a skRLC was engineered that replaced the corresponding residues with their smRLC counterparts (using oligonucleiotide-mediated mutogenesis and overexpressed in *E. coli*). The purified mutant protein was exchanged into and functionally tested with gizzard smooth muscle myosin. Our results indicate that these residues can render phosphorylation-induced regulation capacity to the skRLC. Thus these six residues, including four basic arginine residues located in the E, F, G and H helices which are missing in skRLC, may be the structural coordinates for the phosphoryl serine in the N-terminus.

M-Pos198

PHOSPHORYLATION OF THR18 ALONE OF MII REGULATORY LIGHT CHAIN IS SUFFICIENT FOR ACTIN FILAMENT MOTILITY. ((A.R. Bresnick, V.L. Wolff-Long, O. Baumann and T.D. Pollard)) Department of Cell Biology and Anatomy, Johns Hopkins University School of Medicine, Baltimore, MD 21205.

The full-length myosin II regulatory light chain (XRLC) cDNA was cloned from a stage II *Xenopus* oocyte library. The XRLC is 94% identical with chicken gizzard RLC, and the PKC and MLCK consensus phosphorylation sites are conserved. Recombinant XRLC is purified from *E. coli*, with a yield of 10-20 mg protein per liter of culture. Three mutant XRLCs have been engineered and expressed in *E. coli* in which the serine and threonine residues of the consensus MLCK site were replaced with alanine. The wt and mutant XRLCs bind to gizzard myosin denuded of endogenous RLC, demonstrating that wt and mutant XRLCs can adopt a properly folded structure. Denuded MHC was reconstituted with mutant XRLCs which were singly phosphorylated by MLCK. Although S19A XRLC/MII has only 50% the actin-activated ATPase of wt and T18A XRLC/MII hybrids, all three move actin filaments at the same rate of 0.7μm/s. However, the percentage of filaments in the field and the quality of movement of those filaments varied significantly between the S19A and wt or T18A XRLC/MII hybrids.

M-Pos199

THE DISTANCE BETWEEN RESIDUES 28 AND 108 IN A MUTANT OF CHICKEN GIZZARD MYOSIN REGULATORY LIGHT CHAIN BY RESONANCE ENERGY TRANSFER TECHNIQUE.((L.J. Ruan, T. Tao, and R.C. Lu)) Muscle Research Group, Boston Biomedical Research. Inst. Boston, MA 02114.

In order to use resonance energy transfer technique to study the effects of phosphorylation on the structure of smooth muscle myosin, a series of regulatory light chain (RLC) mutants that contain two Cys's are being made. In this report the RLC mutant (RLC28/108) which contains Cys instead of Ser at position 28 in addition to the endogenous Cys-108 was generated by site-directed mutagenesis and expressed in E.*coli* using the vector pAED4. This mutant retained the functional properties of the wild type RLC such as re-association with heavy chain, ability to be phosphorylated and regulation of ATPase activity. RLC28/108 was labeled with the donor 1,5 -IAEDANS to ~ 1 mole/mole and then labeled with the acceptor N-(4-dimethylamino-3,5 di-nitrophenyl) maleimide (DDPM). In the free state the fluorescence lifetime of the donor was measured to be 7.9 and 13.0 ns in the presence and absence of the acceptor, respectively. Application of the Förster Equation yielded an interprobe distance of 31 Å. Similar results were obtained when the light chains were associated with heavy chains. Our results showed that the distance between Cys-28 and 108 of the regulatory light chain is not affected by the association of the heavy chains and work is in progress to measure the distance between these two sites when light chains are phosphorylated. Supported by NIH PO1 AR41637.

M-Pos200

CONFORMATION OF A REGULATORY LIGHT CHAIN MUTANT OF CHICKEN GIZZARD MYOSIN. ((R.C. Lu, A. Wong, & L.J. Ruan)) Muscle Research Group, Boston Biomed. Res. Inst., Boston, MA 02114

To study effects of light chain phosphorylation on the smooth muscle myosin, both the wild type regulatory light chain (RLC) and mutant (RLC28/108) were expressed in E.*coli* using vector pAED 4. RCL has a single Cys at 108 whereas RLC28/108 has an additional Cys at 28. After incubation with 5,5'-dinitrobis(2-nitrobenzoic acid) (Nbs2) most of RLC28/108 formed an intramolecular disulfide bond, which appeared as a band with a higher mobility on non-reducing SDS-PAGE. To study the light chain- heavy chain interactions, a peptide, CG-A821, with amino acid sequence corresponding to the portion of heavy chain containing the light chain binding region (Ala821-Val847 with a Cys at 824) was synthesized. CG-A821 formed a single crosslinked product with RLC while two kinds of products formed with RLC28/108, suggesting that Cys824 of the heavy chain can form a disulfide bond with either Cys28 or Cys108. Energy resonance transfer (ERT) measurements showed that the distance between the probes at residues 28 and 108 is 31Å whether or not light chain is bound to heavy chain (Ruan et al. see above) whereas disulfide bond spans only 2Å. The seeming discrepancy is attributable to the facts that ERT measurements represent the average distance between the probes whereas crosslinking results reflect the possible minimum distance between the two thiols. Taken together, RLC is a flexible molecule whether or not it is bound to the heavy chains. Supported by NIH AR41637, AR28401.

M-Pos201

NUCLEOTIDE SEQUENCE OF 3 REGULATORY LIGHT CHAIN ISOFORMS PRESENT IN STRIATED AND CATCH *PLACOPECTEN* MYOSINS. ((C.L. Perreault, A. Jancso, L. Nyitray and A.G. Szent-Györgyi)). Brandeis University, Waltham, MA 02254

Catch myosin has lower actin-, K- and Ca-activated ATPase activity than striated myosin. To determine the regulatory light chain (RLC) composition of these two myosins, we cloned and sequenced the cDNA encoding the RLC of *Placopecten* catch and striated myosins. We found 3 different isoforms of the RLC, 2 from catch cDNA, which differed by 5 amino acids (156 and 161) and 1 from striated (156). In contrast, the essential light chains (ELC) from striated and catch myosin had identical nucleotide and deduced protein sequences. The longer RLC isoform of catch, representing about 40% of the RLC population, has an N-terminus extended by 5 amino acids and can be readily detected by urea gels. We were unable to detect the presence of a longer RLC isoform by urea gels of myosin from *Mercenaria, Ostrea* or *Argopecten* catch or non-catch muscles. However, a similar isoform has been found previously in *Patinopecten* and reported to be phosphorylated at serine 11 (Sohma et al., 1985). Differences between all 3 isoforms are restricted to the first third of the RLCs and they are most likely produced by the alternative splicing of a single RLC gene. We have expressed these RLC isoforms and are testing their function by RLC hybrids. Supported by NIH AR15963 and MDA.

M-Pos202

Characterization of Smooth Muscle Myosin Which Has Been Crosslinked in the 10S Conformation. ((Jennifer Olney and Christine Cremo)) Department of Biochemistry and Biophysics, Washington State University Pullman WA. 99164-4660

Smooth muscle myosin was labeled with a photoreactive crosslinker on Cys-108 in the C-terminal domain of the regulatory light chain (RLC). The RLC crosslinked to the heavy chain (HC) after irradiation of the folded 10S monomer, but not the extended 6S monomer. The 10S crosslinked myosin was purified based on its inability to form filaments. Gel analysis showed that purified crosslinked myosin contains a single crosslink between one HC and one RLC; the second HC remains unreacted. Salt-dependent changes in shape and ATPase activity of the crosslinked species were examined. Based on gel filtration analysis of conformation, the crosslinked myosin was incapable of adopting the 6S extended conformation. At all salt concentrations tested the crosslinked myosin eluted as though it were in a 10S folded conformation. Western blot analysis of proteolytic digestions showed that the crosslink was specific to the LMM region of the tail. Single turnover experiments showed that the crosslinked myosin had two populations of heads; one that retained a slow turnover rate and another which was partially activated by increasing salt concentration. These results suggest that the crosslinked head is locked in a "slow" conformation which is insensitive to salt concentration. In contrast, the uncrosslinked head can be partially activated by high salt even though it possesses a folded tail. In conclusion, the C-terminal portion of the RLC has been shown to interact with the LMM portion of the rod in the 10S state. The reason this interaction generates a salt-independent inactive state is under investigation. These studies may lend insight to the significance of the C-terminal portion of the RLC in phosphorylation-dependent regulation.

M-Pos203

EXPRESSION OF BOVINE ADRENAL GLAND MYOSIN I IN BACULOVIRUS-INFECTED SF9 CELLS.
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Previously we isolated a new cDNA clone encoding one of the non-conventional myosins, myosin I, from bovine adrenal gland (FEBS Letters 339 (1994) 31-36). The deduced amino acid sequence was highly homologous to other known myosin Is in the N-terminal 2-kb region which corresponds to myosin head domain, while no strong homology was detected in the tail region. To biochemically characterize this myosin I, we attemped to functionally express this myosin I using baculovirus expression system. Insect SF9 cells were transfected with recombinant baculovirus containing the myosin I gene. A large amount of a 108 kDa polypeptide corresponding to the myosin I heavy chain was detected in the total cell homogenate, however, the peptide could not be well extracted from the cell homogenate, presumely forming an insoluble aggregate. We found previously that calmodulin can bind the recombinant myosin I, suggesting that calmodulin serves as subunits. Therefore, we co-expressed calmodulin and myosin I to overcome the problem. A large fraction of expressed myosin I can be extracted from cell homogenate now, and the supernant displayed high K^+, EDTA ATPase activity. The recombinat myosin I co-sedimented with actin in an ATP dependent fashion. The partially purified myosin I showed high Ca^{2+}-ATPase activity. We are currently further characterizing the recombinant myosin I (supported by NIH).

M-Pos204

Use of Two New Sulfhydryl Reactive Fluorescent Probes to Examine Effects of Phosphorylation on the Regulatory Light Chain of Smooth Muscle Myosin ((Derek J. Pouchnik‡, Leroy L. Laverman‡, Gregory D. Reinhart#, Fabiola Janiak-Spens#, David M. Jameson⊥, and Christine R. Cremo‡)) ‡Washington State University, Department of Biochemistry and Biophysics, Pullman, Washington 99164-4660, #University of Oklahoma, Department of Chemistry, Norman, Oklahoma 73019- 0370, and ⊥University of Hawaii, Department of Biochemistry and Biophysics, Honolulu, Hawaii 96822-2233

We report the synthesis and characterization of two new fluorescent probes, 2-amino-2(bromoacetyl) hydrazidebenzoic acid (BAH) and N-[2-[(bromoacetyl)amino]ethyl]-2 methylamino)-benzamide (NBMB). These small anthraniloyl derivatives contain a sulfhydryl reactive haloacetyl group that reacted specifically with Cys-108 of the smooth muscle myosin regulatory light chain. In addition to having a high quantum yield and good sensitivity to solvent polarity these probes have relatively long excited state fluorescence lifetimes (~8 ns), useful for rotational studies of proteins. Time-resolved decay of anisotropy of the regulatory light chain was examined in the frequency domain. The effects of calcium and phosphorylation upon the global and local motions of these probes were investigated. The bimolecular acrylamide quenching constants have also been determined. These studies are designed to address the structural basis of the phosphorylation-dependent regulation of smooth muscle myosin.

M-Pos205

PROBING METAL BINDING TO MYOSIN LIGHT CHAIN TWO.

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Myosin light chain 2 (MLC2) is a 18.7 kDa protein involved in the regulation of muscle contraction. In skeletal muscle, MLC2 appears to be involved in modulating the Ca^{2+}-sensitivity of crossbridge cycling[1] . Previous data have suggested that MLC2 may bind two moles of Ca^{2+} per mole of MLC2[2]. One site, located at the functional EF-hand I in the N-terminal domain, is believed to be a physiologically relevant site and binds metal non-specifically in the μM range. The second site, which may not be physiologically relevant, binds metal in the mM range. We have used fluorescence resonance energy transfer (FRET) to show that this site is very close (<20 Å) to three residues located in the C-terminal domain of MLC2, demonstrating that the low affinity Ca^{2+}-binding site most likely resides in this domain. Additionally, NMR and circular dichroic spectroscopy have been used to monitor the conformational changes which occur in MLC2 upon metal binding. A novel pulsed-field-gradient NMR spectroscopy approach has also been used to probe the polydispersity of MLC2; we have used this technique to find suitable conditions for studying the structure of MLC2 using NMR spectroscopy.

1. Sweeney, Bowman & Stull, 1993 *Am. J. Physiol.* **264**:C1085
2. Hardwicke & Huvos 1988 *Biochim Biophys Acta* **957**:352
3. Supported by the NHMRC of Australia.

M-Pos206

Transcriptional Alterations in Myosin Heavy Chain Gene Expression are an Early Response to Hyperglycemia in Rats.

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Diabetic cardiomyopathy is a chronic pathologic adaptation to a complex neurohumoral and metabolic load. It is unknown whether cardiac muscle alterations are a primary component of the disease process or if they develop secondary to other metabolic abnormalities. To explore this, we looked at steady state mRNA levels of α and β myosin heavy chain (MHC) in control (C) and diabetic (D) rats early (3days; 3d) and late (1 month; 1 mo) following treatment with 65mg/kg streptozotocin (STZ). To control for acute STZ effects, a separate group of STZ animals was rendered euglycemic with insulin for the initial 3d (D + I). mRNA data are expressed relative to an oligo dT signal.

	3d			1 mo	
	C	D	D + I	C	D
α(MHC)	2.60	1.10*	2.23	2.62	0.81*
β(MHC)	1.30	2.00	1.93	0.42	1.87*

*$p < 0.05$ vs. matched C (n=4-7). Our results show that α- and β-myosin mRNAs are altered in 3d D hearts and these changes are further exaggerated in the chronic D animals in parallel with the protein data shown earlier. Thus, transcriptional changes in contractile protein gene expression are an early and persistent response of the heart to diabetes.

M-Pos207

SMOOTH MUSCLE MYOSIN ISOFORM mRNA AND PROTEIN LEVELS IN SMOOTH MUSCLE TISSUES AND CELLS. ((D.P. Meer and T.J. Eddinger)) Biology Department, Marquette University, Milwaukee, WI 53233.

This study was designed to examine smooth muscle myosin heavy chain (SM-MHC) content and regulation in single smooth muscle cells. cDNA produced via reverse transcription (RT) from single smooth muscle cells or SM tissue was utilized in the polymerase chain reaction (PCR) using two oligonucleotide primers from the known sequence of rabbit uterus myosin (nucleotide positions 5627-5646 and 5917-5898; Babij *et al.*, 1991). These primers span the alternative splice site which generates the difference between the 3' ends of the SM1 and SM2 smooth muscle myosin isoforms. Densitometric analyses of the PCR products corresponding to the mRNA of SM1 and SM2 were compared to the relative proteins levels of SM1 and SM2 in adjacent tissue samples generating a correlation of $r^2 = 0.91$. Duplicate RT reactions performed on the same extracted RNA yielded PCR amplified SM1:SM2 ratios which are not significantly different ($P<0.05$, n=6) indicating reproducibility of the methods. RT-PCR performed on single SM cells from the same tissue show a 4.5-fold range in SM1:SM2 content. These results suggest the relative content of SM1 and SM2 MHC is regulated at the transcriptional level and that the SM-MHC content of individual cells is not uniform.

M-Pos208

2'- AND 3'- CARBAMOYL DERIVATIVES OF ADENINE NUCLEOTIDES: THEIR RATES OF INTERCONVERSION AND ACTIVITY AS SUBSTRATES IN ATP AND ADP UTILIZING ENZYMES.

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2'- and 3'-*O*-*N*-methylanthraniloyl (mant) esters of ribonucleotides equilibrate rapidly ($t_{½}$ = 7 min at pH 7.4 and 22°C)[Eccleston et al. (1991) Biochem. Soc. Trans. **19**, 432-437]. This can be disadvantageous if the mant group is to be used as a fluorescent probe. As a model for further work we have synthesized 2'- and 3'-*O*-[*N*-2-[acetamido]ethylcarbamoyl] ADP and ATP derivatives based on established methods [Cremo et al. (1994) Biochemistry **29**, 3309-3319],separated them by HPLC, characterized them by ^{1}H-NMR and investigated their rate of equilibration. The ratio of 2'- to 3'- substituted nucleotide is 0.7 at equilibrium with $t_{½}$ for equilibration equal to 44 h and 0.3 h at pH 7.1 and pH 9.4 respectively. We have also studied properties of the triphosphates as substrates for myosin subfragment 1 and the diphosphates as substrates for creatine kinase and 3-phosphoglycerate kinase. Both triphosphates are satisfactory substrates for subfragment 1 but the diphosphates are poor substrates for the kinases. The 3'-derivative was a substrate for 3-phosphoglycerate kinase (1% of kinase activity compared to that with ADP), but otherwise activity was barely detectable.

MUSCLE MECHANICS AND ULTRASTRUCTURE

M-Pos209

ELECTRON MICROSCOPY OF ELASTICITY IN TITIN MOLECULES

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The titin molecule spans between the Z- and M-lines in striated muscle myofibrils. In the I-band it forms an elastic connection between the ends of the thick filament and the Z-line. At long sarcomere lengths the A-band part of the molecule also appears to become elastic. The mechanism of elasticity is not known. In the present experiments, electron micrographs were obtained of purified titin molecules staightened by a flow of liquid and after rotary shadowing. Frequently the molecules showed a segmented appearance, which suggests they were not only straightened but extended. This behaviour was accentuated in high salt. The segments had a width of ~4 nm and were joined by thinner connections. Segmented molecules were longer than unsegmented ones. Segmentation is unlikely to result from proteolysis, since it could also be seen in molecules renatured after exposure to 6M GuHCl, which would have allowed cleaved peptides to separate. The propensity to form segments was greater in monomers than in side-by-side dimers, possibly as a result of increased viscous drag during flow-straightening. The width of the monomers measured by unidirectional shadowing appeared more variable and was slightly less than in the molecules in dimers. Taken together, the data suggest that titin can be extended by shearing forces and that this probably reflects the elasticity of the molecule.

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M-Pos210

INTERACTION BETWEEN CARDIAC TITIN AND ACTIN?

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I-band titin is broadly viewed as a functional spring, independent of other of sarcomere's elements. Evidence for this has, however, been conflicting. Results in skeletal muscle suggested that titin was free and spring-like, while a recent cardiac study claimed interaction between titin and actin at the N2-line level (Funatsu et al. J. Cell Biol.120: 711-724). To follow up, we carried out "freeze-break" experiments on papillary muscles of dog heart. Narrow strips of stretched muscles were quickly frozen and broken under liquid nitrogen to fracture in planes perpendicular to the filament axis. Then mucle fragments were labelled with monoclonal antibody specific to cardiac titin (9D10), which labels the I-band near the A-I junction. Titin epitope position was normally symmetrical about the Z-line. In sarcomeres, broken at the A-I junction, however, the epitopes no longer remained symmetrical: titin filaments in the broken half-sarcomeres retracted, independently of the thin filaments forming dense band just near the Z-line. In sarcomeres, broken at the Z-line level, the titin filaments retracted to the ends of the thick filaments. In sarcomeres (or half-sarcomeres) slightly remote from the break, the titin-filament set was occasionally broken by the fracture, and retracted. Such sarcomeres remained elongated. This effect show that the titin filaments are important in returning the sarcomere to its resting lenght.
Conclusion: titin breakage and retraction occurs the same way in heart as skeletal muscle. The presence or absence of nebulin does not appear to influence titin behavior in the I-band. If an interaction between titin and actin exists in heart muscle, then the forces involved are insufficient to prevent titin-filament retraction after breakage.

M-Pos211

PASSIVE TENSION IN CARDIAC MUSCLE: THE CONTRIBUTION OF COLLAGEN, TITIN, MICROTUBULES AND INTERMEDIATE FILAMENTS. Henk Granzier & Tom Irving. Dept. of VCAPP, Washington State Univ., Pullman, Wa 99164-6520. The passive tension - sarcomere length relation of rat cardiac muscle was investigated by studying passive single myocytes and trabeculae. The contribution of collagen, titin, microtubules, and intermediate filaments to tension and stiffness was investigated by measuring (1) the effects of KCl/KI extraction, (2) the effect of trypsin digestion, and (3) the effect of colchicine. It was found that over the working range of the heart (sarcomere lengths ~1.9 - 2.2 μm) collagen and titin are the main contributors to passive tension with titin more dominant at the shorter end of the working range and collagen at the longer end. Microtubules contributed by a modest degree to passive tension in some cells, but on average their contribution was not significant. Finally, intermediate filaments contributed about 10% to passive tension at sarcomere lengths from ~1.9 to ~2.1 μm, and their contribution dropped to a few percent at longer lengths.

At physiological sarcomere lengths of the heart, cardiac titin developed much higher (>20 times) tensions than skeletal muscle titin did at comparable lengths. This might be related to the finding that cardiac titin has a Mr of 2.5 MDa, 0.3-0.5 MDa smaller than titin of mammalian skeletal muscle, which is predicted to result in a much shorter extensible titin segment in the I-band of cardiac muscle (Wang et al., 1991). Passive tension plotted vs. the I-segment extension ratio of titin showed that the differences between cardiac and skeletal muscle at the sarcomere level predominantly resulted from much higher strains of the I-segment of cardiac titin, and not from intrinsic differences in the capacity of titin to generate tension. By expressing a smaller titin isoform, without changing the properties of the molecule itself, cardiac muscle is able to develop significant levels of passive tension at physiological sarcomere lengths.

M-Pos212

CHARACTERIZATION OF A NOVEL 5.4 kb cDNA FRAGMENT CODING FOR THE N-TERMINAL REGION OF RABBIT CARDIAC TITIN **((M.G.Sebestyen, J.A.Wolff and M.L.Greaser)) University of Wisconsin-Madison, Madison, WI 53706**

Titin is an approximately 3 MDa elastic protein, which extends from the M-line to the Z-line in sarcomeres of striated muscle. In spite of the efforts of several laboratories, barely more than the half of the cDNA (about 45 kb) has been published, encoding primarily the C-terminal half of the polypeptide chain, corresponding to the A-band and M-line region. In order to obtain data from the unknown N-terminal part, a special cDNA library was constructed using size-selected total RNA from adult rabbit cardiac muscle. The T12 monoclonal antibody, which binds to an epitope close to the N-terminal end, was used to obtain initial cDNA clones. Additional overlapping clones have been isolated and sequenced yielding a 5.4 kb contig. Analysis of the predicted amino acid sequence revealed the presence of 16 Type-II (immunoglobulin-like) motifs and 4 unique interdomain segments. The highest amino acid sequence homology was with a previously described chicken embryonic skeletal muscle titin fragment. One of the interdomain segments carries potential phosphorylation sites for proline-directed protein kinases, similar to the ones found close to the C-terminal end of the human cardiac titin.

M-Pos213

CLONED TITIN FRAGMENTS CONTAINING A CLASS I AND A CLASS II MOTIF, INHIBIT ACTIN SLIDING IN THE *IN VITRO* MOTILITY ASSAY. (Quanning Li, Jian-Ping Jin, and Henk Granzier. Department VCAPP, Washington State University, Pullman, Wa and Dept. of Medical Biochemistry, University of Calgary, Calgary, Alta.).

Titin, a giant protein in the sarcomere of skeletal and cardiac muscles, consists mainly of repeats of two types of about 100-amino acid motifs (class I and class II motifs). Single class I, class II and a coupled class I-II motifs have been cloned from rat cardiac muscle (Jin, 1994. Submitted). To investigate the function(s) of titin, we studied the effect of the class I, class II and class I-II titin motifs on the actomyosin interaction using an *in vitro* motility assay.

The sliding velocities of fluorescently labeled actin filaments on rat skeletal myosin were measured in the absence of these titin fragments and after adding the fragments to the ATP containing assay buffer. In the absence of titin fragments, more than 95% of the actin filaments in the field of view moved, and their average velocity was 4.2 μm/s (29 ± 1.0 °C). It was found that the class I-II fragment inhibits the sliding movement of actin filaments; class I and class II, however, did not affect motility. In the presence of class I-II fragment, the ratio of the number of sliding actin filaments to the total number of filaments in the field of view decreased both with increasing concentrations of the fragment and with time. Actin filaments stopped moving completely ~6 minutes after the addition of 5 μM of the class I-II titin fragment.

M-Pos214

SEGMENTAL EXTENSION OF TITIN FILAMENTS IN ISOLATED CARDIAC MYOFIBRILS. ((W.A. Linke‡, M.L. Bartoo¥ and G.H. Pollack¥)) ‡Physiology II, Univ. Heidelberg, Im Neuenheimer Feld 326, D-69120 Heidelberg and ¥Bioengineering WD-12, Univ. of Washington, Seattle, WA 98195.

Single myofibrils were isolated from chemically skinned rabbit ventricle and investigated in an apparatus described previously (Linke *et al., Circ. Res.* 73:724-734, 1993). In relaxed myofibrils (room temperature; 200 mM ionic strength), we measured both passive tension and stiffness (determined from the response to 500 or 1300 Hz sinusoidal oscillations) over a wide range of sarcomere lengths (SLs), from 2 to 6 μm. We tested whether the segmental extension model of resting tension proposed for skeletal muscle (Wang *et al., Proc. Natl. Acad. Sci. USA* 88:7101-7105, 1991) is also applicable to cardiac muscle. According to this model, resting tension at small to moderate degrees of stretch is supported by the elastic I-band segment of titin; at larger degrees of stretch, a titin segment ordinarily bound along the thick filament becomes unbound and adds to the free I-band segment.

Stretch of cardiac myofibrils up to SLs of approximately 3 μm resulted in a steady increase of both passive tension and stiffness. With further stretch, however, the slope of the passive length-tension curve was greatly reduced and the length-stiffness curve even became flat. Clearly, an elastic limit was reached at those extended lengths. In addition, between 3.0 and 4.3 μm SL, both curves usually showed a series of mostly four inflections, spaced regularly at intervals of 0.3 to 0.35 μm. We assume that those inflections result from micro-release of titin segments, at a specific locus (bond) along the thick filament. From the spacing of the inflections, we calculated the distance between two such bonds to be 38 to 44 nm (*cf.*, Linke *et al., Biophys. J.* 67:782-792, 1994). Finally, beyond an SL of approximately 4.3 μm, passive tension and stiffness increased again, likely due to strain of endosarcomeric intermediate filaments (Wang *et al., Biophys. J.* 64:1161-1177, 1993).

We conclude that (i) both myofibrillar passive tension and stiffness are determined by the properties of the titin (connecting) filament; (ii) the segmental extension model of resting tension appears to be valid for cardiac muscle, with an elastic limit occurring at shorter SLs than in skeletal muscle; (iii) under physiological conditions, titin may be bound along the thick filament with a distinct spacial periodicity near 40 nm.

M-Pos215

SHORT TIME SPECTRAL ANALYSIS OF MUSCLE FIBER IMAGES AS A MEANS OF ASSESSING LOCAL SARCOMERE HETEROGENEITY **((M.P. Slawnych, L. Morishita and B.H. Bressler)) Department of Anatomy, University of British Columbia, Vancouver, B.C., Canada.**

Sarcomere length heterogeneity in psoas muscle fiber segments was examined by employing a short time spectral analysis method. Muscle segment images were partitioned into several, overlapping sub-images spanning a small fraction of the full image, and the associated power spectra were calculated. Centroid frequencies of the first order diffraction lines were calculated and inverted to yield the sarcomere lengths. In general, fiber segments exhibited little initial pre and post activation heterogeneity. However, this heterogeneity increased both during activation and after many activation cycles.

This method offers a number of advantages over the more commonly employed "hill/valley" analysis of luminance lines. Firstly, line luminance patterns are often distorted by other cell structures, which complicates the process of identifying each series sarcomere. Secondly, in order to get a good representation of sarcomere heterogeneity in the direction normal to the fiber axis, several luminance lines spanning the fiber width have to be examined. Since the sub-images extend in both the axial and normal fiber directions, the spectral based sarcomere measures give a two-dimensional representation of regional sarcomere length.

(Supported by the Medical Research Council of Canada).

M-Pos216

EXCHANGE OF FLUORESCENTLY LABELLED MOLECULES IN SKINNED MUSCLE FIBERS STUDIED BY CONFOCAL MICROSCOPY. ((*T.Kraft, +J.M. Chalovich, $Th. Wallimann, *B.Brenner)) Medical School, Hannover, FRG; $IZB, ETH-Hönggerberg, Zürich, Switzerland; +ECU, Greenville, NC.

Previously we observed that Rh-labelled creatine kinase (Rh-M-CK) after diffusion into a skinned muscle fiber is bound rather tightly to the M-line of each sarcomere and cannot be removed even within several hours by incubating the fiber in relaxing solution. However, during incubation in a relaxing solution containing unlabelled M-CK the bound Rh-M-CK could be exchanged within a few minutes.

We now extended our studies on removal and exchange of Rh-phalloidine and Rh-troponin after equilibrating fibers with these molecules. Although Rh-phalloidine, due to its tight binding to actin, could not detectibly be removed by incubation in relaxing solution for 10 hours, about 80% of the fluorescently labelled phalloidine could be replaced within the same period in the presence of unlabelled phalloidine, indicating significant dissociation of Rh-phalloidine from actin. A similar behaviour was found for Rh-Tn, that could be replaced almost completely within 12 hours by unlabelled troponin without the need of first extracting the fluorescently labelled troponin under quite rigorous experimental conditions. (Supported by DFG Br849/1-4).

M-Pos217

STRUCTURAL CHANGES IN FLUORESCENTLY LABELLED TNC CORRELATE MORE WITH [Ca^{2+}] THAN FORCE IN PHOSPHATE ANALOGUE INHIBITED SKINNED SKELETAL FIBERS. ((D.A. Martyn, P.B. Chase, L.L. Huntsman and A.M. Gordon)) Center for Bioengineering, Dept. of Radiology, and Dept. of Physiology & Biophysics, University of Washington, Seattle, WA 98195

The effects of Ca^{2+} and actomyosin crossbridges on troponin C (TnC) structure were measured in single glycerinated and detergent treated fibers from rabbit psoas muscle. Endogenous TnC was extracted and fibers were reconstituted with skeletal TnC labelled at methionine 25 with the fluorophore (N-((2-(iodoacetoxy)ethyl)-N-methyl)amino-7-nitrobenz-2-oxa-1,3-diazole (TnC-NBD). The NBD-label was excited by illumination of the fiber at 480 nm and the resulting emission collected though a 500-570 nm filter. At maximal Ca^{2+} activation (pCa 4.6) fiber fluorescence (FL) was enhanced 40 ± 18% (mean ± SD, n = 4 fibers), relative to FL at pCa 9.2. Maximum Ca^{2+}-activated force was inhibited with phosphate analogues, either vanadate (Vi; 1 mM) or aluminofluoride (AlF_x; 0.5 mM Al^{3+}, 10 mM F; Chase *et al.*, J.P. 460, 231, 1993). With Vi at pCa 4.6, force was 13 ± 1% of maximum and FL was enhanced 43 ± 11% (mean ± SD, n = 3 fibers), relative to pCa 9.2. When force was inhibited with AlF_x the corresponding values were 44 ± 29% for FL and 4.3 ± 0.5% for force (mean ± SD, n = 3 fibers). When Vi or AlF_x inhibited fibers are exposed to pCa 4.6 without inhibitors, tension recovered to near control level with a slow time course, being slower for AlF_x than for Vi (Chase *et al.*, J.P. 460, 231, 1993). On the other hand, FL was maximal throughout recovery from force inhibition. To test if "weakly binding" crossbridges contribute to structural changes in TnC-NBD, fibers were exposed to low ionic strength relaxing solution known to promote weak actomyosin interaction; fiber FL was unaffected relative to that found in normal ionic strength relaxing solution. These results suggest that structural changes in TnC-NBD correlate more with Ca^{2+} binding to TnC than to direct effects of crossbridges in skeletal muscle fibers. Supported by NIH HL 51277 and HL52558.

M-Pos218

AN OPTICAL BIOSENSOR OF SMOOTH MUSCLE REGULATORY LIGHT CHAIN PHOSPHORYLATION IN SKINNED SMOOTH MUSCLE FIBERS (W. Glenn L. Kerrick, Kathleen M. Trybus*, Young Soo Hann, Jung Ren Chen and Pei-Ren Wang) Department of Physiology and Biophysics, Univ. of Miami, Miami, FL 33101 and *Rosenstiel Basic Medical Sciences Research Center, Brandeis Univ., Waltham, Mass.

A genetically engineered monocysteine derivative of the chicken gizzard regulatory light-chain (Cys18/RLC) when labeled with acrylodan (AC) acts as an optical biosensor of light-chain phosphorylation in purfied myosin (Post, P.L., Trybus K. M., and Taylor, D.L., J.B.C. v.269, 12880-7, 1994). In the present study approximately 30 percent of the endogenous RLC was substituted with Ac-Cys18/RLC in skinned chicken gizzard fibers in order to determine the feasibility of measuring RLC phosphorylation simultaneously with force. Activation of the skinned fibers by Ca^{2+} caused an increase in acrylodan fluorescence which preceded the activation of force. Removal of Ca^{2+} from the fibers resulted in a rapid decrease in acrylodan fluorescence which also preceded a slower decline in force. When the skinned fibers were transferred to a rigor solution, only a small calcium sensitive increase in fluorescence was observed, less than 10% of maximal change. These results in rigor solutions suggest that only a small fraction of the Ca^{2+}-activated fluorescence change in the presence of $MgATP^{-2}$ can be

M-Pos219

ORIENTATION OF ACETAMIDOTETRAMETHYLRHODAMINE (ATR) PROBES COVALENTLY BOUND TO SINGLE CYSTEINE MUTANTS OF REGULATORY LIGHT CHAIN (RLC) IN RABBIT PSOAS MUSCLE FIBERS. **((Cibele Sabido-David, Lakshmi D. Saraswat[1], Susan Lowey[1], James S. Craik[2] and Malcolm Irving))** Randall Institute, King's College London, London WC2B 5RL, U.K., [1]Rosenstiel Center, Brandeis University, Waltham, MA 02254, USA, [2]National Institute for Medical Research., Mill Hill, London NW7 1AA, U.K.

Mutants of chicken skeletal RLC containing single cysteine residues at positions 2, 73, 94, 126 and 155 (Cys2 etc., Saraswat et al., *J. biol. Chem.* **267**, 21112-8, 1992) were modified with either the 5- or 6- isomer of iodo-ATR (Corrie & Craik, *J. Chem. Soc., Perkin Trans.* 1, 2967-74, 1994). The labelled RLC was exchanged into single glycerinated muscle fibers. Steady state fluorescence polarization ratios parallel ($P_\parallel$) and perpendicular ($P_\perp$) to the fiber axis were measured under conditions of relaxation, rigor and isometric contraction (10°C). For most of the labelled RLCs the separation between $P_\parallel$ and $P_\perp$ was greatest in rigor. Thus, orientational order of the RLC-binding domain of the myosin head is greater in rigor than in either relaxation or activation, as found previously with ATR probes on the native cysteines of skeletal and smooth muscle RLCs (Shrimpton et al., *Biophys. J.* **57**, 545a, 1990; Sabido-David et al., *Biophys. J.* **66**, A234, 1993). For 5- and 6-ATR-labelled Cys2, 73, 94 and 126 and for 5-ATR-labelled Cys155, $P_\perp$ was greater than $P_\parallel$ in rigor, but for 6-ATR-labelled Cys155, $P_\parallel$ was the larger ratio. In isometric contraction, probes on all the RLC mutants were highly disordered, but in some cases the observed polarised intensities could not be fitted by a linear combination of the relaxed and rigor values, suggesting that the orientation of the RLC-binding domain during contraction is distinct from that in either rigor or relaxation. Supported by Wellcome Trust and MRC, UK.

M-Pos220

MICROSECOND ROTATIONAL DYNAMICS OF MUSCLE CROSS-BRIDGES DETECTED AT TWO DISTINCT SITES IN ORIENTED FIBERS. ((David W. Hayden, Sampath Ramachandran and David D. Thomas)) Dept. of Biochemistry, University of Minnesota Medical School, Minneapolis, MN 55455.

Recent models of crossbridge motion suggest independent movement of domains within the myosin head, so a complete description of the molecular dynamics requires probes at several distinct sites. Therefore, we have studied the microsecond rotational motions of phosphorescent probes **attached specifically to two distant sites on the myosin head** in **oriented** rabbit psoas fibers. Motions of the light-chain-binding region were detected with phosphorescent probes bound to Cys108 of chicken gizzard regulatory light chains (RLC), which were incorporated into RLC-depleted rabbit psoas fibers. We labelled SH1 (Cys707) directly in fibers, to look at the ATP-binding region. **Time-resolved phosphorescence anisotropy** (TPA) was performed under physiological ionic conditions at 4 °C. Both sites were rigid in rigor in the 2 to 800 microsecond time scale of the experiment. The addition of ATP induced large-amplitude microsecond rotational motion both in the presence and absence of calcium, but **substantial differences in the amplitudes and rates of motion suggest different molecular dynamics in the two regions of myosin.**

M-Pos221

SATURATION TRANSFER EPR OF SPIN-LABELED LIGHT CHAINS IN CONTRACTING MUSCLE FIBERS. ((Josh E. Baker, *Osha Roopnarine, Sampath Ramachandran, David D. Thomas)) University of Minnesota Medical School, Minneapolis, MN 55455, *Albert Einstein College of Medicine, Bronx, NY 10461

Using EPR spectroscopy, we have studied the orientation and dynamics of three different spin labels (FDNASL, MSL, and InVSL) covalently attached to the single Cys (108) of gizzard regulatory light chains (RLC), functionally incorporated into the crossbridges of both rabbit and scallop muscle fiber bundles. Conventional EPR shows that both MSL and FDNASL are significantly oriented but weakly immobilized in both fiber types, while InVSL is orientationally disordered and strongly immobilized. Therefore, InVSL is ideal for the detection of microsecond rotational dynamics with saturation transfer (ST) EPR. ST-EPR spectra show that InVSL-RLC is more dynamically disordered in relaxation and contraction than in rigor. A comparison of these ST-EPR results with those of InVSL bound to Cys 707 (SH1, on the catalytic domain) indicates that the light chain probe is more mobile in rigor, has similar mobility in relaxation, and is less mobile in contraction. These results support the proposal that the light chain binding domain undergoes rotational motions distinct from those of the catalytic domain during force generation.

M-Pos222

ORIENTATIONAL DYNAMICS OF SPIN LABELED MYOSIN HEADS IN MUSCLE FIBERS IN THE PRESENCE OF NUCLEOTIDES. ((Osha Roopnarine and David D. Thomas)) Albert Einstein College of Medicine, Bronx, NY 10461 and University of Minnesota Medical School, Minneapolis, MN 55455

We have used conventional EPR to study the orientation of InVSL-labeled myosin (at Cys 707) heads in the presence of nucleotides designed to trap states intermediate in the actomyosin ATPase cycle. We previously showed that the spin labels are well-oriented in rigor, disordered in relaxation and contraction. The addition of 5 mM MgADP to rigor fibers, inducing a state very late in the force-generating cycle, induces a small axial rotation (approximately 7°) of the myosin head on actin. 5 mM K_4PPi or 16 mM AMPPNP (inducing a state with intermediate actin affinity) introduces a disordered population of detached heads, but the actin-bound heads are oriented as in rigor. 5 mM orthovanadate has negligible effect in rigor, but in 5 mM MgATP (partial relaxation), vanadate induces substantial disorder. In 25 mM MgATPγS the myosin heads are disordered as in relaxation, and the addition of Ca^{2+} resulted in less than 5% in a rigor-like state. The results are consistent with a model in which the myosin heads are disordered early in the power stroke (weak-binding states) and rigidly oriented as in rigor late in the power stroke (strong-binding states).

M-Pos223

ORIENTATION OF PARAMAGNETIC PROBES ATTACHED TO GIZZARD LIGHT CHAIN-2 EXCHANGED ONTO MYOSIN HEADS OF RABBIT SKELETAL MUSCLES ((Jeremy Gollub, Liang Zhao and Roger Cooke)) Dept. of Biochemistry, & Biophysics, Cardiovascular Research Institute, & Grad. Group in Biophysics, University of California, San Francisco, CA 94143.

Theories of muscle cross-bridge function suggest that force generation results from a change in the orientation of the neck region of myosin. To test this hypothesis, we have studied the orientation of the neck region using electron paramagnetic resonance (EPR) spectroscopy. A paramagnetic probe, 4-(2-Iodoacetamido)-TEMPO (IASL), was covalently attached to cys-108 on LC-2 of chicken gizzard myosin. Labeled gizzard LC-2 was exchanged for native skeletal LC-2 in rabbit psoas muscle fibers. EPR spectra of rigor fibers showed that the probes as well as the myosin neck region were ordered in the rigor state. The spectra of labeled S1 and HMM bound to fibers showed a similar orientation as rigor fibers. Spectra of relaxed fibers showed that the myosin neck region was disordered in this state. Active fibers produced spectra identical to relaxed fibers, suggesting that the neck region was also disordered. In order to test the response of the myosin head neck region to a static external force, passive tension was applied to rigor fibers. At all loads from 0 to 4 N/mm^2 the EPR spectra were essentially identical, providing no evidence of a change in the orientation of the neck region. The data show that the myosin neck region is disordered during active force generation, but not by a static passive external force. This observation is compatible with the above model, but suggests a more flexible lever arm than had been expected. Supported by AR30868, AR42895, and 5T32CA09270 (LZ).

M-Pos224

MOBILITY OF MYOSIN HEAD MONITORED BY A SPIN PROBE ATTACHED TO THE ALKALI LIGHT CHAINS IN MYOSIN FILAMENTS.
((B. Adhikari, D. Szczesna, K. Hideg, and P. Fajer)) Inst. of Mol. Biophys., Biol. Sci. Dept., Florida State University, Tallahassee, FL.; University of Pecs, Pecs, Hungary.
Fundamental to understanding the mechanism of muscle contraction is characterization of the internal flexibility of the myosin head. In this study, we have measured the mobility of the alkali light chains using saturation transfer electron paramagnetic resonance (ST-EPR). Alkali light chains bind to the regulatory domain of the myosin head, proposed to be a lever in transmitting mechanical force (*Raymont et. al., 1993*).
The light chains have been purified according to Wagner (*1982*) and labeled with maleimide, iodoacetamide, and indane-dione (InVSL) spin labels. In all cases the labeled site was Cys-177 in LC1 or Cys-136 in LC3 (*Bhandari et. al., 1991*). The light chain isozymes were not separated but the cysteines are situated at identical location within the myosin head (S1). Rigidity of the labels with respect to the protein was determined by immobilization of the protein on glass beads and monitoring the mobility by ST-EPR. Among the above spin labels only InVSL was found to be rigidly bound to the protein. The 90% InVSL labeled alkali light chains (InVSL-alkLC) were subsequently exchanged into myosin (*Wagner, 1982*). The efficiency of exchange was between 40 and 55%. The observed rotational correlation time (τ_R) of InVSL-alkLC in myosin filaments was ~16-20 μs. This mobility is significantly lower than the mobility of myosin filaments labeled with InVSL at the reactive cysteines (SH1, SH2) of the catalytic domain (τ_R ~ 2 - 4 μs). The difference in mobility implies a putative hinge between regulatory and catalytic domain within S1, consistent with the model of Raymont et. al. (*1993*).
Raymont, I et. al. Science. vol 261. pp. 58-64. 1993.
Wagner, PD. Methods in Enzymology. vol. 85. pp. 73-81. 1982.
Bhandari DG. et. al. Biochim Biophys Acta. 1077. pp. 385-91. 1991.

M-Pos225

Abstract Withdrawn.

M-Pos226

THE THREE-DIMENSIONAL STRUCTURE OF THE Z BAND FROM SKELETAL MUSCLE IN RIGOR. ((J.P. Schroeter*+, R.J. Edwards**, and M.A. Goldstein*)) *Baylor College of Medicine, Houston, TX., 77030, +University of Texas Health Science Center at Houston, *Rice University, Houston, TX., and **Duke University, Durham, NC.

Three dimensional reconstructions of several regions of the Z band and the nearby I band have been produced from electron micrographs of rat soleus muscle in rigor. These reconstructions were calculated using the method of weighted back-projection on EM tilt series of a longitudinal section rotated about two orthogonal axes. The resolution of the resulting three-dimensional structures ranged from 4.5 to 5.5 nm.

The Z band reconstructions reveal a complex set of cross-connecting Z-filaments joining interdigitating axial filaments which enter the Z band from opposite sarcomeres. Projections of cross sections of the reconstructed Z bands exhibit the basket-weave form of the Z band lattice, with a Z band spacing of 27 ± 4 nm. This form and spacing for the lattice are consistent with results from electron micrographs of untilted cross sections of this muscle[1].

1. R.J. Edwards, M.A. Goldstein, J.P. Schroeter, and R.L. Sass, J. Ultrastructure Mol. Struct. Res. 102:59-65 (1989)

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M-Pos227

EQUATORIAL X-RAY DIFFRACTION INTENSITIES AND LATTICE SPACING IN INTACT AND SKINNED MUSCLE FIBRES.
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The effect of lattice spacing changes on the equatorial diffraction pattern of single, intact muscle fibres from *Rana temporaria* was studied using synchrotron radiation (λ=0.15nm, beam dimensions 0.3x4mm, source DORIS). In hypertonic saline (1.4x), lattice spacing was 37.66nm, and the intensity of the 10 and 11 reflections was reduced (I_{10} to 51.9%; I_{11} to 59.2%). In hypotonic saline (0.8x), lattice spacing was 43.87nm and I_{10} increased to 198.8%, I_{11} to 179.1%. When the fibre was subsequently chemically skinned, (lattice spacing 44.59nm), an even larger intensity increase was observed for the 10 reflection (I_{10} to 382.3%; I_{11} to 168.8%). The effect of skinning was not completely reversed on lattice recompression by dextran, I_{10} falling to 195.9% of intact cell intensity, I_{11} to 142.0%, hence lattice spacing only partially accounts for intensity changes on skinning. We examined the effect of soluble scattering molecules on I_{10} and I_{11} by exposing the skinned fibre to different concentrations of glycerol during lattice compression with dextran. A 15% solution of glycerol restored the equatorial intensities to their intact cell values (lattice spacing constant). Since this corresponds in density to a protein solution of 150mg.ml^{-1}, we examined various soluble proteins for a similar effect on intensities (BSA, parvalbumin, aldolase, troponin, myoglobin, and others) in this concentration range. Loss of soluble scattering proteins on skinning may in part account for the intensity difference between intact and skinned fibres at a given spacing. Their dilution may account for some of the intensity increase in hypotonic cells.

M-Pos228

TIME COURSE OF TENSION AND INTENSITIES OF THE INNER EQUATORIAL X-RAY REFLECTIONS ON THE PHOTOLYSIS OF DMB-CAGED ATP IN APYRASE-TREATED RABBIT SKELETAL MUSCLE FIBRES. ((P.D. Brown, M.A. Ferenczi, M. Irving*, I. Dobbie*)) *National Institute for Medical Research, Mill Hill, London NW7 1AA, UK. *The Randall Institute, King's College London, WC2B 5RL, UK.*

The intensities of the inner equatorial X-ray reflections from skeletal muscle fibres are related to the structural attachment of subfragment 1 (S1) of myosin to actin in the thin filament. The intensities of these reflections were recorded from single glycerinated fibres of rabbit psoas following ATP release from the *P*^{3}-3',5'-dimethoxybenzoin ester of ATP (DMB caged-ATP) at an ionic strength of 0.15 M and 5°C. DMB caged-ATP photolyzes with a rate constant >10^{5}s^{-1}, at least 2 orders of magnitude faster than conventional caged-ATP (NPE-caged ATP) (Thirlwell *et al.*, 1994, *Biophys. J.* In press).. The fibres were put into rigor using a solution containing apyrase and were then placed in a 10 mM DMB-caged ATP solution that also contained apyrase. This protocol simplifies the relaxation process by removing acto-S1 ADP states (Thirlwell *et al.*, 1994). Laser flash photolysis yielded 1 mM ATP as determined by HPLC analysis. In the absence of calcium the intensity of the 1,1 reflection (I(1,1)) fell with a rate constant of 120±7s^{-1} (30) (Mean±SEM (n)) while tension fell at 55±2s^{-1} (14). In the presence of 32 μM free [Ca^{2+}], tension initially fell partly towards zero before rising to the isometric plateau at 7±2s^{-1} (15). The initial tension drop was characterized by a rate constant of 47±6s^{-1} (13) whereas I(1,1) fell at 112±2s^{-1} (13). No change in I(1,1) was associated with the rising phase of tension. The 1,0 reflection has a more complex time course in all cases, which was not fitted well by a single exponential. The time courses of the initial tension fall and of the I(1,1) indicate that following ATP binding, initial cross-bridge detachment occurs with a rate independent of the presence or absence of calcium. The rate of tension fall observed here is faster than that reported previously (Poole *et al.*, 1991, *Adv. Biophys.* 27: 63-75), but still slower than the rate of decrease of I(1,1). The cross-bridge detachment rate deduced from these measurements is not limited by the rates of ADP release or by that of DMB-caged ATP photolysis.

M-Pos229

Millisecond time-resolved lattice spacing measurements accompanying length changes during tetani in single intact muscle fibers. ((Eric F. Eikenberry, Frederik Osterberg*, G. Cecchi†, M.A. Bagni†, C.C. Ashley‡ and P.J. Griffiths‡.)) Robert Wood Johnson Medical School, Piscataway, NJ; *Princeton University, Princeton, NJ; †Universita degli Studi di Firenze, Florence; and ‡Oxford University, Oxford.

Force development in muscle is believed to be caused by transient formation of cycling cross-bridges between actin and myosin filaments. Force recovery following fast release (200 μs, 1% fiber length) at the tetanus plateau during time-resolved sarcomere length measurement in single fibers *(Rana temporaria*, tibialis anterior muscle, sarcomere length 2.2 μm, 4 °C) showed a multiphase response (Huxley & Simmons, *Nature* **233** (1971), 533-538). The first phase has been interpreted as arising from the structural change in attached cross-bridges responsible for the 'power stroke'. These phases of the axial force response may be associated with changes in the spacing of the myofilament lattice, brought about by the alteration of the radial force component of the cross-bridge tension vector (Cecchi, *et al., Science* **250** (1990), 1409-1411).

We have studied lattice parameters during rapid length changes by time-resolved x-ray diffraction using a charge-coupled device (CCD) x-ray detector operated as a streak camera at station A1 of the Cornell High Energy Synchrotron Source (CHESS, 13.55 keV, 1.75 m small angle camera). Time resolution of *ca.* 1 ms was achieved by clocking the CCD lines at 5 kHz past a 10 pixel-wide aperture. The time resolution of the experiment was limited by the flux in the collimated beam at the specimen plane (*ca.* 6×10^{11} photons/s). Preliminary data analysis has yielded lattice spacings, intensity, and disorder parameters at millisecond time resolution.

M-Pos230

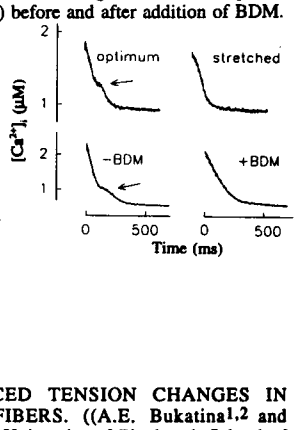
SARCOMERE LENGTH VS. INTERFILAMENT SPACING AS DETERMINANTS OF CARDIAC MYOFILAMENT Ca^{2+} SENSITIVITY. ((Yi-Peng Wang and Franklin Fuchs)) Dept. Cell Biology and Physiology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261.

The Ca^{2+} sensitivity of skinned cardiac muscle can be increased either by increasing the sarcomere length (Hibberd and Jewell, 1982) or by osmotically compressing the myofilament lattice (Harrison, et al, 1988). Since an increase in sarcomere length is accompanied by a reduction in interfilament distance it is not clear whether length-dependent changes in Ca^{2+} sensitivity are related to changes in length or changes in interfilament spacing. To obtain quantitative information on the relative contribution of these two parameters skinned bovine ventricular muscle bundles of varying sarcomere length (1.7-2.3 μm) were exposed to varying concentrations (0-5%) of Dextran T-500. Measurements were made of changes in muscle width in response to Dextran addition and force-pCa curves were determined as a function of both sarcomere length and lattice compression. The data obtained made it possible to 1) determine the effect of osmotic compression at different sarcomere lengths on Ca^{2+} sensitivity, and 2) compare force-pCa curves as a function of sarcomere length under conditions in which interfilament spacing was relatively constant. The estimated pCa_{50} values correlated more closely with change in interfilament spacing than with change in sarcomere length alone. Thus length-dependent force generation in cardiac muscle may be based primarily on length-dependent changes in actin-myosin separation. Supported by NIH grant AR10551.

M-Pos231

CALCIUM RELEASE TO THE CYTOSOL DURING SKELETAL MUSCLE RELAXATION DEPENDS ON FORCE RELAXATION. ((A.J. Baker and M.W. Weiner)). Univ. Calif. San Francisco CA 94121.

During skeletal muscle relaxation, the decline of intracellular calcium ($[Ca^{2+}]_i$) is interrupted by a phase where the fall of $[Ca^{2+}]_i$ is markedly slowed (phase 2). Phase 2 was suggested to represent a co-operative Ca^{2+} release from troponin when cross-bridges depopulate actin. **Goal:** To test this suggestion by determining if phase 2 is abolished when cross-bridge interactions are inhibited by: a) stretching muscles to eliminate filament overlap; and b) using 2,3-butanedione monoxime (BDM) to eliminate strongly bound cross-bridges. **Results:** The figure shows the time course of $[Ca^{2+}]_i$ decline, measured from indo-1 fluorescence, following a brief tetanus of frog muscle: a) at optimum muscle length and after stretching; and b) before and after addition of BDM. Phase 2, the shoulder marked by the arrows on the control records, was abolished when force development was prevented by stretching or BDM. **Conclusion:** Phase 2 represents an addition of Ca^{2+} to the cytosol which occurs only in the presence of relaxation of developed force. This is consistent with co-operativity between Ca^{2+} release from troponin and detachment of strongly-bound cross-bridges.



M-Pos232

CALCULATION OF INTERNAL LOADING IN HIGHLY SHORTENED SMOOTH MUSCLE. ((R. A. Meiss)) Indiana University School of Medicine, Indianapolis, IN 46202

Smooth muscle slows continuously as it shortens. During the major portion of a lightly-loaded isotonic contraction of electrically stimulated ovarian ligament smooth muscle, the principal force opposing shortening was provided by the external afterload. Muscle stiffness during this phase of shortening was proportional to the afterload force. It varied only slightly, taking on a value predicted by the force-*vs*-stiffness relationship measured during isometric contraction. As the muscle length approached its minimum value for a lightly-loaded contraction, stiffness rose markedly and velocity decreased more rapidly. The length-dependent stiffness increase has been interpreted as arising from crossbridges working against an internal load due to the extracellular matrix (Meiss, R.A., *J. Musc. Res. Cell Motil.* 13: 190-198, 1992). Using this assumption, a series of afterloaded contractions covering the range from zero to maximum force was recorded. From the traces of length-*vs*-time, force-velocity curves were constructed at a series of instantaneous lengths. The force equivalent of the extra isotonic stiffness was computed and added to the isotonic load. This correction indicated that V_{max} values at the shortest lengths would have been approximately 50% higher in the absence of an internal load whose magnitude was on the order of 10% of F_{max}. Supported by the Dept. of OB/GYN, I. U. School of Medicine.

M-Pos233

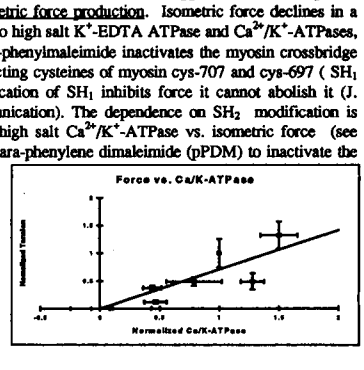
ORIGIN OF PHALLOIDIN-INDUCED TENSION CHANGES IN SKELETAL MUSCLE SKINNED FIBERS. ((A.E. Bukatina[1,2] and F. Fuchs[1])) [1]Dept. Cell Biol. Physiol., University of Pittsburgh School of Medicine, Pittsburgh, PA 15261, [2]Inst. Theor. Exp. Biophysics, Russian Acad. Sci., Pushchino, Russia 142292 (Spon: B.T. Ameredes).

Activated rabbit psoas skinned fibers respond to phalloidin (PH) by changes in isometric force which appear to be the sum of several (usually 2-3) exponentials with the same origin [Bukatina & Morozov, 1979; Son'kin, et al., 1983]. These changes might reflect both 1) binding of PH, and 2) slow isomeric conformational changes taking place after binding of PH. To assess the role of these processes the influence of preliminary partial saturation with PH on the response to PH was studied under conditions where the response of control fibers consisted of a fall in tension followed by an increase in tension with pronounced minimum between them at about 5 min after PH addition. For partial saturation with PH fibers were incubated in rigor or relaxing solution with PH for different times (3-10min). As the time of the pretreatment was increased the following types of response were obtained: 1) a response resembling the control but with reduced magnitude of fall in tension and with minimum of tension at an earlier time, 2) increase in tension after a delay, 3) only an increase in tension with maximal rate of tension increase immediately after PH addition. Thus, the response was changed as if it began from some point of the control response, and this point would be shifted to the right (to a longer time in the control response) as the pretreatment time was increased. The results suggest that PH-induced tension changes reflect the amount of bound PH. We propose that the qualitatively different changes in tension (increase and decrease) are caused by PH bound in different regions of sarcomere. Supported by NIH grant AR10551.

M-Pos234

COOPERATIVITY OF MYOSIN CROSSBRIDGES DURING ISOMETRIC MUSCLE CONTRACTION. ((Cheryl A. Miller and Vincent A. Barnett)) Department of Physiology, University of Minnesota.

Most models for the molecular mechanism of force generation in skeletal muscle do not address the question of cooperativity of the myosin crossbridges. We have used selective modification of myosin with N-phenylmaleimide (NPM) to knock out varying percentages of the crossbridges in glycerinated rabbit psoas fibers. ATPase and isometric tension were measured as a function of the extent of modification. The data suggest that crossbridges do not function cooperatively during isometric force production. Isometric force declines in a roughly linear fashion when compared to high salt K^+-EDTA ATPase and Ca^{2+}/K^+-ATPases, and physiological ATPases ± Ca^{2+}. N-phenylmaleimide inactivates the myosin crossbridge via modification of both of the fast reacting cysteines of myosin cys-707 and cys-697 (SH_1 and SH_2 , respectively). While modification of SH_1 inhibits force it cannot abolish it (J. Matta & D. Thomas, personal communication). The dependence on SH_2 modification is clearly demonstrated in a plot of the high salt Ca^{2+}/K^+-ATPase vs. isometric force (see inset). Previous studies, which used para-phenylene dimaleimide (pPDM) to inactivate the myosin heads, have suggested a cooperative mechanism of force production and unloaded shortening velocity (Chaen et al., 1986 *J.Biol.Chem.* **261**:13632-6). Further studies using NPM-modification will be required to determine if crossbridge cooperativity comes into play during isotonic or unloaded shortening.



M-Pos235

OPTIMIZING EM FIXATION OF MgATP-RELAXED CROSSBRIDGES IN INSECT FLIGHT MUSCLE (IFM). ((M. K. Reedy, C. Lucaveche, M. C. Reedy)) Dept. of Cell Biology, Duke Univ. Med Center, Durham, NC 27710

For electron microscope studies of freeze-substituted fibers of insect flight muscle (IFM) from *Lethocerus* that have been quick-frozen during contraction, we rely on the MgATP-relaxed myosin crossbridge pattern as a closely related functional precursor and reference structure for the force-generating state we want to analyze. However, in our use of the TAURAC method (Biophys J.59:579a) for both aqueous chemical fixation and freeze-substitution of relaxed IFM from Lethocerus, two problems have been non-uniform preservation of the 14.5 nm crossbridge repeat and variable thickening of myofilaments by a coating of excess tannic acid (TA). Systematic chem-fixing and cryofixing trials using TAURAC on relaxed IFM have shown choice of buffer solutes and TA to be critical. Among 6 TA products tested, "Special EM grade" from EM Sciences and Mallinckrodt gave best results. A different Mallinckrodt TA, superb in previous batches, now filled freeze-substituted fibrils with a dense matrix that hid myofilaments. Preserved uniformity of 14.5 nm striping improved from ≤50% (using the other 4 TAs) to ~70% when EM Grade TA was used, and reached ≥90% when 3% dextran T-500 and 5 mM extra Mg^{2+} were added to buffers. Relaxing buffer includes (in mM) MgATP 5, EGTA 5, NaN_3 5, K•MOPS 20, pH 6.8). Relax-fixation buffer has 0.2% TA added for aqueous TAURAC, or 15% glycerol as cryoprotectant for plunge-freeze cryofixation. Excess TA coating of myofilaments still afflicts patches of some freeze-substituted fibers, where fixation time, TA concentration and freeze-substitution solvent conditions are being varied in efforts to overcome this. Supported by NIH AR-14317.

M-Pos236

THE CONTRACTILE APPARATUS IN ULTRARAPIDLY FROZEN SMOOTH MUSCLE: FREEZE-FRACTURE, DEEP ETCH AND FREEZE-SUBSTITUTION STUDIES. ((J.L. Hodgkinson, J. Reckless, N.J. Severs and S.B. Marston)) Dept. of Cardiac Medicine, NHLI, Dovehouse St., London, SW3 6LY, UK.

The structure of the smooth muscle contractile apparatus was studied using ultrarapid freezing followed by longitudinal freeze-fracture, deep etch and platinum-carbon replication, eliminating the use of chemical fixatives and revealing the ultrastructure in three dimensions. Unidirectionally shadowed replicas of ultrarapidly frozen relaxed, intact smooth muscle showed well preserved actin filament structure with a 5.5nm repeat of the actin sub-units along the length of the filament. In obliquely fractured tissue the thick filaments were revealed and their distribution was comparable to that seen in transverse sections of freeze substituted muscle. Relaxed muscle permeabilised using Triton-X-100 (using a method which preserves the contractility of the muscle) showed similar structure to that of intact tissue after ultrarapid freezing; the ratios of actin to myosin were comparable. Deeper etching, enabled by permeabilisation prior to fixation, revealed lateral interactions between actin filaments in freeze fractured muscle. In permeabilised, rigorised tissue the structure of the actomyosin complex was clearly observed, both in longitudinal sections from freeze substituted muscle and in freeze-fractured, deep etched tissue. A cross-bridge spacing of 38nm was measured in the deep etched tissue. The structural detail revealed has led to the development of a model of the acto-myosin interaction in rigor smooth muscle incorporating side polar thick filaments. Currently we are studying thin sections of freeze substituted, rigorised smooth muscle in which the actin has been labelled with S1. This will indicate the polarity of actin filaments interacting with thick filaments and thereby clarify the structure of the thick filaments.

M-Pos237

Studies of actin-myosin interactions: modelling of 2D x-ray diffraction patterns from rabbit psoas muscle at different states.
((S.Malinchik and L.Yu)) NIH, Bethesda, MD.

We modelled 3D structure of unit cell in sarcomere using available biochemical and stereo-geometrical parameters of the thin and thick filaments with S1 shape approximating that found by Rayment et al.(1993). The crossbridge interactions with actins was simulated by means of actin target areas (Holmes et al., 1980; Squire and Harford, 1988) assuming that each G-actin monomer has one specific region for S1 binding. Unlike previous studies (Squire and Harford, 1988) our model allows to calculate the intensities of layer lines, meridional and equatorial regions. For non stereo-specific binding states the model shows some features of 2D diffraction pattern from relaxed muscle at low ionic strength: myosin based (c = 430Å) system of layer lines, strong "215" and weaker "143"Å meridional reflections. For stereo-specific attachments the calculated diffraction pattern shows the appearance of actin based system of layer lines, similar to rigor patterns, with meridional reflections at "215" and "143"Å. The model can be used also in the interpretation of some features of the X-ray diffraction patterns from actively contracting muscle.

M-Pos238

INTERMITTENT-CONTACT SCANNING FORCE MICROSCOPY OF MYOFIBRILS IN SOLUTION. ((D.P. Braunstein, J.A. Spudich)) Dept of Biochemistry , Stanford Univ. School of Medicine, Stanford CA 94305

Rabbit muscle myofibrils were imaged using intermittent-contact scanning force microscopy (ICSFM). ICSFM probes the surface topography of a sample with little applied lateral force, by modulating the cantilever amplitude during imaging. Triton-100X extracted myofibrils in low salt buffer were applied to a poly-lysine coated silicon surface, then spun in a Cytospin® centrifuge to ensure good adhesion. A commercial SFM equipped for non-contact SFM was modified for liquid ICSFM operation by gluing a small glass coverslip on the back of the glass chip containing the cantilever. This arrangement allows a small drop of buffer solution to be trapped between the cover-glass and the sample surface. Prior to imaging, the cantilever/liquid cell resonance response is mapped out by sweeping over the 10-300 kHz range. Resonant peaks were typically found in the 70-100 kHz range. The oscillation frequency was adjusted to be at the greatest slope on either side of the chosen resonant peak for maximum sensitivity. Feedback control is similar to contact SFM except that the piezo height is adjusted to changes in the cantilever amplitude, in response to changes in surface topography. The images show the general myofibril sarcomere ultra-structure. The components of sarcomere, the Z-line H-, I- and A-bands, are distinguishable. The small volume of the liquid cell should allow rapid exchange of the sample buffer and observation of changes in the myofibril structure in response to different buffer conditions. This work is supported by NIH fellowship PHS AR08202-02

BIOCHEMISTRY AND MECHANICS OF MUSCLE CONTRACTION I

M-Pos239

ENERGY CONSUMPTION DURING ISOMETRIC AND ISOTONIC CONTRACTION OF CHEMICALLY SKINNED CANINE AIRWAY SMOOTH MUSCLE ((Jizhong Wang and Newman L. Stephens)) Department of Physiology, University of Manitoba, Canada R3E 0W3

We have reported that intra-cellular Ca^{+2} concentration and myosin light chain phosphorylation level are lower in isometric contraction compared with isotonic shortening for canine tracheal smooth muscle. These studies seem to suggest that crossbridge recruitment proceeds only until the muscle starts to shorten, hence more crossbridges become activated in isometric contraction where no shortening occurs. In this study, we examined ATPase activity by measuring NADH fluorescence of chemically skinned canine tracheal smooth muscle under isometric and isotonic conditions. In the chemically skinned muscle we found: 1) As expected, no evidence showing existence of slowly cycling/latch crossbridges; 2) Maximum isotonic afterloaded shortening capacity was as high as 0.83 ± 0.05 l_o, compared to only 0.67 ± 0.04 l_o in the intact muscle; 3) ATPase activity (related to crossbridge cycling) under isotonic conditions was found to be greater than that under isometric conditions. This suggested isotonically contracted smooth muscle also demonstrated a Fenn effect, i.e. energy consumption during shortening was greater than in an isometric contraction. Our data suggested that though a large number of crossbridges were phosphorylated due to the higher level of intra-cellular Ca^{+2} in isometric contraction, not all of the crossbridges cycled and utilized ATP. Some phosphorylated crossbridges could remain phosphorylated and yet not interact with actin at all during isometric contraction due to mis-alignment with their potential binding sites. In an isotonic contraction, because of filament sliding, these bridges could attach and cycle thus accounting for the Fenn effect. (This work is supported by a operating grant from INSPIRAPLEX, Canada; J. Wang is the recipient of a studentship from the Medical Research Council of Canada).

M-Pos240

THE INABILITY OF TRIMETHYLAMINE N-OXIDE (TMAO) AND BETAINE TO AMELIORATE THE EFFECTS OF INORGANIC ORTHOPHOSPHATE (Pi) ON FORCE GENERATION OF CARDIAC MUSCLE: A POSSIBLE MECHANISM FOR THE DIFFERENTIAL EFFECTS OF Pi FORMS IN SKELETAL AND CARDIAC MUSCLE: ((M.A. DeRosa and M.A. Andrews)) Division of Physiology, NY College of Osteopathic Medicine, Old Westbury, NY 11568.

It has been shown that the protein stabilizer TMAO has the capacity to reverse or ameliorate Pi effects on Fmax of skinned skeletal muscle fibers (Andrews 1993. *Biophys. J. 64*:A361). The present experiments were run to determine whether TMAO or betaine (another stabilizer), or both, can likewise ameliorate the effects of Pi on Fmax of Triton X-100 skinned rat cardiac papillary muscle bundles (150-200 μm diameter). Experiments were run at pH = 7, and 22° C, with solutions formulated according to a program which solves the set of simultaneous equations describing the multiple equilibria of ions in solution. Solutions contained (mM): 5 EGTA, 20 imidazole, 2 Mg^{2+}, 5 MgATP, 15 phosphocreatine, appropriate KH_2PO_4, and 100 u/ml CPK. Total ionic strength was maintained at 200 mM ($KMeSO_3$ added). The pCa of maximal activation was determined for each bundle, then it was activated at that pCa in solutions containing 0 to 30 mM Pi (in 5 mM steps), relaxed, then reactivated in a set of similar solutions containing TMAO or betaine (100 or 300 mM). Results indicate that, unlike its effect on skeletal muscle preparations, there is an inability of TMAO (and betaine) to ameliorate Pi effects on Fmax of cardiac muscle. This is interesting in light of the fact that the inhibitory effects of Pi on Fmax of skeletal muscle are related to $[Pi^{-1}]$ and such effects in cardiac muscle are related to total [Pi]. Given that TMAO and betaine work via stabilization of proteins, results indicate that the difference in sensitivities to Pi forms may be related to the tendency of skeletal muscle to be more sensitive to the destabilizing effects of Pi^{-1}. TMAO, which stabilizes proteins, is then able to ameliorate this effect of Pi^{-1}.

M-Pos241

TRIMETHYLAMINE N-OXIDE (TMAO) AMELIORATES DECREASED CALCIUM-SENSITIVITY OF THE CONTRACTILE APPARATUS ONLY UNDER SPECIFIC SOLUTE CONDITIONS. ((C. Lauder, V.S. Tivakaran and M.A. Andrews)) Division of Physiology, NY College Osteopathic Medicine, Old Westbury, NY 11568.

It is known that the protein stabilizer TMAO increases force generation of skeletal muscle even in the presence of high salt concentrations (Godt, et al. 1993. *Adv. Exp. Med. Biol. 332*: 763), while decreasing the sensitivity (increasing Ca_{50}) of the contractile apparatus to calcium under physiological conditions (Tivakaran, et al. 1994. *Biophys. J. 66*:A302). The present experiments used single Triton X-100 skinned fast-twitch rat extensor digitorum longus muscle fibers under physiological and high (300 mM) salt conditions to further elucidate the effects of TMAO, salts, and TMAO-salt interactions on the Ca^{2+}-sensitivity of the contractile apparatus. All experiments were run at pH 7, and 22° C, using solutions formulated by programs which solve the set of simultaneous equations describing the multiple equilibria of ions in the solutions. All solutions contained (mM): 5 EGTA, 20 imidazole, 2 Mg^{2+}, 5.MgATP, 15 phosphocreatine, with 100 u/ml CPK. Such a solution had a total ionic strength (μ_S) of 90 mM. Control solutions had 110 mM $KMeSO_3$ added to attain physiological μ_S (200 mM), while experimental solutions had either 300 mM $KMeSO_3$ or KCl added to attain a μ_S of 390 mM. TMAO was added to appropriate solutions in concentrations of 50, 100, or 300 mM. Fibers were activated in a step-wise manner through solutions of pCa 8.5 to 4.0. Results indicate that: 1) Ca_{50} increases in direct proportion to the increase in TMAO; 2) high salt conditions increase Ca_{50}, KCl causing a greater increase than $KMeSO_3$; 3) addition of 300 mM TMAO to the 390 mM μ_S $KMeSO_3$ solutions increased the Ca_{50} even further, while its addition to the 390 mM μ_S KCl solutions ameliorated the effects of KCl on Ca_{50}, supporting the proposed capacity of TMAO to ameliorate the effects of solutes found to be most deleterious to contractile protein function.

M-Pos242

EFFECTS OF REPLACEMENT OF MgATP WITH MnATP ON ACTO-HMM ATPase, ACTIN FILAMENT SLIDING VELOCITY, AND UNLOADED SHORTENING VELOCITY OF RABBIT SKELETAL MUSCLE. ((M. Regnier, A. Bobkova, B. Kim, E. Reisler*, and E. Homsher)) Physiol. Dept, Medical School, *Chemistry Dept., UCLA, Los Angeles, CA 90024.

Myosin hydrolyzes MnATP at 20°C an order of magnitude faster than MgATP. This rate (like that of MgATP hydrolysis) is limited by Pi release from the Enz.ADP.Pi complex. However, as the temperature is lowered to < 10°C, the steady state ATPase for MnATP becomes rate- limited by ADP release from the Enz.ADP complex. To learn whether the change in rate limiting step was manifested in a temperature dependent change in the maximally activated HMM ATPase rate (V_{max}), f-actin sliding velocity in motility assays (V_f) or in the unloaded shortening velocity of glycerinated single muscle fibers (V_u). At 20°C V_{max}, V_f, and V_u in MgATP (9.1 s^{-1}, 2.1 μm/s, and 5.9 ML/s respectively) were greater than those in MnATP (6.8 s^{-1}, 1.4 μm/s, and 4.9 ML/s). As temperature was lowered, V_{max}, V_f, V_u all decreased, but those in MgATP declined more rapidly than those in MnATP. Arrhenius plots of V_{max}, V_f, and V_u yielded activation energies, E_a, of ca. 70 kJ in MnATP and 80-95 kJ in MGATP. Thus, as temperature is reduced, the difference between a pair of velocities in MgATP and MnATP diminishes and at a specific temperature (ranging from 5-13°C for V_u,V_{max}, and V_f) the velocities in MnATP and MgATP are the same. With a further decline in temperature, the velocities in MgATP fall below those in MnATP. Isometric force at all temperatures is about 30% less in MnATP than in MgATP. Because the rates of ATP binding to HMM, ATP cleavage, and ATP induced acto-HMM dissociation are practically identical for the Mn and Mg nucleotide forms, the rate limiting step controlling V_{max}, V_f, and V_u must involve product release steps or related isomerizations. The differential temperature sensitivity of these steps suggest a means to identify the specific step(s) involved.
(Supported by NIH grants AR-39088 [EH] and AR-22031 [ER]).

M-Pos243

PHOSPHORYLATION OF cTnI FULLY EXPLAINS THE DIMINISHED Ca^{2+}-SENSITIVITY INDUCED BY cAMP-DEPENDENT PROTEIN KINASE IN SKINNED CARDIOTRABECULAE.
((Xiao-Ling Ding, A. Babu Akella, Edmund H. Sonnenblick and Jagdish Gulati)) The Molecular Physiology Lab, Albert Einstein College of Medicine, Bronx, NY 10461

There is evidence that phosphorylation of myofibrillar proteins induced by cAMP-dependent protein kinase (PKA) is coupled with regulation of cardiac Ca^{2+}-sensitivity. But the mechanisms underlying this remain obscure. In this study, Ca^{2+}-sensitivity of force development was investigated in rat skinned cardiac trabeculae with TnC- or TnI•TnC-exchange. At 2.4μm sarcomere length, treatment of native trabeculae with PKA resulted in a rightward shift of force-pCa relation (ΔpCa_{50}=0.15±0.02pCa unit, n=8). A similar ΔpCa_{50} shift was also observed at 1.9μm SL (0.14±0.03, n=5). Trabeculae depleted of endogenous TnI and reconstituted with purified, phosphorylated cTnI displayed a similar shift in Ca^{2+}-sensitivity (0.14±0.03 at 2.4μm SL, n=4) as seen in the PKA-treated trabeculae, suggesting that TnI~P was sufficient to account for the PKA effect in cardiac muscle. To see whether isoform-specific interactions between the phosphorylated TnI and TnC were necessary for the modulation of Ca^{2+}-sensitivity, unitary TnC-exchange was performed on the PKA-treated trabeculae. The substitution of sTnC for cTnC did not alter the affinity shift attributed to TnI phosphorylation (0.16±0.02, n=3). These results demonstrate that phosphorylation of cTnI fully explains PKA-induced change in Ca^{2+}-sensitivity and the effect of phosphorylation would be transduced to either sTnC or cTnC. However, the length dependent shifts in Ca-sensitivity underlying the Frank-Starling mechanism were uninfluenced by cTnI phosphorylation. *Supported by NIH & NY Heart Association*

M-Pos244

ROLE OF CA^{2+}, TNC, AND CROSSBRIDGES IN DETERMINING THE RATE OF FORCE DEVELOPMENT IN FROG SKELETAL MUSCLES. ((Philip A. Wahr and Jack A. Rall)) Department of Physiology, Ohio State University, Columbus, OH 43210.

Force traces from skinned fibers activated by photo-release of Ca^{2+} were fit by a double exponential equation with rate constants k_1=44.5 ± 10.0 and k_2=5.7 ± 1.8 s^{-1} (mean ± S.D., N=77) at 10 °C. Upon repeated contractions the amplitude of k_2 increases relative to k_1 with no change in the rates. Force traces from intact fibers were fit by a single exponential with a rate constant of 37.4 ± 13.1 s^{-1}, N=7. Thus, k_1 is the physiologically relevant rate constant while k_2 is unique to skinned fibers. Experiments were performed to determine if k_1 and k_2 are controlled by TnC interactions, crossbridges, and/or Ca^{2+}. The rates were unaffected by TnC extraction to 42% of F_{MAX} or 5 μM calmidazolium used to increase TnC-Ca affinity. Decreased Ca^{2+} release decreased k_1 by ~7 fold, decreasing the number of cycling crossbridges with 200 μM vanadate increased k_1 by 67%. Altering the cross-bridge cycling rate with the ATP analog CTP caused a 100% increase in k_1 and a 50% decrease in k_2, but 2-deoxy-ATP had no effect. Thus, the rate of force development is affected by the crossbridge cycling rate and the Ca^{2+} level but not by thin filament cooperativity. NIH AR20892 and AHA, Ohio Affiliate.

M-Pos245

EFFECTS OF HDTA PRE-SOAKS ON ACTIVE TENSION GENERATION IN SKINNED RABBIT PSOAS FIBER SEGMENTS
((M.P. Slawnych, L. Morishita and B.H. Bressler)) Department of Anatomy, University of British Columbia, Vancouver, B.C., Canada.

The role of HDTA pre-soaks on active tension generation was examined in short segments of skinned rabbit psoas muscle fibers. Tension was recorded using an Akers strain gauge (AE801) and sarcomere length was obtained by off-line Fourier analysis of video images of the fiber. Contractions in pCa 4.6 were preceded by variable duration pre-soaks in HDTA. Segment lengths were typically below 400 μm., thus allowing the majority of the sarcomeres to be visualized in the field of view (200X and 400X magnification).

HDTA pre-treatment resulted in the following: 1) a significant reduction in the time required to reach the 50% level of maximum tension, 2) no significant change in the maximum tension level, and 3) less deterioration of fiber quality with repetitive activation, as measured by evaluating the Fourier spectra of muscle segments. These results were independent of pre-soak duration for durations greater than 10 seconds. The results support the premise that HDTA (which has a significantly lower affinity for Ca^{2+} than EGTA) works by decreasing the overall Ca^{2+} buffering capability of the fiber and hence increases the Ca^{2+} gradient when the fiber segment is exposed to the activating solution.

(Supported by the Medical Research Council of Canada).

M-Pos246

THE MYOFILAMENT Ca^{2+}-SENSITIZING EFFECT OF LEVOSIMENDAN, WHICH BINDS TO THE NH_2-TERMINUS OF CARDIAC TROPONIN C (TNC), IS BLOCKED BY PHOSPHORYLATION OF THE NH_2 TERMINUS OF TROPONIN I (TNI). ((Y. Kitada and R. J. Solaro)), University of Illinois at Chicago, College of Medicine, Chicago, IL 60612-7342.

Levosimendan (L) was rationally designed by Orion-Farmos Pharmaceuticals to bind to the NH_2-region of TnC involved in structural changes associated with Ca^{2+}-binding to the regulatory site. We investigated the effects of L on force developed by saponin permeabilized fiber bundles of guinea pig ventricle. L, in a concentration dependent manner (0.1-10 μM), increased force development at submaximally activating pCa values. At maximally activating pCa values, there was no significant effect of L. Therefore, the pCa-force relation was significantly shifted to the left in the presence of L. The effect of 10 μM L was completely reversed by phosphorylation of TnI at its NH_2-terminus by PKA-dependent (Ser 23/24) or PKC-dependent (Ser 43/45) pathways. These effects were reversed by KT 5720, a PKA inhibitor and calphostin C, a PKC inhibitor, although these inhibitors had no effect when added alone. Our results indicate that despite the fact that TnI and TnC are arranged anti-parallel, the NH_2-terminus of cTnI affects the region of docking of L at the NH_2-terminus of cTnC.

M-Pos247

CALCIUM BINDING TO TROPONIN C IN SKINNED SKELETAL MUSCLE ASSESSED WITH CAGED Ca^{2+} AND A Ca^{2+} FLUOROPHORE. ((R.L. Moss, J.R. Patel, K.S. McDonald, and M.R. Wolff)) Dept. of Physiology, Univ. of Wisconsin, Madison WI 53706.

Calcium sensitivity of tension varies with length in both cardiac and skeletal muscles. It has been hypothesized that this effect involves changes in the affinity of troponin C (TnC) for Ca^{2+}. The extent of Ca^{2+} binding changes as a function of sarcomere length in cardiac muscle (Hofmann & Fuchs *AJP* 1987;253:C90), while this does not appear to be the case in skeletal muscle. To examine Ca^{2+} binding to TnC, we developed a technique by which changes in sarcomere length, tension, and Ca^{2+} concentration can be simultaneously monitored in skinned muscle following flash photolysis of caged Ca^{2+}. Small bundles of skinned psoas fibers were initially loaded with the Ca^{2+} fluorophore, Fluo-3 and caged Ca^{2+} (DM-nitrophen or NP-EGTA). Following photolysis of caged Ca^{2+}, both tension and fluorescence rapidly increased from pre-flash levels. After extracting TnC, the fibers did not generate tension following a flash and the steady-state fluorescence signal was significantly greater than in the presence of TnC. This result indicates that the difference in fluorescence before and after TnC extraction is primarily determined by Ca^{2+} binding to TnC. Experiments are now underway to investigate whether changes in sarcomere length alter the apparent affinity of TnC for Ca^{2+} in either skeletal or cardiac muscle.

M-Pos248

CALCIUM SENSITIVITY OF FROG ATRIAL MYOFIBRILS. ((P.W. Brandt, F. Colomo*, N. Piroddi*, C. Poggesi* & C. Tesi*)) Department of Anatomy and Cell Biology, Columbia University, New York. *Dipartimento di Scienze Fisiologiche, Università di Firenze, Italy.

The serial dilution method (Brandt et al. Proc. Natl. Acad. Sci., 1980, 77, 4717-4720) has been used to describe, with a very high resolution, the pCa-force relation of skinned frog atrial myocytes. This preparation, that contains only one to five myofibrils 200-300 μm long, is a close approach to a thin bundle of isolated myofibrils. Enzymatically isolated frog atrial cells were held between a force transducer and a length control motor (Colomo et al. 1994, J. Physiol, 475.2, 347-350), bathed in a pCa 8 relaxing solution (20 °C), and skinned by jetting on them from a pressure micropipette relaxing solution added with Triton X-100 0.05 %. A series of pCa concentrations was perfused past the preparation by pumping a pCa 4.75 solution into the myofibril chamber in increments of fixed volume. Because the volume of fluid in the chamber was kept constant the pCa at each addition could be calculated (Brandt et al. 1980). A small Ca^{2+} electrode sitting near the myofibril continuously monitored pCa. At each step in the pCa series, the length of the myofibril bundle was rapidly released to obtain a true zero force baseline and active force was recorded both before the release and following the restretch of the preparation. The pCa-force relations of several preparations were defined with a very high density of pCa points and these were fit to the Hill equation which yielded the pK that ranged between 5.6 and 5.8 and the Hill coefficient that ranged between 3 and 4. The Hill coefficient found is rather large implying a high degree of cooperativity of Ca^{2+} activation that probably involves at least several neighbouring TnC's.

M-Pos249

THE ROLE OF THE Ca^{2+}-Mg^{2+} SITES OF TROPONIN C IN THE REGULATION OF MUSCLE CONTRACTION. ((James D. Potter, Todd Miller, Georgianna Guzman, Alan Mandveno, Dore Meinholz and Jiaju Zhao)) Dept. of Molecular & Cellular Pharmacology, Univ. of Miami Sch. of Med., Miami, FL 33136

To study the role of the two Ca^{2+}-Mg^{2+} sites (sites III and IV) of STnC in the regulation of contraction, we have constructed three mutants where we have inactivated Ca^{2+} binding site III (TnC3), site IV (TnC4) and both sites III and IV (TnC3,4) by replacing the first Ca^{2+} coordinating residue, aspartic acid, with alanine. Our working hypothesis has been that these sites are crucial for maintaining the structural integrity of the troponin complex when either Ca^{2+} or Mg^{2+} is bound to them, and that alteration of them would change the interaction of TnC with the thin filament. To study these mutants, we have examined their ability to reconstitute TnC depleted skinned skeletal muscle fibers. We found that all three mutants were able to fully restore force development. The concentration dependence of rebinding of wild type TnC (WT) TnC3 & TnC4 were the same. Higher concentrations of TnC3,4 were required to restore force. We also found that if we let the fibers, reconstituted with the mutant TnC's, incubate in relaxing solution for differing lengths of time between contractions, that the maximal force declined in a time dependent manner. Fibers reconstituted with WT, native TnC and TnC4 under the same conditions did not. TnC3 and TnC3,4 did dissociate with TnC3,4 dissociating the fastest. This drop in maximal force seen in these mutant substituted fibers could be restored by the addition of wild type TnC. Thus, although these mutants appeared to be normal, their ability to remain bound to the fibers was altered, consistent with our hypothesis. When we examined the Ca^{2+} dependence of force development in these fibers (without allowing the TnC's to dissociate) the Ca^{2+} dependence was not altered. Thus these results indicate that the major role of the Ca^{2+}-Mg^{2+} sites is to maintain the integrity of the Tn complex.

M-Pos250

TROPONIN-I ISOFORM EXPRESSION IN IMMATURE CARDIAC MYOYTES: EFFECT OF BETA-ADRENERGIC STIMULATION ON CALCIUM SENSITIVITY OF TENSION. ((S.H. Buck, K.T. Strang, R.L. Moss)) Depts of Pediatrics and Physiology, Univ of Wisconsin, Madison WI 53706

During cardiac development, there is a switch in TnI from slow skeletal TnI (ssTnI) to cardiac TnI (cTnI). ssTnI lacks PKA-phosphorylatable serine residues believed responsible for the rightward shift of Ca^{2+} sensitivity of tension in mature heart following β-adrenergic stimulation. We have previously shown that in contrast to mature cardiac myocytes which exhibit a rightward shift of the tension-pCa relationship following PKA treatment, no such shift to the right was evident in immature cardiac myocytes from 13-15 day-old rat (Biophys. J. 66(2):A404). To further investigate the ssTnI to cTnI transition, we have performed PAGE, silver-staining, and western blot analysis of mature and immature skinned cardiac myocytes in conjunction with autoradiography of control and PKA-treated myocytes incubated with [γ-^{32}P]ATP. Our results indicate that by 13-15 days in developing rat heart, the majority of TnI is cTnI; however, the myocytes do not demonstrate a rightward shift in the tension-pCa relationship following PKA. An alternative explanation for the lack of rightward shift, that basal cTnI phosphorylation is greater in the immature heart at this age than in adult heart, is being investigated with the use of serine phosphatase.

M-Pos251

NUMERICAL MODELS OF CALCIUM ACTIVATION CAN ACCOUNT FOR DIFFERENCES BETWEEN SKELETAL AND CARDIAC FORCE REDEVELOPMENT KINETICS ((W.O. Hancock, L.L. Huntsman, and A.M. Gordon)) Center for Bioengineering and Dept. of Physiology and Biophysics, University of Washington, Seattle, WA 98195.

To explain differences in the activation dependence of force redevelopment kinetics between cardiac and skeletal muscle, two numerical models of contractile regulation by Ca^{2+} were investigated. A continuous population of thin filament units (1 tropomyosin : 1 troponin : 7 actin) was examined, and Ca^{2+} binding and force production each modeled as two state processes with forward and reverse rate constants from the literature. The first model incorporates 4 possible thin filament states. In the second model Ca^{2+} is assumed to not dissociate from a thin filament unit in the force generating state, resulting in 3 states. To study the redevelopment of force at various [Ca^{2+}], the crossbridge detachment rate was momentarily elevated to drop force, and the rate constant of tension redevelopment (k_{tr}) was calculated. The 4 state model can account for the activation dependence of k_{tr} seen in skeletal muscle without requiring that Ca^{2+} directly modulates the kinetics of any step in the crossbridge cycle. Using identical kinetic parameters, the 3 state model shows no activation dependence of k_{tr}, consistent with our results in intact and skinned cardiac muscle. The models therefore present possible differences in the Ca^{2+} activation scheme between cardiac and skeletal muscle that can account for the contrasting activation dependencies of force redevelopment kinetics. Supported by NIH Grants HL31962 and HL07403.

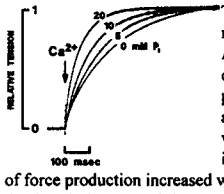
M-Pos252

INORGANIC PHOSPHATE INCREASES THE RATE CONSTANT FOR THE DISSOCIATION OF MYOSIN CROSS-BRIDGES FROM ACTIN IN SKINNED RAT SOLEUS FIBERS. ((W. G. L. Kerrick and P. Wang)) Dept of Physiol. and Biophys. Univ. of Miami, Miami, FL 33101 (Spon. by P.E. Hoar) The mechanism by which inorganic phosphate (Pi) causes a decrease in maximal Ca^{2+}-activate force and Ca^{2+} sensitivity was investigated using skinned rat soleus fibers. According to the 1957 Huxley model the ratio of actomyosin ATPase/Force would be proportional to the rate constant for dissociation of myosin cross-bridges from actin. In this study the ATPase rate and force were measured simultaneously at different Ca^{2+} and Pi concentrations. It was found that in all cases increasing Pi causes a larger percentage decrease in force than ATPase rate. At submaximal Ca^{2+} activation of force a fixed amount of Pi caused a larger increase in actomyosin ATPase/Force ratio (rate of dissociation of myosin cross-bridges from actin) than it does at maximal Ca^{2+} activation of force. In conclusion inorganic phosphate decreases both maximal Ca^{2+} activated force and Ca^{2+} sensitivity of force activation in part by increasing the rate constant for dissociation of myosin cross-bridges from actin.

M-Pos253

EFFECTS OF INORGANIC PHOSPHATE ON THE KINETICS OF SKINNED CARDIAC MUSCLE ACTIVATION INITIATED BY THE PHOTOLYSIS OF NP-EGTA. ((H. Martin, G.C.R. Ellis-Davies*, J. H. Kaplan* & R.J. Barsotti))
Bockus Research Institute, Graduate Hospital Phila., PA and *Department of Biochemistry & Molecular Biology, OHSU, Portland, OR

We previously reported (Barsotti *et al.*, Biophys. J. 66:A5, 1994) that the rate of force development of Triton-skinned Guinea-pig trabeculae initiated by laser photolysis of NP-EGTA (2mM NPE, 1.65mM Ca^{2+}, 5mM MgATP, I=200mM, 23°C) was biphasic.



The rapid component was significantly faster than the rate of force development initiated by photolysis of caged ATP (pCa 4.5), suggesting that ATP hydrolysis, or some other step in the cross-bridge cycle prior to the force producing transition was rate limiting during ATP activation. This was tested by determining the effects of varying [Pi] on the biexponential time course of Ca^{2+} induced force production. The rate of the fast component of force production increased with increasing [Pi]. In 20mM Pi, the rate was 2.9 times that in solutions containing no added Pi: 98 ± 15 sec^{-1} *vs.* 34 ± 4 sec^{-1} (mean ± s.e., n = 6), while the steady-state force declined by 56 ± 6%. The rate of the slow component of force production was less sensitive to [Pi]. In 20mM Pi, the rate of the slow component increased significantly to 1.6 times that in the absence of added Pi: 8.5 ± 1.8 sec^{-1} *vs.* 5.2 ± 0.4 sec^{-1} (n = 6). The increase in the rate of force development by Pi suggests that the slower rate of force production in activations induced by photolysis of caged ATP is not limited by the ATP hydrolysis step. Supported by HL40953 to RJB; HL30315 & GM39500 to JHK.

M-Pos254

COMPARISON OF PHOSPHATE ADDITION AND SUBSTITUTION OF UTP FOR ATP ON THE CONTRACTILE PROPERTIES OF SKINNED MUSCLE FIBERS. ((C.Y. Seow & L.E. Ford)) Univ. of Chicago, Chicago, IL 60637

UTP is hydrolyzed by actomyosin at about half the rate as ATP (H.D. White, personal communication). Substitution of UTP for ATP is therefore expected to mimic the effects of increased phosphate that are due to slowed phosphate hydrolysis or release but not those due to phosphate re-binding. As indicated in the Table, adding 40 mM phosphate and substituting UTP for ATP have similar effects on isometric force (Po) and stiffness (S), but different effects on maximum shortening velocity (Vmax) at higher pH.

	Po	S	S/Po	Vmax
40 mM Pi, pH 6.35	-47%	-31%	+30%	-11%
40 mM Pi, pH 7.35	-40%	-31%	+15%	+29%
UTP, pH 7.0	-42%	-20%	+38%	-56%
UTP, pH 7.35	-34%	-14%	+30%	-49%

The increased velocity caused by phosphate at the higher pH is correlated in tension transient experiments with more extensive force recovery during late Phase 3 and early Phase 4. This more extensive force recovery is nearly absent from the transients recorded in the presence of UTP and at low pH in the presence of ATP. These comparisons suggest that phosphate release occurs before low force bridges can undergo the power stroke to high force states and that phosphate re-binding at high pH facilitates a transition that occurs after a lag, as would be imposed by the power stroke.

M-Pos255

ACTIVATION- AND TENSION-RELATED HEAT IN SINGLE SLOW AND FAST TWITCH MUSCLE FIBRES FROM *XENOPUS LAEVIS*. ((H.P.J. Buschman, G.J.M. Stienen, W.J. van der Laarse and G. Elzinga)) Laboratory for Physiology, Vrije Universiteit, van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands

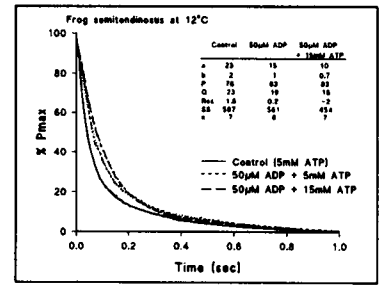
The economy of cross-bridge interaction in isometric contractions in single type 1 (fast-twitch) and type 3 (slow-twitch) iliofibularis muscle fibres from *Xenopus laevis* was determined by measuring the contribution of the heat production by the sarcoplasmic reticulum calcium-pumps (activation heat) to the total heat production (stable maintenance heat). This was done during fused and unfused contractions (10 Hz stimulation) by variation in sarcomere length and in 2,3-butanedione 2-monoxime (BDM) concentrations.

No difference was found in the activation heat determined by fibre stretching or with BDM. The activation heat in type 1 fibres for fused contractions was 0.187 ± 0.020 W/g dry weight (mean ± S.E.M.; n = 13), i.e. 34 ± 4 % of the stable heat rate, while this was 0.078 ± 0.008 W/g dry weight (n = 13), i.e. 48 ± 5 %, for unfused contractions. In type 3 fibres the activation heat was 0.127 ± 0.010 W/g dry weight (n = 15), i.e. 52 ± 4 %, for fused contractions, and 0.050 ± 0.006 W/g dry weight (n = 11), i.e. 36 ± 3 %, for unfused contractions. The economy of actomyosin interaction (isometric force production rate and actomyosin heat rate ratio) in type 1 fibres was 1.97 ± 0.13 for fused contractions and 0.71 ± 0.09 for unfused contractions. In type 3 fibres the values were significantly higher, 5.04 ± 0.12 and 1.43 ± 0.13 for fused and unfused contractions.

M-Pos256

DOES ATP REVERSE THE SLOWING EFFECT OF ADP ON FROG SKELETAL MUSCLE RELAXATION PRODUCED BY DIAZO-2 PHOTOLYSIS? ((S.Lipscomb, E.Johns, I.P. Mulligan and C.C.Ashley)) Univ. Lab. of Physiol., Parks Rd., Oxford OX1 3PT. U.K.

50 μM ADP has been shown to slow the rate of relaxation of frog skeletal muscle produced by photolysis of diazo-2 (Palmer et al). In this experiment we examine whether there is any competition between ADP and ATP, using Triton-skinned single fibres from frog (Rana temporaria) semitendinosus muscle. Control solution contained 5mM MgATP and no added ADP, (+ADP) solution contained 50μM added ADP and 5mM MgATP whilst (+Both) solution contained 50μM added ADP and 15 mM MgATP. The (+ADP) and (+Both) solutions contained no added CPhos or CPK, whereas the control contained 10mM CPhos and 15U/ml CPK. Relaxations were produced by photolysis of 2mM diazo-2 solutions for each condition. Average traces with curve fit (double exponential) values are shown in the diagram (a and b = rate constants for each exponential component, P and Q = percentage of each component). Mean half times for relaxation were also calculated, and are (ms±SEM); control 46±7.7, (+ADP) 77±11.3, (+Both) 74±11.0. These results show that ATP does not reverse the slowing of relaxation produced by ADP.



M-Pos257

CROSS-BRIDGE KINETICS IN SINGLE FIBERS OF HUMAN SOLEUS MUSCLE. ((T. Kraft, F. Becker, G. Regel, B. Brenner)) Medical School, 30625 Hannover, FRG.

To provide the basis for investigating the effects of mutations in the ß-myosin isoform seen in familial hypertrophic cardiomyopathies and also expressed in slow skeletal muscle, we studied several mechanical parameters of chemically skinned slow twitch fibers of human soleus muscle. These studies allow to characterize both, actin binding kinetics and kinetics of active cross-bridge cycling. At 5, 10 and 20°C relaxed and active fiber stiffness, isometric force and fiber ATPase activity, the rate constants for force redevelopment, and maximum isotonic shortening velocity (V_{max}) were determined.

Two somewhat surprising results were found: (i) Unlike rabbit psoas muscle (Brenner, B., PNAS 88, 1991), the increase in isometric force with increasing temperature in the soleus results from changes in cross-bridge cycling kinetics (f_{app} increases severalfold more than g_{app}), leading to an increase in the fraction of cross-bridges contributing to active force. (ii) Between 10 and 20°C, the Q_{10} for the increase in V_{max} is approximately 5fold larger than the Q_{10} of tension cost (ATPase/isometric force). This may indicate that g_{app} for strained and unstrained cross-bridges (g_1 and g_2 of A.F. Huxley, 1957) are dominated by different reaction steps. (Supported by DFG Br849/1-4).

M-Pos258

A DIAGONAL MOVEMENT MODEL OF MUSCLE CONTRACTION AND A DOUBLE HYPERBOLIC LOAD-VELOCITY RELATIONSHIP IN SINGLE MUSCLE FIBERS ((T. Majima)) Electrotechnical Laboratory, Tsukuba, Ibaraki 305, JAPAN

A model of muscle contraction based on diagonal movements of myosin head on a thin filament was proposed (Majima 1993, J. Muscle Res. Cell Motil. 14:361 (Abstr.)). Basic assumptions of the model are as follows: 1) Sliding direction of a myosin head on actin molecules are not necessarily parallel to the axis of thin filament and it changes depending on load; 2) Working time (power stroking time) of actomyosin ATPase is constant except < 0.2 Vmax (at slow and isometric shortening conditions); and 3) Myosin head performs many power strokes during ATPase cycle.

A double hyperbolic load-velocity relationship in single muscle fibers (Edman 1988, J. Physiol. 404:301-321) is analyzed in the light of this model. The result shows that the relationship at slow sliding conditions implies restricted movements of myosin head on a large interaction area which is assumed on an actin molecule. A hypothetical interpretation is that different types of myosin heads (myosin isomers) become to be dominant actuators depending on load.

M-Pos259

CROSS-BRIDGE ATTACHMENT AND DETACHMENT OBSERVED BY STIFFNESS MEASUREMENTS. ((T. Blangé [1], U.A. v.d. Heide [1,2], E.L. de Beer [2], F.A.M. v. Kaam [1] and B.W. Treijtel [1])) [1]Dept. of Physiology, University of Amsterdam, Meibergdreef 15, Amsterdam, The Netherlands. [2]Dept. of Medical Physiology, Utrecht University, Universiteitsweg 100, Utrecht, The Netherlands.

In this study we investigated the high-frequency stiffness of skinned frog muscle fiber during fast length changes in order to reveal the attachment and detachment of cross-bridges. To this end a 10 kHz sinusoidal modulation was superposed upon a length step of 1 nm/half sarcomere, completed in 30 μs, while the resulting tension was recorded. By subtracting the tension transient after a single step, the response to the 10 kHz modulation was obtained. In activated fibers a fast increase of stiffness after a stretch and a decrease after a release were observed. A maximum deviation of about 5 % is reached at 0.3 to 0.5 ms after the step. With the muscle fiber in rigor, changes in stiffness were observed too, but took place well within 0.1 ms. This implies that the 0.3 to 0.5 ms duration of the change in stiffness of the activated fiber clearly exceeds time resolution of the experiment. At a timescale extended to 10 ms a partial recovery of stiffness is found. This behaviour of stiffness indicates changes in the number of cross-bridges present.

Results obtained from the fiber in rigor suggest that the changes in stiffness are not delayed with respect to the changes in length evoked by the step. It is unlikely that rigor bridges attach or detach that quickly. The results therefore indicate that in rigor the changes in stiffness originate from a non-linear force-length relationship of the cross-bridge itself.

M-Pos260

SARCOMERE LENGTH DEPENDENCE OF THE RATE OF FORCE REDEVELOPMENT IN RAT SKINNED SOLEUS FIBERS. ((K.S. McDonald, M.R. Wolff, and R.L. Moss)) Dept. of Physiology and Section of Cardiology, University of Wisconsin, Madison WI 53706

Twitch tension changes as a function of sarcomere length (SL) in mammalian cardiac and skeletal muscle. One mechanism that may contribute to this phenomenon is a SL-dependent change in isometric cross-bridge kinetics. We tested this idea by examining the rate of force redevelopment (k_{tr}) following a slack-restretch maneuver at varying SL in skinned rat soleus fibers. At SL 2.30 μm, k_{tr} was 2.99 ± 0.54 s^{-1} during maximal activation at pCa 4.5. Reducing SL to 2.0 μm decreased k_{tr} to 2.28 ± 0.31 s^{-1} and was associated with a 10 ± 2% decline in peak tension. During submaximal activation (pCa 5.8; $P/P_{4.5}$ = 0.64 ± 0.02), k_{tr} was 1.67 ± 0.22 s^{-1} at SL 2.30 μm and fell to 1.41 ± 0.04 at SL 2.0 μm, while tension declined 27 ± 5% over this SL range. Following osmotic compression with 4% dextran, tension at short SL during submaximal activation increased to levels obtained at long SL of uncompressed fibers. Osmotic compression at short SL increased k_{tr} to 94% (1.57 ± 0.08 s^{-1}) of the rate at long SL. These results indicate that the kinetics of isometric cross-bridge interactions change as a function of SL, an effect that appears to be mediated by changes in interfilament lattice spacing.

M-Pos261

SLOWING OF RELAXATION IN FATIGUED SKELETAL MUSCLE FIBERS OF XENOPUS. ((H. Westerblad, J. Lännergren and D.G. Allen)) Karolinska Institute, S-171 77 Stockholm, Sweden; University of Sydney, NSW 2006, Australia.

Fatigue in skeletal muscle is generally accompanied by a slowing of relaxation. This slowing can in principle be caused by a reduced rate of Ca^{2+} removal from the myoplasm or slowed cross-bridge kinetics. We have used two methods to study the relative importance of these two mechanisms for the slowing of relaxation observed in single muscle fibers of *Xenopus* during fatigue produced by repeated short tetani. The first method involves measurement of the free myoplasmic $[Ca^{2+}]$ ($[Ca^{2+}]_i$) with indo-1 and construction of Ca^{2+}-derived force records by means of the steady-state $[Ca^{2+}]_i$-force relation (Westerblad & Allen, J Physiol 466: 611, 1993); these Ca^{2+}-derived force records represent a situation where tension reacts instantaneously to changes of $[Ca^{2+}]_i$. The second method involves a rapid slackening of the fiber during early relaxation and measurement of the time to peak force redevelopment. The rationale behind this method is that force redevelopment will occur as long as $[Ca^{2+}]_i$ is high enough to promote net cross-bridge attachment. These two methods gave very similar results and showed that about half of the slowing of relaxation in fatigued *Xenopus* fibers is caused by slowed Ca^{2+} handling and the other half can be ascribed to slowed cross-bridge kinetics. This result contrasts to our previous results in fatigued mouse fibers (Westerblad & Allen, J Physiol 468: 729, 1993) where the slowing of relaxation seemed to be exclusively due to slowed cross-bridge kinetics.

M-Pos262

COMPARISON OF THE EFFECTS OF FDNB AND NPM ON ISOMETRIC FORCE IN SKINNED RABBIT PSOAS FIBERS. ((Mark Schoenberg)) LPB, NIAMS, NIH, Bethesda, MD, 20892.

Using NPM to link phenylmaleimide groups to myosin's two most reactive sulfhydryls, SH1 and SH2, locks crossbridges in a weakly-binding configuration resembling that of the normal myosin.ATP crossbridge (Ehrlich et al., BJ 64:A360:'93). 2,4-dinitro-fluorobenzine (FDNB), a reversible thiol-alkylating agent may be useful in linking phenylmaleimide groups to SH2 alone since treating myosin in solution with FDNB causes dinitrophenylation of SH1 which can be reversed with DTT; while SH1 is dinitrophenylated, SH2 is available for reaction with other agents (Reisler, et al., Biochem. 13:2014:'74). Using techniques developed by Roopnarine and Thomas (BJ 67:1634:'94) for dinitrophenylation and reversal in muscle fibers, I examined the effect of FDNB on isometric force in skinned rabbit psoas fibers. FDNB at a concentration one-half that which selectively alkylates SH1 to 96% over 60 m led to a substantial, rapid decrease in force; 70% of the force disappeared with a time constant of 2.4 m, and 30% disappeared with a time constant of 62 m. Most of the force loss was reversed by DTT. Contrastingly, the decrease in rigor stiffness caused by NPM as a function of treatment time is fit by a single time constant (10.5 m) and none of the force loss is recoverable by DTT. Treating FDNB-treated fibers with 0.1 mM NPM for 60 m totally inhibits force production, with DTT giving no recovery. One interpretation of these results is that a phenylmaleimide at SH2 or a dinitrophenyl at SH1 is sufficient to inhibit force production, but the interpretation is clouded by the complex kinetics of the FDNB response.

M-Pos263

MAXIMUM VELOCITY OF UNLOADED SHORTENING AND MYOSIN ISOFORM COMPOSITION IN SINGLE SKELETAL MUSCLE FIBRES FROM AGEING RATS. ((X.Li and L.Larsson))Dept. of Clinical Neurophysiology and Neurology, Karolinska Hospital, Stockholm, Sweden.

The maximum velocity of unloaded shortening (Vmax) and the composition of myosin heavy (MyHC) and light chain (MyLC) isoforms were determined in 155 single fibres of freeze-dried soleus and EDL muscle samples from young (3-6 months) and old (20-24 months) male albino rats. Type I, IIA, IIX and IIB MyHCs were isolated in single fibre segments on 6% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Essential (MyLC1 and MyLC3) and regulatory (MyLC2) were resolved on 12% SDS-PAGE. In the soleus, the Vmax, measured with the slack-test, was significantly ($p<0.01$) lower in type I MyHC fibres from old rats (0.59 ± 0.28 ML/s, n=55) than in this type from young animals (1.12 ± 0.46 ML/s, n=48). The compositions of MyLCs in the type I MyHC fibres were identical in the two age groups. In the EDL, the Vmax in single fibres in the young and old animals were 2.26 ± 0.66 ML/s (n=27) and 2.35 ± 0.57 ML/s (n=22), respectively. In the young animals, there was a significant correlation between Vmax and MyLC3 content in both type IIB ($p<0.05$) and IIXB fibres ($p<0.001$). Also, the MyLC3 content was related to the composition of MyHC isoforms in the IIXB fibres and a significant ($p<0.01$) correlation was found between Vmax and type IIB MyHC content in this fibre type. In the old rats, the relations between Vmax and MyLC or MyHC content were lost in type IIXB fibres, as well as the relation between the relative contents of MyLC3 and IIB MyHC. The strong influence of MyHC and MyLC isoforms on the Vmax was confirmed in single muscle fibres from young rats. This study also demonstrated significant slowing of Vmax in soleus fibres in old age without any age-related differences in myosin isoform compositions, and loss of both the co-ordinated expression of MyHC and MyLC isoforms and the relation between Vmax and myosin isoforms in type IIXB fibres in the old animals.

M-Pos264

MICROFABRICATED CANTILEVER BEAMS FOR FORCE MEASUREMENTS ((M. Fauver, C. Galambos, M.L. Bartoo, G.H. Pollack)) University of Washington Center for Bioengineering Seattle, WA 98195

Microfabrication processes allow for easy mass production of mechanical structures with reproducible feature sizes as small as 100 nanometers. Optical lithography was applied to fabricate cantilever beams of radically different compliances for use with two small scale preparations-- the single myofibril, and the single actin filament. To measure force generation in single isolated myofibrils, a cantilever beam was fabricated from a 625 nm thick silicon nitride membrane. Beam widths were 2 μm, and lengths ranged from 100 to 250 μm. A myofibril was attached to the end of one of the beams (at a right angle), and the cantilever beam and myofibril were imaged. Normal contraction of the myofibril, and bending of the lever were observed, indicating that there were no problems with biocompatibility, and that force measurements were possible. To resolve the piconewton forces produced by single actin-myosin interactions, cantilever beams were made from a silicon nitride film 100 nm thick . The width of the levers was approximately 1 micron, and the lengths ranged from 75 to 250 μm. The theoretical compliance for the longest lever (in its most compliant direction) is on the order of 100 nm/pN, allowing for easily measured displacements using video microscopy. Coating the tip with NEM treated myosin provides adequate "glue" for sticking an actin filament to the lever. The next step, which is underway, is to place the other end of the actin filament onto a surface with functional myosin. When ATP is added, the deflection of the lever tip will allow for sensitive, reliable measurements of force generation. Features and measurements made with these devices will be presented.

M-Pos265

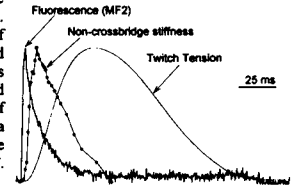
STIFFNESS OF SINGLE FROG MUSCLE FIBRES DURING THE LATENT PERIOD. ((M.A. Bagni, G. Cecchi, E. Cecchini, F. Colomo and P. Garzella)). Dipartimento di Scienze Fisiologiche, Università di Firenze, ITALY.

Early effects of activation on the mechanical characteristics of single intact fibres from lumbricalis frog muscle were studied by applying ramp stretches of various amplitude (1.8-6%l_0) and velocity (40-80$l_0 s^{-1}$) to one fibre end and by measuring the force response at the other fibre end. Stretches were applied at increasing times after a single stimulus. Force responses show the presence of a fibre "static" stiffness, previously unrecognized, not attributable to the presence of attached cross-bridges. The static stiffness time course follows approximately the internal Ca^{++} concentration, starting to increase about 2 ms after the stimulus, reaching a peak 8ms later and returning to the resting level well before the peak of the twitch tension. Static stiffness accounts for all the fibre stiffness increase during the latent period while crossbridge stiffness predominates during tension development. These results indicate that the lead of stiffness over force during the latent period is not due to the formation of non-force generating crossbridges but to a stiffening of some unidentified elastic structures of the fibre.

M-Pos266

THE INTRACELLULAR Ca^{2+} TRANSIENT CAUSES A TRANSITORY INCREASE IN NON-CROSSBRIDGE STIFFNESS IN FROG SKELETAL MUSCLE. ((P Garzella, DR Claflin & FJ Julian)) Dept. of Anesthesia, Brigham & Women's Hospital, Boston, MA 02115.

Depolarization of an intact frog skeletal muscle fiber with a single electrical stimulus results in a large, abrupt, transient increase in intracellular Ca^{2+} (ICT). This increase in intracellular Ca^{2+} ultimately results in the attachment of crossbridges in a strongly-bound state that causes an increase in fiber stiffness and leads to the generation of tension. The aim of this study was to test whether, after detaching crossbridges using large rapid stretches, a non-crossbridge stiffness remains in the fiber that exhibits a Ca^{2+}-dependency. Large (approximately 110nm/sarcomere, 5.5ms duration) ramp stretches were applied to intact single fibers dissected from the tibialis anterior muscle of the frog (*R. temporaria*). Experiments were performed at 15°C at an average sarcomere length of 2.2μm and the Ca^{2+}-sensitive fluorescent dye mag-fura-2 (MF2) was used to report the time-course of the ICT. The non-crossbridge stiffness was measured at the end of the ramp by subtracting the stretch response of the resting fiber as well the twitch tension in the absence of stretch from the stretch response during the twitch. Measurements were made at different times during the twitch. Results from one experiment are shown below (normalized traces). From the Figure it can be seen that the fiber undergoes a transient increase in non-crossbridge stiffness that follows the ICT but precedes tension. The non-crossbridge stiffness reaches a peak of about 5% of the maximum fiber stiffness (obtained with 0.1% l_0 steps) at the time that twitch tension is approximately 13.7±2.6% (mean ± SEM, n=3) and returns to resting level shortly after the peak of twitch tension. We conclude that there is a Ca^{2+}-dependent stiffness that differs from the crossbridge stiffness and follows closely the ICT.



Supported by NIH HL35032.

M-Pos267

Myopathic Length-Dependent Contractile Ca-sensitivity Changes Appear to be correlated with TnT Alterations but not with Myosin Isozyme Shifts
((*A. Babu Akella, Xiao-Ling Ding and Jagdish Gulati*)) The Molecular Physiology Lab, Einstein College of Medicine, Bronx, NY

Despite evidence on isoform switching, and genetic mutations of cardiac regulatory proteins in hypertrophic cardiomyopathy (Gulati et al, BBRC, 202: 384,1994; Seidman & coworkers, Cell, 77:701,1994), the pathophysiological correlations have remained tenuous. To explain these uncertainties, we investigated the length-dependent contractile modifications and TnT alterations in experimental cardiomyopathies in the rat. Sarcomere length (SL) dependent pCa-tension responses of skinned trabeculae from control, hypothyroid and diabetic rats were compared. Ca-sensitivity at 2.4μm was unaltered in diabetes: pCa_{50} was 5.75±0.03 for controls and 5.77±0.03 in diabetes. At 1.9μm, pCa_{50} was 5.57±0.03 for controls and 5.43±0.04 in diabetes, indicating a two-fold greater effect of SL in disease. Comparable experiments with hypothyroid rats, in which a majority of the V1 myosin was converted to V3, revealed no change in length-dependence. In diabetes, in addition to V1→V3 shifts, TnT was also altered. While three TnT isoforms (TnT1, 2 & 3) were evident in immunoblots of both normal and diabetic tissues, there was a marked mass intensity transfer (65±4%) between TnT1 & TnT3 in diabetes. Compressing the myofilament lattice with Dextran at 1.9μm had identical effect on sensitivity in control and diabetic trabeculae. The findings suggest a vectorial modification of TnT subunits on the diabetic thin-filament, which regulates the TnC length-sensing function and results in a modified Starling's law of the heart.

Supported by NIH & NYHA

M-Pos268

AN AGE-RELATED β-SLOW MYOSIN HEAVY CHAIN ISOFORM TRANSITION. ((L. Larsson[1], X. Li[1], U. Müller[1] and S. M. Hughes[2])) Departments of Clinical Neurophysiology and Neurology[1], Karolinska Hospital, Stockholm, Sweden, MRC Muscle and Cell Motility Unit[2], King's College, London, UK.

Slowing of movement is a prominent feature of old age and increased contraction or half-relaxation times, or both, of the isometric twitch have been reported in various mammals. Studies on the effects of ageing on shortening velocity, on the other hand, are sparse. Recently we have shown an age-related decrease ($p < 0.01$) in the maximum speed of unloaded shortening in single skinned soleus muscle fibres from 1.12 ± 0.46 (SD) ML/s in young rats (3-6 months) to 0.59 ± 0.28 ML/s in old (20-24 months) ones (Li & Larsson 1994). All these fibres appeared homogeneous with respect to their myosin heavy chain (MyHC) and myosin light chain (MyLC) isoform compositions, i.e. they expressed the β-slow (type I) MyHC isoform and slow isoforms of $MyLC_1$ and $MyLC_2$, according to silver stained SDS-PAGE. However, a heterogeneity of type I MyHCs has been reported in mammalian skeletal muscle by various groups and Hughes et al. (1993) recently observed at least three isoforms of type I MyHC in rat and human skeletal muscle, by using a set of monoclonal antibodies (mAbs) that recognize different epitopes on the MyHC. By using these mAbs on immunoblotts, a sub-set of type I MyHC fibres from old rats was identified which had a pattern of immunoreactivity that has not been observed in any soleus fibres from adult rats. These fibres had shortening velocities which were slower than any type I MyHC fibre from adult animals. Thus, the present results suggest an age-related isoform transition of different type I MyHCs which may play an important role in determining the contractile properties of the muscle cells. Preliminary results from skinned human muscle cells show a similar slowing with age in type I MyHC fibres.

M-Pos269

ACTIVATION OF MITOGEN-ACTIVATED PROTEIN (MAP) KINASE IN INTACT AND DETERGENT SKINNED SWINE CAROTID ARTERY. ((S.S. Katoch and R.S. Moreland)) Bockus Research Institute, Graduate Hospital, Philadelphia, PA 19146. (spon. by S. Moreland)

MAP kinase activation is a potentially important step in signal transduction pathways. The goal of this study was to determine if MAP kinase(s) is activated during contractile stimulation of vascular smooth muscle. To achieve this goal, MAP kinase phosphotyrosine levels and MAP kinase activity were measured during membrane depolarization and histamine induced contraction of intact and Ca^{2+}-dependent contraction of Triton X-100 skinned swine carotid artery. Two immunoreactive proteins (42 and 44 kDa) to anti-MAP kinase antibodies were detected in the artery. Phosphotyrosine levels of both the 42 and 44 kDa MAP kinases were elevated during stimulation of intact as well as skinned strips. Both isoforms of MAP kinase phosphorylated myelin basic protein (MBP) in an "in-gel" assay, thus demonstrating stimulation-induced kinase activation. Intact and skinned arteries were contracted, homogenized and assayed for MAP kinase activity using a specific substrate for MAP kinase, $MBP_{95\text{-}98}$. MAP kinase activity increased monotonically in both membrane depolarization and agonist-stimulated intact strips; the increase in MAP kinase activity was tightly coupled to the stimulation-induced increase in force. MAP kinase activity also increased during Ca^{2+}-stimulation of skinned strips, however the increase was transient, falling with time to suprabasal steady state values. Our results clearly demonstrate that 42 and 44 kDa isoforms of MAP kinase are activated in response to all forms of contractile stimulation. Because MAP kinase is activated in response to a simple increase in the $[Ca^{2+}]$, we suggest that activation of MAP kinase may play an important role in the basic regulation of a smooth muscle contraction. Supported in part by NIH HL37956 and HL46704.

M-Pos270

PKCε BUT NOT ζ CAUSES A Ca^{2+}-INDEPENDENT CONTRACTION OF SMOOTH MUSCLE CELLS THAT IS REVERSED BY CALPONIN ((A. Horowitz[1,2], O. Chomienne[3], M. P. Walsh[3], T. Tao[2], H. Katsuyama[1], and K. G. Morgan[1])) [1]Harvard Medical School, Boston, MA 02215; [2]BBRI, Boston, MA 02214; [3]Dept. of Medical Biochemistry, University of Calgary, Canada, T2N 4N1.

PKC causes a Ca^{2+}-independent contraction of aortic smooth muscle cells (Collins et al., AJP, 1992), with PKCε being the hypothetical isoform involved. To directly test this hypothesis we used the two Ca^{2+}-independent PKC isoforms expressed in aortic smooth muscle cells, ε and ζ. PKCζ has been localized to the nuclear area, whereas PKCε appears to be cytosolic (Khalil et al., AJP, 1992), suggesting spatial segregation. When applied consecutively to the same saponin-skinned cell, PKCζ caused no significant force response. PKCε, however, induced contraction of a magnitude (89±13 μg) similar to that induced by phenylephrine (PE; 89±9 μg), a known PKC agonist. This is the first direct evidence that PKCε is the isoform involved in regulation of contractility in smooth muscle. Since PKC may activate smooth muscle via phosphorylation of the thin filament-associated protein calponin (CaP), we tested the effect of CaP on PKCε-induced contraction. Application of 5 μM exogenous CaP reduced the magnitude of PKCε-induced contraction by 40%. A similar effect was observed on basal tone and PE-induced contraction at pCa 9.0. These results are compatible with a signal transduction pathway in which PKCε induces contraction possibly by direct phosphorylation of CaP, thereby alleviating CaP inhibition of cross-bridge cycling. (Supported by P01-AR41637, HL42293, HL31704, and MRC Canada).

M-Pos271

LOCALIZATION OF CALPONIN RELATIVE TO THE α AND β ISOFORMS OF ACTIN IN RESTING AND STIMULATED SMOOTH MUSCLE CELLS.

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We have previously reported that agonist stimulation of smooth muscle cells (SMCs) causes the redistribution of the thin filament associated protein calponin (CaP) from the cytosolic core (C) to the surface membrane area (S) in ferret portal vein cells (Parker et al., Am. J. Phys., in press). In our present study we have attempted to determine the structural basis for this redistribution by relating it to the distribution of actin isoforms. Observations were quantitated using a digital imaging algorithm, whereby the ratio S/C was calculated. In resting SMCs α-actin immunofluorescence colocalized with filaments which ran parallel to the long axis of the cell throughout the cell. The S/C ratio for α-actin was 0.80 and did not change upon stimulation with phenylephrine. In contrast, β-actin immunofluorescence was discretely distributed near the surface membrane in both resting and stimulated cells (S/C=1.12). The S/C ratio for CaP increased from 0.64 to 1.18 upon stimulation as reported previously (ibid). The percent overlap of CaP and α-actin in cells colabeled for both proteins significantly decreased with stimulation (59.1% to 35.1%), while that between CaP and β-actin significantly increased (41.5% to 62.9%). Thus the distribution of CaP, by ratio analysis, in the resting cells appears similar to α actin whereas the distribution of CaP for the stimulated cells was similar to that for β actin. These results are consistent with the hypothesis that agonists cause a preferential redistribution of CaP to β-actin. Support: HL31704, HL42293, PO1-AR41637.

M-Pos272

PHOSPHOTYROSINE-DEPENDENT TARGETING OF MAP KINASE TO CALDESMON TRIGGERS A Ca^{2+}-INDEPENDENT CONTRACTION IN DIFFERENTIATED VASCULAR CELLS.

((R.A. Khalil, C-L.A. Wang and K.G. Morgan)) Harvard Medical School and Beth Israel Hospital, Boston, MA 02215 and Boston Biomedical Research Institute, Boston, MA 02114.

Tyrosine phosphorylation has been linked to plasmalemmal targeting of SH2-containing proteins, nuclear targeting of MAP kinase and proliferation of cultured cells. Significant tyrosine phosphorylation and MAP kinase activities have also been reported in differentiated cells, but the signaling role of tyrosine-phosphorylated MAP kinase in these cells is unclear. The spatial and temporal relationship between phosphotyrosine and MAP kinase immunoreactivity was quantitated in freshly isolated contractile ferret aorta cells using digital imaging microscopy. An initial association of MAP kinase with the plasmalemma (peak at 4 min) required upstream protein kinase C activity but occurred in a tyrosine phosphorylation-independent manner. Subsequent to membrane association, a delayed redistribution of MAP kinase, colocalizing with the actin-binding protein caldesmon, occurred in a tyrosine phosphorylation-dependent manner. The apparent delayed association of MAP kinase with the contractile proteins (steady-state at 10 min) coincided with a Ca^{2+}-independent contractile activation (maximum at 10 min). Thus in contrast to nuclear targeting of MAP kinase in undifferentiated cells, tyrosine phosphorylation appears to target MAP kinase to cytoskeletal proteins and to mediate a Ca^{2+}-independent contraction in contractile vascular cells. Supported by HL-31704, HL-42293 & AR-41637.

M-Pos273

CALPONIN INHIBITS SHORTENING VELOCITY AND ISOMETRIC FORCE IN SKINNED TAENIA COLI SMOOTH MUSCLE.

((K. Obara, P.T. Szymanski*, T. Tao* & R.J. Paul)) Dept. of Molecular and Cellular Physiology, University of Cincinnati, College of Medicine, 45267-0576 and * Muscle Research Group, Boston Biomedical Research Institute, Boston, MA 02114 USA.

Calponin, a thin filament-associated protein, inhibits actomyosin ATPase in solution and has been suggested to modulate smooth muscle contractility. We used permeabilized guinea pig taenia coli smooth muscle to investigate whether calponin can modulate actin-myosin interaction in a more organized contractile system. Fibers were permeabilized with Triton X-100 and glycerol, which permits access of large macromolecules to the contractile apparatus. For contractures elicited by Ca^{2+} (6.6 μM +0.1 μM calmodulin), the recombinant α-isoform of chicken gizzard calponin (RαCaP) decreased isometric force (F_o) and unloaded shortening velocity (V_m) in a dose-dependent manner; 10 μM RαCaP reduced F_o to 27% of control and V_m to near zero levels. In fibers actived by thiophosphorylation with ATPγS in which myosin light chain kinase/phosphatase effects are minimized, F_o was not as strongly inhibited (74% of control) by RαCaP, whereas V_m was reduced to 32%. A similar inhibition was obtained with a mutant RαCaP (ser^{175}→ cys) that retains the ability to bind to actin. RαCaP phosphorylated by PKC and the cys^{175} mutant labeled with 1,5-IAEDANS, which bind actin poorly, were not effective inhibitors. Our results indicate that calponin more strongly inhibits V_m (~crossbridge cycle rate), than F_o (~number of activated crossbridges) and further implicate calponin as a modulator of smooth muscle contractility. Supported by NIH HL23240 (RJP), PO1-AR41673 (TT) and AHA 13-523-934 (PTS).

M-Pos274

LINEAR FORCE RISE OBSERVED IN RAMP STRETCHES OF FAST MAMMALIAN SKELETAL MUSCLE.

((E. Burmeister, R. Cooke and S. Lehman)) Bioengineering Grad. Group, Dept. of Human Biodynamics, UCB, and Dept. of Biochem/Biophysics, UCSF. (Spon. by E. Pate)

Muscles in intact animals are often stretched while activated, at velocities up to 10 lengths/sec (L/s). We stretched skinned rabbit psoas fibers using laser diffraction to produce constant sarcomere velocities of 0.5-3.5 L/s for excursions of 20% initial length. Initially, force rose abruptly to about 2 times isometric tension (Po) in approximately 10 nm. Fibers stretched at more than 1.2 L/s yielded, then increased force linearly for the duration of the stretch. Fibers stretched at less than 1.2 L/s did not yield before the linear phase. At each velocity, the final value of force was about 2.5 times Po. The linear increase in force was not due to changes in filament overlap, as the same behavior was observed in fibers stretched from both long and short initial lengths. Nor was the increase due to passive forces, which were much smaller than the observed total force changes. The observed force increase must be a property of individual sarcomeres, as the diffraction pattern of the sampled portion (more than half) of the fiber showed no evidence of increased sarcomere inhomogeneity during the stretch. The spring-like mechanics observed here in activated lengthening fibers provides an explanation for the spring-like behavior observed during braking of human limb movements. Supported by UCB Graduate Opportunity Fellowship and HL32145.

M-Pos275

INJURY TO FIBERS FOLLOWING SINGLE STRETCHES OF EXTENSOR DIGITORUM LONGUS MUSCLES OF YOUNG, ADULT, AND OLD MICE.

((Susan V. Brooks and John A. Faulkner)) Institute of Gerontology and Department of Physiology, University of Michigan, Ann Arbor, MI, 48109-2007.

Contraction-induced injury is most likely when muscles are stretched. Following a protocol of injurious contractions, an initial injury is observed, which is predominantly mechanical in nature, followed by a delayed secondary injury. Identical protocols of repeated contractions produce more severe damage in muscles of old compared with young or adult mice. This conclusion is based on evaluations made at the peak of the secondary injury. Consequently, whether muscles of old animals are more severely damaged by the same initial event, are damaged equally but undergo more severe secondary injury, or both, remains unknown. For extensor digitorum longus (EDL) muscles in young mice, the magnitude of the initial injury is well predicted following single stretches by the work done. For maximally activated muscles of old compared with young mice, isometric specific force is 20% lower, while specific forces developed during stretches are not different. We hypothesized that for maximally activated muscles of old compared with young or adult mice, the work required for stretches of any given strain is not different, but for any given work input, the magnitude of the injury is greater. Isolated EDL muscles in young (2-6 months), adult (12 months), and old (25-28 months) mice were exposed to a single stretch *in situ* during the plateau of a maximum isometric contraction. Stretches were initiated at optimum length for force (L_o) and were of 10% to 60% strain relative to L_f at 2 L_f/s. The magnitude of the resultant injury was assessed by the force deficit [(1 - post-stretch maximum isometric force/pre-stretch maximum isometric force) x 100] one minute following the stretch. No differences were observed for data from young and adult mice and these data were pooled. In support of our hypotheses, the relationship of strain with work (J/kg) was not different for any age group, while the relationship of work with force deficit was ~10% steeper for the muscles of old compared with young and adult mice. We conclude that the muscles of old mice are more susceptible to the initial mechanical injury induced when maximally activated muscles are stretched than are the muscles of young or adult mice. Supported be AG-06157.

M-Pos276

SARCOMERE DYNAMICS AND CONTRACTION-INDUCED INJURY TO ACTIVE SINGLE MUSCLES FIBERS.

((P.C.D. Macpherson & J.A. Faulkner)) Department of Physiology, and Institute of Gerontology, University of Michigan, Ann Arbor, MI. 49109

Following stretches of contracting muscles, direct morphological evidence of damage is localized to small groups of sarcomeres. Our purpose was to determine the relationship between sarcomere lengths and contraction-induced injury. Experiments were performed on 1.6 to 2.5 mm long permeabilized single fiber segments from soleus muscles of rats. The average sarcomere lengths of each of five discreet regions along the length of a given fiber segment were determined by transilluminating the fiber with an HeNe laser diode. The beam width was focused to ~200 μm. We tested the hypothesis that the regions of sarcomeres that increased in length during a maximum isometric contraction are the regions injured during a subsequent stretch. Injury to specific regions of the fiber were identified by the complete loss of a dominant peak in the 1st order diffraction pattern and confirmed with light microscopic evaluation of fiber morphology. Fiber segment length was adjusted to the optimum length for force development (average sarcomere length 2.55 ± 0.01). Maximum isometric force (P_o) was achieved by immersing fibers in a solution with a pCa of 4.5 at 15°C. With a fiber at P_o, a ramp stretch of 40% strain was imposed at a velocity of 0.5 fiber lengths per second. Since the injury is focal, the magnitude of injury is best evaluated by the deficit in the force developed immediately after a given stretch expressed as a percentage of the P_O prior to the stretch (force deficit). Single stretches of single fibers (n = 12) resulted in an 11 ± 1% force deficit. Just prior to stretching maximally activated single fibers, average sarcomere lengths ranged from 2.11 μm to 2.91 μm. Following single stretches, regions of sarcomeres with the longest average sarcomere lengths prior to the stretch produced 1st order diffraction patterns that were severely attenuated and contained no dominant peaks. In contrast, in regions of the fiber that shortened initially 1st order peaks were observed. Morphological evaluation confirmed that regions of long sarcomeres were damaged. We conclude that heterogeneities in sarcomere lengths develop in maximally activated muscle fibers and when muscles fibers are lengthened the longer, weaker, sarcomeres are stretched excessively and damaged. (Supported by NIH grant AG06157)

M-Pos277

EFFECT OF INITIAL MUSCLE LENGTH ON INJURY INDUCED BY A SINGLE LENGTHENING CONTRACTION. ((KD Hunter and JA Faulkner)) Department of Physiology and Institute of Gerontology, Univ. of Michigan, Ann Arbor, MI 48109. (Spon. by T Coffin)

Single lengthening contractions injure muscle fibers mechanically, resulting in an immediate disruption in the ultrastructure of single sarcomeres and decreased force development (force deficit). For a maximally activated muscle with fibers at optimal length for force production (L_o), the most important factor in determining the magnitude of the injury produced after a lengthening contraction is the strain on the muscle. Our working hypothesis is that the critical variable that determines the magnitude of contraction-induced injury is the length to which the muscle is stretched rather than the displacement the muscle undergoes. We tested the hypothesis that regardless of the initial muscle length, the force deficit is not different when maximally activated muscles are stretched to the same final length. Mice were anesthetized and secured to a platform maintained at 37° C. The extensor digitorum longus muscle was attached to a servomotor. Each muscle was maximally activated and stretched at a velocity of 2 L_o/sec from an initial fiber length of either 90%, 100%, or 120% of L_o. Of fifteen muscles studied at each initial length, five were stretched to a final fiber length of 150% L_o, five to 160% L_o and five to 170% L_o. Isometric force production was measured prior to and 2 minutes after the lengthening contraction. For each final length examined, muscles exposed to lengthening contractions starting at the longest initial fiber length (120% L_o) showed a smaller force deficit than those exposed to contractions which started at 100% L_o (0.4% ± 1.3% vs. 8.4% ± 1.2% for final fiber length 150% L_o, 7.8% ± 2.3% vs. 19.7% ± 6.6% for 160% L_o, 30.7% ± 11.5% vs. 47.2% ± 3.9% for 170% L_o, mean ± 1 SEM). With fiber lengthening to 170% L_o, muscles with an initial fiber length of 90% L_o had a smaller force deficit, 29.5% ± 1.6%, than those with an initial fiber length of 100% L_o, 47.2% ± 3.9%. We conclude that although final length is the single best predictor of the magnitude of contraction-induced injury (r^2 = 0.49), initial length and displacement are also important determinants. Supported by AG-06157.

M-Pos278

INCREASES IN LABILE FRACTIONS OF MYOFIBRILLAR PROTEINS IN CHRONICALLY STIMULATED SKELETAL MUSCLE. ((William O. Kline[1] and Peter J. Reiser[1,2])) [1]Exercise Science and [2]Oral Biology, The Ohio State University, Columbus, OH 43210.

We observe a marked decrease in the total amount of protein between adult rabbit tibialis anterior (TA) muscle that has been chronically and continuously stimulated electrically (10 Hz, 3 weeks) compared to control TA muscles when glycerinated (G) samples of both are examined with SDS-PAGE. We tested in the present study whether there is a greater loss of protein during glycerination in stimulated TA compared to control TA. Fresh (F) samples were dissected from stimulated and control muscles, macerated, weighed, placed in gel samplebuffer (8 M urea, 2 M thiourea, .05 M tris, .075 DTT, 3% SDS, pH 6.8), heated and frozen until run on gels. Bundles of fibers were prepared from strips adjacent to the fresh samples from control and stimulated muscles and stored in glycerinating solution at -20°C for up to one week. The glycerinated samples were then macerated and prepared in the same manner as the fresh samples except they were blotted on filter paper to remove excess fluid prior to weighing. Densitometric scans of gels, on which G and F samples were run, reveal significant differences in the G/F band area ratios for several proteins (myosin heavy chain, actin, beta-tropomyosin) in stimulated, compared to control, TA. There is an ~50-70% labile fraction of each of these proteins, possibly not assembled into filaments, in fresh stimulated TA. This result can explain discrepancies among reports of altered protein expression during stimulation-induced transformation of muscle between studies based on whole, fresh muscle homogenates and those based on glycerinated samples. Supported by NIH Grant AR39652.

COMPUTER SIMULATIONS

(See also M-Pos313)

M-Pos279

ANALYTICAL SOLUTION OF REVERSIBLE THREE AND FOUR STEP REACTIONS. ((R. Rhoads, J.A. Watkins, C-Y. Li, and J. Glass)) Center for Excellence in Cancer Research, Treatment, and Education, Dept. of Medicine, LSUMC-S, Shreveport, LA, 71130.

The analytical solution of the three step reversible reaction given by A ↔ B ↔ C ↔ D has been a significant kinetic problem while the four step reaction, A ↔ B ↔ C ↔ D ↔ E has been considered to be analytically intractable. Using a symbolic logic algorithm, the algebra has been computed and expressions describing the relationships between intrinsic rate constants and eigen values (λ_r) as well as pre-exponential factors has been determined for the general and specific solutions of the form $A_i = \Sigma Q_r B_{ij} \exp(-\lambda_r t)$. The eigen values of the three step reaction were computed from the determinant $\lambda^3 - a\lambda^2 + b\lambda - c\lambda = 0$ while λs for the four step reaction were determined from the determinant $x^4 + px^2 + qx + r = 0$ which is obtained following the substitution of $\lambda = x + d/4$, and the constants a,b,c,d,p,q, and r are sums of products of the intrinsic rate constants (k_{ij}). The amplitude terms were determined, using the method of cofactors, from $Q_r = B_{ir}^{-1} C_i$, where B_{ir}^{-1} is the inverse matrix of B_{ir} and C_i is the matrix of initial conditions. In terms of the k_{ij}s, the observed rate constants and amplitudes are very complex and indicate the formidable nature of the problem when approached without the use of symbolic logic algorithms. Expressions describing the time dependence of the relative concentrations of reactants and products are particularly involved. The equations have been applied to several examples of physical-biochemical significance with satisfactory results. Similar manipulations to those routinely used for the two step reaction have been employed and are discussed. These expressions may provide a basis for more detailed kinetic analysis of complex biochemical reaction networks. Supported by NIH DK-37866

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TOWARDS PHASE TRANSFERABLE COMPUTATIONAL POTENTIAL FUNCTIONS: METHODOLOGY, BIOPHYSICAL IMPLICATIONS AND APPLICATION TO NITROGEN ((Peter C. Jordan[1,2], Paul J. van Maaren[1], Janez Mavri[1,3], David van der Spoel[1] and Herman J.C. Berendsen[1])) [1]Bioson Research Institute, Department of Biophysical Chemistry, University of Groningen, The Netherlands, [2]Department of Chemistry, Brandeis University, Waltham, MA 02254, USA, and [3]National Institute of Chemistry, P.O.B. 30, 61115 Ljubljana, Slovenia

We describe a generalizable approach to the development of phase transferable effective intermolecular potentials (reliable over wide regions of thermodynamic phase space) and discuss its implications for systems of biophysical interest (water, hydrocarbons, polypeptides). It is based on a polarizable shell model description of the isolated molecule and uses experimental data to establish the parameters. We describe a procedure that, using quantum calculations as a guide, permits very accurate modeling of not only a molecule's electrostatic field (i.e., a practical representation of the molecular charge distribution) but also its response to electrical and mechanical stress (polarization and deformation), and apply the method to nitrogen. The shells are surrogate electrons and purely intermolecular terms in our potential function reflect shell-shell interactions. They are determined from properties of low pressure, low temperature solid nitrogen (the cubic α phase). The resulting potential is phase transferable. It accounts for the second virial coefficient, the pressure induced phase transition between nitrogen's cubic and tetragonal phases, and a wide range of liquid properties (pair distribution function, heat of vaporization, self diffusion coefficient and dielectric constant).

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SIMULATING ENERGY FLOW IN BIOMOLECULES. ((Q. Wang*, C.F. Wong* and H. Rabitz^)) *Department of Physiology and Biophysics, Mount Sinai School of Medicine, NY, NY 10029 and ^Department of Chemistry, Princeton University, Princeton, NJ 08544.

By constructing a continuity equation of energy flow, we can utilize results from a molecular dynamics simulation to calculate the energy flux or flow in different parts of a biomolecule. A key objective of this work is to examine whether there are preferred pathways of energy flow in biomolecules and whether one can control or alter the pathways of energy flow in a biomolecule to change the function or activity of the biomolecule in a desired manner. The method was first tested on a cluster of 13 argon atoms. We demonstrated that it was possible to follow the pathways of energy flow after the velocity of an atom in the cluster was perturbed. We then applied the method to study the pathways of energy flow after the iron in the heme of a tuna ferrocytochrome c molecule was oxidized. We found that energy redistributed in an earthquake-like fashion after a cytochrome c molecule was oxidized. There was a hierarchy of time scales that the atoms in the surrounding of the heme group responded to the perturbation introduced to the heme. The time scales range from ~1 fs to ~40 fs.

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BINDING ENERGETICS: MODEL STUDY OF CYCLIC DIPEPTIDES. ((P. Brady, K. Sharp)) U. of Pennsylvania, Philadelphia, PA 19104.

During a bimolecular binding event, three translational and three rotational degrees of freedom are replaced by six internal modes of vibration. To accurately calculate binding energies from structures of complexes, these contributions must be determined. However, calculations of this nature are presently difficult for protein systems. We are using the gas to crystal and water to crystal transfer of cyclic dipeptides as a model for molecular association. We employ a variety of different methods to compute the contributions to binding: We use Finite-Difference Poisson-Boltzmann for the electrostatic contributions, Molecular Mechanics for the internal energies, a Surface Free Energy method to account for hydrophobic solvation, Quasiharmonic Analysis for intramolecular vibrations, and an Einstein Model for crystalline lattice vibrations. The calculations are compared to experimental enthalpies and free energies.