534 - Pos
MOLECULAR BASIS OF CHARYBDOTOXIN AND IBERIOTOXIN INSENSITIVE MaxiK CHANNELS: A NOVEL β SUBUNIT.
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Neuronal MaxiK channels can be classified into two types based on their toxin sensitivity: one sensitive to the Charybdotoxin (CTX) and Iberiotoxin (IBTX) and the other insensitive to both toxins. We have isolated a β subunit (β3) cDNA that is highly expressed in brain (Wallner et al., this issue). Coexpression of this neuronal MaxiK β3 subunit with its pore forming α subunit results in channels that have low apparent toxin sensitivity. Application of 100 nM of CTX or IBTX blocked only 20% of the current within 5 minutes. In contrast, channels formed by MaxiK α subunit alone were completely blocked by both toxins. When the MaxiK channel α subunit was coinjected with the smooth muscle β1 (β3) subunit, CTX completely abolished the currents, whereas IBTX blocked ~60%. We conclude that the association of the neuronal β3 subunit with the MaxiK channel α subunit forms the molecular basis of toxin insensitive channels found in brain. Supported by AHA 9730745N (F.M.), AHA 990020Y (M.W.), NIH HL54970 (L.T.). L.T. is an Established Investigator of AHA.

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6A NEURONAL MAXIK CHANNEL β SUBUNIT.
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Large conductance voltage- and Ca2+ activated (MaxiK) potassium channels are composed of a pore forming α subunit and tissue specific modulatory β subunits (β1 & β2). We found an additional MaxiK channel β-subunit homolog (β3) in the EST database. A full length clone was isolated by cDNA library screening. RNA-seq analysis shows that β3 is highly expressed in the brain with lower levels in other tissues. In contrast to β1 and β2 subunits which dramatically sensitize the pore forming α subunit towards Ca2+ and voltage, functional expression of β3 with its α subunit in oocytes leads to macrocurrents which show extreme variations in their apparent voltage/Ca2+ sensitivities. Currents in 10 µM (Ca2+) are found in different sensitivity modes, varying from a high (Vc= -80 mV) to a low sensitivity mode (Vc= -110 mV) with respect to the α subunit alone (Vc = -110 mV). A similar variability, although to a lesser extent, was observed when channels were expressed in HEK293T cells. Because patches with high and low sensitivity currents have been observed in the same oocyte expressing α β3, we speculate that local differences in membrane or signaling properties may be responsible for this surprising behavior. Supported by AHA 9730745N (F.M.), AHA 990020Y (M.W.), NIH HL54970 (L.T.). L.T. is an Established Investigator of AHA.

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ANALYSIS BY MUTAGENSES OF INACTIVATION MEDIATED BY THE BK CHANNEL β accessory subunit.
Xia-Ming Xia, Jie-Ping Ding, Chris J Lingle, Washington Univ. Sch. Med. Calcium-activated, voltage-gated, big conductance potassium channels (BK channels) are composed of four α subunits encoded by the slo gene along with auxiliary β subunit(s) encoded by separate genes. The diversity of BK channel properties arises not only through numerous slo splice variants but also through coassembly of α and various β subunits. Rat chromaffin and pancreatic β cells express an inactivating BK channel, which results from a unique β subunit (β3) coassassembled with the α subunit. The amino terminal of the β3 subunit is required for BK channel inactivation. However, in comparison with inactivation of the Shaker β, there are striking differences between these two types of inactivation. Specifically, the β3 inactivation domain does not appear to interact directly with the mouth of the channel.

To understand the inactivation mechanism, we have mutagenized both charged and uncharged residues throughout the β3 N-terminal. The results suggest that, unlike blockade by the Shaker N-terminal tail, blockade by the β3 N-terminal is unlikely to involve long-range electrostatic interactions. Furthermore, the charged residues on the β3 N-terminal are unlikely to move through the electric field during the inactivation process. The results are consistent with our earlier model that binding of the inactivation domain occurs at some distance from the mouth of the K+ permeation pathway.

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IS THE BK, CHANNEL S Ca2+ BINDING AFFINITY ENHANCED BY ITS β SUBUNIT? A SIMPLE EXPERIMENT.
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At many membrane voltages the BKα channel is augmented by the expression of an auxiliary subunit (β1). An important question with regard to this effect is whether or not β1 actually increases the affinities of the channel's Ca2+ binding sites, or whether it affects an apparent increase in affinity by altering other aspects of gating. In addition to being sensitive to internal Ca2+ BKα channels are also sensitive to membrane voltage. This allows the experimenter to alter the membrane voltage in the absence of Ca2+ so that the channel's open probability is more energetically sensitive (Popen = 0.5), and then to see how much Ca2+ is required to just begin to activate the channels further. Allosteric models indicate that this critical [Ca2+] is determined almost exclusively by the affinity of the channel for Ca2+ when it is open (Popen). To answer the above question, therefore, we have determined the critical [Ca2+] for both α alone and α + β channels, and have found that, in fact, the critical [Ca2+] changes very little with β1 co-expression. This suggests that Ko is minimally altered by β1 binding and that changes to other aspects of gating must be important. Fits to our data yield estimates of Ko for both channel types.

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A NOVEL FAMILY OF ALTERNATIVELY SPliced BK β-subunit.
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BK channels, in at least some tissues, are composed of two distinct types of subunits. Although functional channels can be expressed from α subunits alone, coexpression of β subunits may alter the biophysical and pharmacological properties of the expressed current. However, the properties of some native BK currents are not well reproduced by currently known combinations of α- and β subunits, suggesting novel homologies of these subunits may still exist.
We identified a novel family of BK β subunits, BKBα3α, which arise by alternative splicing of a single gene that maps to 3q26.3. BKBα3α have predicted molecular weights of 29.1-36.6 kDa and share ~30-40% amino acid identity with BKB1 and BKB2. The 4 variants differ in sequence in their extreme amino terminal 23 residues and exhibit distinct tissue distributions. Coexpression of BKα3α, BKB1 or BKB2 increases the IC50 of Ca2+ from <2 nM (α alone) to >50 nM (α + β). Inactivation rates (IC50 Ca2+) varied with the different beta subunits: BKα3α was the slowest (τ = 5942), BKα3α was intermediate (τ = 5245) and BKα3 was too rapid to resolve, resulting in an apparent rectification. Unlike the BKα or β2 subunits, the β3 subunits do not alter the calcium and voltage sensitivity of the current. Tissue specific coassembly of these novel β subunits with α subunits may contribute to the large functional diversity of native BK currents.

539 - Pos
ACCESSORY β SUBUNITS CAN INCREASE THE APPARENT Ca2+ SENSITIVITY OF LARGE-CONDUCTANCE Ca2+-ACTIVATED K+ (BK) CHANNELS BY A Ca2+-INDEPENDENT MECHANISM.
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Previous studies indicated that the coexpression of the β1 subunit (bovine BKα4β1) with the α subunit (mshβ) of BK channels increases the apparent Ca2+ sensitivity by increasing burst duration 20-100 fold. We now investigated whether the mechanism underlying this increase in Ca2+ sensitivity, in fact, requires Ca2+ by comparing the gating properties of α and α+β channels in = Ca2+. HEK 293 cells were transiently transfected with cDNAs encoding for the α and β1 subunits, and single-channel currents were recorded in inside-out patches. With = Ca2+ at ~30 mV, β1 subunits increased: Po (=10-fold), mean number of openings per burst (=3-fold), mean open interval durations (=10-fold), mean burst duration (=20-fold), and mean gap duration between bursts (=3-fold). Mean closed interval durations remained unchanged. These general effects of the β1 subunit occurred from ~0 to higher Ca2+ and over the examined voltages. (The β1 subunit effect was not equivalent to an increase in membrane potential, as increasing Po with depolarization gave different channel kinetics than increasing Po with the β1 subunit.) Since the β1 subunit had the same general multiplicative effect on channel kinetics in = Ca2+ that it had in the presence of Ca2+, we conclude that Ca2+ is not required for at least a large part of the β1 subunit-induced increase in Po that gives rise to the apparent increase in Ca2+ sensitivity. (Supported by grants from the AHA, NHI, and MDA.)
540 - Pos
BK CHANNEL CARBOXYL TERMINAL TAIL DOMAIN CAN MODULATE GATING PROPERTIES AND CONDUCTANCE.
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To study the contribution of the carboxyl (C)-terminal tail domain to the functional properties of large-conductance calcium-activated potassium (BK) channels, the mSlo1 core domain (SO-S8) was expressed with either the mSlo1 tail (mSlo1 tail channels) or the mSlo3 tail domain (mSlo3 tail channels). Currents recorded from mSlo1 tail and mSlo3 tail channels expressed in Xenopus oocytes using the inside-out configuration of the patch clamp technique. Although mSlo3 tail channels have fewer negatively charged residues in the region of the calcium bowl and lack the high calcium sensitivity of mSlo1 tail channels, mSlo3 tail channels could still be weakly activated by calcium. This observation suggests that the two negative charges remaining in the calcium bowl region and/or residues located elsewhere may still bind calcium. Replacing the mSlo1 tail with the mSlo3 tail also resulted in additional changes in channel properties. Compared to mSlo1 tail channels, mSlo3 tail channels showed a pronounced decrease in burst duration, small decreases in both voltage sensitivity and single channel conductance, and an increase in sensitivity to block by internal TEA. These results suggest modulatory functions of the tail in addition to its key role in calcium sensitivity. cDNAs coding for mSlo1 and mSlo3 were kindly provided by L. Salkoff. Supported by grants from the NIH and MDA.

542 - Pos
CALCULUM BINDING ACTIVITY OF A C-TERMINAL FRAGMENT OF THE DROPHILA BK CHANNEL.
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Large conductance Ca-activated K-channels (BK) are thought to consist of an N-terminal membrane domain (Cor) and a C-terminal Ca-activation domain (Tail). To investigate Ca-binding properties of the Tail domain we expressed the C-terminal 280 residues of the Drosophila BK channel fused to an N-terminal Ompl A-Flag sequence (DSio-C280) in E. coli. DSio-C280 was purified to homogeneity from the inclusion bodies of E. coli and from inclusion protein. Purification involved anti-Flag immuno-affinity and high performance size-exclusion chromatography (HPSEC). Purified DSio-C280 exists as a high MW aggregate that can be dispersed to smaller aggregates with DTT and mild detergents. The smaller aggregate migrates as an apparent mixture of monomer to tetramer species on HPSEC. Dimer formation was demonstrated by chemical crosslinking. Certain Ca-binding proteins such as calmodulin bind Ca2+ when subjected to SDS-PAGE, blotted onto PVDF membrane, and assayed by Ca2+-autoradiography. With this assay DSio-C280 (lane 2) exhibits robust Ca2+-binding activity both in crude and purified states. Specificity is demonstrated by positive Ca-bands for calmodulin (lane 1) and calpain (lane 4) and the absence of such bands for control proteins: MW standards (lane 1), Flag-alkaline phosphatase, trypsin. We conclude that DSio-C280 contains a functional Ca2+-binding site and may autoassociate. Supported by NIH GM-51172 and AHA postdoctoral fellowships (S.B., I.F).

543 - Pos
MODULATION OF Ca2+-ACTIVATED K CHANNELS BY OXIDIZING AGENTS.
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Reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide and hydroxyl radical, are produced as by-products of oxidative metabolism and they are involved in many physiological processes and cellular dysfunction. Large-conductance Ca2+-activated K+ channels in both native and heterologous expression systems are known to be modulated by the cellular redox status. We investigated how different oxidizing treatments altered the human Ca2+-activated K+ channel (hSK3) heterogeneously expressed in HEK 293 cells using symmetrical K+ solutions with 10 mM EGTA and no added Ca2+. Application of H2O2 and tert-butyl hydroperoxide (tBHP) to the bath solution in the cell-attached configuration increased the macroscopic iCl current amplitude at a given voltage and shifted the voltage dependence of activation to more positive voltages. In the inside-out configuration, H2O2 decreased the current amplitude, suggesting that the regulation observed in the cell-attached configuration may be mediated by changes in the intracellular Ca2+. Application of tBHP in the inside-out configuration, however, did not have any obvious effect. Photocatalysis of rose bengal did not produce any noticeable effect on the sIo channel in either the cell-attached or inside-out configuration. Fotofenton (Molecular Probes, Eugene, OR), a hydroxy radical donor, also decreased the current amplitude in most of the inside-out patches. Our preliminary results suggest that the sIo channels in the cells overexpressing the enzyme methionine sulfoxide reductase (MsrA) are more resistant to the oxidizing agents (Supported by NIH GM57654 and HL14388).

544 - Pos
A TWO-TIERED ALLOSTERIC MECHANISM DESCRIBES BOTH THE CALCIUM- AND VOLTAGE-DEPENDENT GATING OF SINGLE CALCIUM-ACTIVATED K CHANNELS.
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The Ca2+-dependent gating of single calcium-activated K channels (BK channels) can be described by a two-tiered allosteric mechanism in which the channel can undergo transitions among five substates, each with five closed and four open states, with each substate identified by 0, 1, 2, 3, or 4 Ca2+ bound to the channel (Rothberg and Magleby, 1999, J. Gen. Physiol. 114: 93-124). In the present study, we tested the ability of this mechanism to describe the voltage-dependent gating of BK channels. Currents through single BK channels were obtained with the patch-clamp technique over a wide range of Ca2+ (0.0003 to 1024 μM) and voltage (-130 mV to +110 mV). Adjacent open and closed dwell times were biased as open-closed interval pairs. The resulting two-dimensional dwell-time distributions were used to estimate the rate constants of their voltage-sensitivity for a simplified form of the two-tiered gating mechanism, in which each of the five substates had only one closed and two open states. By incorporating voltage-sensitivity into the non-calcium-dependent O-O, C-C and C-O transitions, the simplified two-tiered gating mechanism could describe both the Ca2+- and voltage-dependence of the gating of single BK channels over the wide range of examined voltage, Ca2+, and Fm. Supported by: NIH NS007944, AR32807 and the MDA.

545 - Pos
FUNCTIONAL CHARACTERIZATION OF A CLONED LARGE-CONDUCTANCE Ca2+-ACTIVATED K CHANNEL.
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The Ca2+-activated K+ channels (BKCa) play important roles in the regulation of action potential and in the maintenance of resting membrane potential of bladder smooth muscle cells. To characterize BKCa, a 3 kb cDNA encoding the α subunit was cloned from the human bladder. Currents were measured by two-electrode voltage clamp from oocytes injected with cRNA or by whole-cell patch clamp from HEK293 cells transfected with cDNA of the α subunit. Under the normal physiological solutions, the currents expressed in oocytes or HEK293 cells were voltage-dependent, sensitive to tetraethylammonium and barium blockade, and activation by NS1619, a BKCa channel opener. However, during the 400 ms depolarization pulse, currents inactivated in both oocytes and HEK293 cells unlike the BKα currents recorded from bladder smooth muscle cells that did not inactivate under similar conditions. When BKα is subunit was co-expressed with the β1 subunit in HEK293 cells, the currents showed properties resembling native BKCa currents recorded from bladder smooth muscle cells. These studies demonstrate that inactivation is an intrinsic property of the BKα subunit.
546 - Pos
REDUCTION OF SINGLE CHANNEL CONDUCTANCE BY D TO N MUTATION IN THE hSlo POTASSIUM CHANNEL PORE
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The outer pore region of K+ channels contains a highly conserved aspartic acid (D), located at residue 219 in the hKv2.1 channel. While a consensus substitution of D447 with asparagine (N) produces a nonconducting channel with apparently normal gating currents (Hurst et al. FERS Lett. 1996, 389-395). Residue D447 corresponds to D292 in the pore of hKCl, a calcium and voltage activated K+ channel. The equivalent mutation in the hSlo pore (D292N) did not prevent conductance. Instead, the hSlo mutant D292N showed a 40% reduction in single channel conductance compared to the wild-type. The channels (wild-type and mutant) were expressed in Xenopus oocytes and recorded under voltage clamp conditions. Membrane patches excised in symmetrical 120 mM K+ and 5 mM Ca2+ solutions, in inside-out configuration, exhibited single channel activity with a typical conductance of ~25pS for the wild-type. However, D292N mutants had a single channel conductance of ~15pS. It is notable that the D to N mutation in Shaker K+ channels prevents conductance while in hSlo allows reduced ion conduction suggests different molecular arrangements of the pore region in both channels. Supported by NIH grants. LT is Established Investigator of AHA.

547 - Pos
LOW CONCENTRATIONS OF ETHANOL INHIBIT A SMOOTH MUSCLE MAXI-K+ CHANNEL (Kv2.1) IN A LIPID BILAYER.
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We investigated the physiologically relevant concentrations of 10 to 20 mM ethanol on single channel kinetics of the Maxi K+ channel from bovine aortic sarcolemmal membrane reconstituted in a lipid bilayer composed of POPG-POPC (1:1). Ethanol did not affect ion selectivity or channel conductance (249 pS ± 11) compared to controls (243 pS ± 16, n=6). By contrast, 10 and 20 mM ethanol caused a 75% ± 20.3% decrease in the open probability (P_o) in 11 channels. The decrease in P_o was due mainly to an increase in the mean closed time (ρ 82-9600, ρ = 7). Ethanol affected the mean open time by decreasing it significantly in some channels and altering it little in others to produce an average decrease of ~40.8% ± 22.5. At 20 mM ethanol, the voltage dependence of the channels exhibited a rightward shift of 10 to 25 mV and a decreased slope factor of ~1.4 to ~6.5. The maximum P_o at 20 mM ethanol was decreased by 50% compared to controls. Inhibition occurred when ethanol was added to either the cytosolic or extracellular side of the channel. These results suggest that alcohol consumption at clinically relevant amounts may alter the effects of Maxi K+ channels on arterial tone. (Support: AA07106 and AA07463)

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CA2+ / CALMODULIN-GATED AND VOLTAGE-GATED K+ CHANNELS IN MICROGLIA: EXPRESSION AND ROLES.
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Microglia, the endogenous immune cell of the brain, undergo complex activation processes following CNS damage or disease. We have investigated the role of specific potassium channels in the microglial respiratory burst that underlies their anti-microbial function but, paradoxically, can damage bystander cells. We examined purified cultured microglia isolated from the neonatal newborn rats. RT-PCR analysis identified mRNA for the voltage-gated Kv1.3 and Kv1.3 channels, and the Ca2+/calmodulin-gated SK2 and SK4 channels, and Western blots showed Kv1.3 and 1.5 protein in the lysate. These immunoreactive K+ currents were present in whole-cell recordings, identified as Kv1.3, SK2- and SK4-like, based on their biophysical and pharmacological properties. To determine whether these currents are important for microglial activation, we used phospholipase A2 to activate an NADPH oxidase-mediated respiratory burst, which was measured in individual cells using a fluorescence-based dityrodrhonadine-123 assay. Several K+ channel blockers significantly inhibited the respiratory burst: by 83% for spiperone (blocks SK2), by 93% for clofilamine (blocks SK4), by 41% for apomorphine-2 (blocks Kv1.3), and by 70% for charybdotoxin (blocks Kv1.3 and SK4). Thus, we identified K+ currents corresponding with three channel types and found that all three modulate the ability of microglia to produce reactive oxygen intermediates. Inhibiting these channels may allow therapeutic control of microglial functions that exacerbate brain damage.

549 - Pos
CHANGE OF MAXIK CHANNEL DENSITY IN CORONARY ARTERY WITH AGING
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Coronary artery smooth muscle cells have abundant MaxiK channels. We investigated the aging-associated change of MaxiK channels in coronary artery from F344 rats using isotonic contractile force recording, whole-cell recording and immunobiochemistry methods. Exposure to Berberitoxin (100 nM), a selective blocker of MaxiK channels, produced a significant increase of contractile tension in the young group (3 months), compared to the old group (27-30 months). Using non-stationary variance analysis, we observed that the density of active MaxiK channels in the old group was diminished -3 fold. Immunofluorescence staining with the anti-cxKv2.1 antibody in coronary artery showed that the expression of MaxiK channels in old rats was lower than in young rats. Using fluorescent confocal microscopy, we found that the pixel intensity in young rats was 40.18 ± 5.78 Pi vs. 21.10 ± 9.11 Pi in old rats. We confirmed this result in single smooth muscle cells. Western blot analysis of coronary artery membranes demonstrated equivalent results to the immunocytochemical and electrophysiological experiments. In summary, MaxiK channels in rat coronary artery diminish dramatically with aging. (Supported by NIH, LT/AHA-EE).

550 - Pos
EFFECTS OF MEMBRANE TENSION ON S3-44 LINER DELETION VARIANTS OF SHAKER-IR.
Iustia V Tabarasea, Peter F Juraska, Catherine E Morris, Loeb Health Research Institute
We have shown that the open probability of Shaker-IR channels expressed in oocytes is sensitive to membrane tension: near the "foot" of the g(V) curve, tension (patch suction) reversibly increases NPopen. We are now examining macroscopic patch currents from Shaker-IR variants whose mechanical degrees of freedom are reduced by the partial or full deletion of the S3-44 linker (Gonzalez et al 1999, Biophys J 76:478). As anticipated, the exceptionally slow kinetics of these deletants (compared to wild-type Shaker-IR) make the effects of tension on kinetics easier to quantify, but qualitative differences have not been detected in tension responses when comparing wildtype to deletants. Tensions accelerated the activation kinetics of the currents, an effect more pronounced close to the foot of the g(V) curve. A slow partial inactivation was also enhanced. Gd(III) (20 micromolar) caused a rightward shift of the g(V) curve and further slowed the kinetics but did not prevent the acceleration. Of tension on burst. Burst effects were wholly reversible or partially reversible in most experiments. In contrast, patch excision irreversibly accelerated kinetics. The mechanism-susceptibility of Shaker-IR is not dependent on the mechanically flexible 31 residue S3-44 linker. Support: NSERC, Canada

551 - Pos
SLOW ONSET OF INHIBITION BY PCMBs APPLIED TO S4 CYSTEINE MUTANTS OF THE HUMAN POTASSIUM CHANNEL Hkv2.1
Carol J Milligan, Dennis Gressy, Letha University, UK
Previous studies have shown that the fourth transmembrane region (S4) of the voltage-sensitive Shaker K+ channel is translocated outwards upon depolarization. In this study, we have used the slow activating human hKv2.1 channel. Cysteine residues were introduced into the S4 segment at positions 296, 298, F299, I301 and M302, which align with Shaker residues L361, V363, L364, L366 and V367 respectively. These mutants were expressed in Xenopus oocytes and the effects of parachloromercuribenzene-sulfonate (PCMB), a membrane-impermeable sulphydryl reagent, were then examined using the two-electrode voltage clamp technique. For the hKv2.1 channel, PCMBs (100 mM) inhibited K+ currents for mutants V296C, F299C, F299C and I301C, but not those for M302C, demonstrating that the S4 region is exposed up to residue D301C but not M302C, which parallels similar results for Shaker. For application of PCMBs during repetitive depolarizations, the time taken to reach 80% inhibition of currents was 6.9±0.9 min (V296C), 55.9±2.3 min (I298C) 21.5±1.6 min (F299C), 22.5±2.1 min (I301C). Inhibition of all hKv2.1 mutant K+ channel currents by PCMBs was therefore slower than that observed in corresponding Shaker channel mutants. These data indicate that the S4 region of the hKv2.1 S4 residues, may be associated with the slower S3-44 linker and slow activation properties found in hKv2.1.
554 - Pos
MODULATION OF THE SHAKER K+ CHANNEL ACTIVATION KINETICS BY THE S3-S4 LINKER
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To investigate the effect of the S3-S4 linker length on gating charge movement, we constructed S3-S4 linker deletion mutants in ShakerH46(-46). Deletions were: from Val 330 to the C-terminal alanine (V330A), from Val 330 to 331 (VV330V), from Val 330 to 332 (VVV330V) and from Met 356 to the C-terminal alanine (M356A). Activation parameters were: the relative slope of activation, the time constant for half activation (t1/2) and the relative initial kinetic rate (V112). A 1 A mutant, which has been noted to disassemble complete alpha helix turns of the S4 segment. Supported by Cátedra Presidencial (RL) and Fondecyt grant 1970739.

554 - Pos
VOLTAGE-DEPENDENT ACTIVATION IN K+2.1/KVL2-CHIMERIC CHANNELS WITH SUBSTITUTED EXTRACELLULAR AND INTRACELLULAR ELEMENTS
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Among the various Kv channels, steady-state activation and gating kinetics vary markedly. To investigate the role of all intracellularly and extracellularly located elements in the control of channel activation, we studied K+2.1 channels in which each of these elements was substituted by the respective element of KVL2. Substitution of the N-terminus, the S4/S5 linker and the C-terminus yielded chimeras with extremely slow activation, whereas substitution of the S2/S3 linker affected channel activation only negligibly. The voltage of half-maximum steady state activation (V1/2) of all these chimeras was shifted by about 11 mV compared to K+2.1 (V1/2= -3.5 mV). Regarding the extracellular elements, substitution of the S1/S2 linker (Ch1/2) shifted steady-state activation (V1/2-- 37 mV) to a value even more negative than that of K+2.1 (V1/2 = -17.8 mV). Compared to K+2.1, current activation was accelerated. Substitution of the S5/S6 linker also accelerated activation, whereas steady-state activation was practically unchanged (V1/2= 0.5 mV). Substitution of the S2/S3 linker and the S6/P linker had no relevant effects on activation properties. We conclude that in K+2.1 structural elements on both, the intracellular and the extracellular side are involved in the control of channel activation. We propose that substitution of the N-terminus, C-terminus or the S5/S6-linker disrupts interactions essential for normal (fast) activation. On the extracellular side, interactions controlling activation include the S1/S2 linker and the S5/S6-linker.

555 - Pos
MEASURED DISTANCE CHANGES IN THE VOLTAGE-SENSING REGION OF A POTASSIUM CHANNEL USING LRET
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Although the voltage-sensing regions have been identified in voltage-gated ion channels, little is known about the structural changes of these proteins in response to voltage. Using lanthanide-based resonance energy transfer (LRET), distances were measured between Shaker-potassium channel subunits at specific residues. Voltage-dependent distance changes of up to 3.2 Å were measured at several sites near the voltage-sensing S4 segment. At site S346C, the voltage-dependent distance changes correlated directly with gating charge movement, establishing the link between physical changes and charge movement. In addition, this close correlation suggests strong coupling between movement of the S4 segment and the S3-S4 linker. Voltage-dependent distance changes from -120 mV to 50 mV at sites S351C (+1 Å), S352C (0 Å) and N353C(-1.2 Å) are consistent with a rotation and possible tilt of the S3-S4 linker and S4 segment. Furthermore, these measured distance changes argue against a large transmembrane movement of the S4 segment in response to voltage, as suggested by previous models. Voltage-dependent distance changes obtained with LRET suggest that the region associated with the S4 segment undergoes a rotation possible tilt, rather than a large transmembrane movement, in response to voltage. Supported by NIH grants GM30376, GM068042, MH12187, AR44420, GM08276, and Research Corp. R10213.

555 - Pos
EFFECT OF SOLUTION VISCOSITY ON GATING CURRENT KINETICS MEASURED FROM THE SHAKER POTASSIUM CHANNEL
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We postulate that friction plays a role in protein conformational changes associated with ion channel gating. There are two sources of friction to consider. Motion of protein domains through solvent causes Stokes friction. In K+ channels, the relative rate of transition events as well as friction similar to the friction coefficient. Motion of friction in channel gating may be represented by the early fast component of gating, whereas activated transition events generate the main component of gating currents. Friction may also arise from internal sources, in which case, gating currents should be independent of viscosity. Intermediate cases are possible, such as a viscosity dependence of the opening and closing of the pore not the early steps in the activation sequence. Preliminary experiments performed on a nonconductive Shaker K channel (444:W434F) revealed little change in the ON gating current with moderate (10 to 30 percent) increases in external solution viscosity (500 mPa s 1 M sucrose, respectively). The OFF current experienced an initial kinetic component, which has been noted at low temperature, but was not seen at regular viscosity and room temperature. There was otherwise no significant reduction in the rate of the OFF current with increase in viscosity. Supported by NIH- GM30376.

557 - Pos
ESCAPE OF AN ORGANIC K+ BLOCKER FROM ENCLOSURE IN THE CHANNEL CAVITY.
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S-nitrosothiol (SNDDT) is a selective blocker of K+ channels that, like quaternary ammonium (QA) ions, produces a reversible exponential decrease in I<sub>K</sub>, following channel activation. Our earlier work using the non-inactivating Shaker channel (SHB3x6-46 4749) C30t1, C30t8 in whole-cell-voltage-clamped HER293 cells, showed that channels can close with SNDDT bound, and that SNDDT can subsequently unbind from closed channels. Two new results with this system suggest the binding site for SNDDT lies in the channel's "QA trapping" cavity. First, blocking concentrations of internal (2.5 mM), but not external (120 mM) TEA markedly slow the macroscopic SNDDT blocking rate. Second, mutations at residue 470, which lies in the cavity, alter the SNDDT affinity and escape the escape rate. Despite binding in the cavity, SNDDT shows no evidence for trapping in three systems where trapping of QA ions has been demonstrated: squid giant axon I<sub>K</sub>, I<sub>K</sub> in squid giant fiber neuronal cell bodies, and ShB3x6-4749 expressed in HEK293 cells. In experiments using a conventional two-state recovery protocol, all show complete recovery that is accelerated by increasing hyperpolarized voltages. Together these results suggest that SNDDT has a unique ability to "escape from the trap" after being enclosed in the cavity of the channel. (Supported by NIH 5 NS 75150)
558 - Pos
MODIFICATION OF K CHANNEL GATING BY A GASTROPOD SECRETION.
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The marine gastropod, Calliostoma crassum, deters predators by secreting a brilliant yellow mucus from its hypobranchial gland. When applied externally, extracts of this yellow secretion (YS) reversibly modify gating of Shaker B (C301S; C308S; T449V) K+ channels expressed in HEK293 cells and studied with whole-cell voltage-clamp. YS only mildly shifts the steady state conductance-voltage relation to more positive voltages but greatly slows activation of K currents in a voltage-dependent manner. Single exponentials can fit to the final 50%-90% of current rise associated with activation during a depolarizing voltage step. The time constants from these fits exhibit a similar voltage dependence in YS and control solutions. Upon repolarization, tail kinetics are not detectably altered by YS. After a brief repolarization, reopening is slowed by YS to a lesser degree than is the original time course of activation. These results indicate YS may act by slowing the same gating transitions that normally limit the rate of Shaker activation. The bioactivity of YS can be eliminated by treatment with the alkyd disulfide reducing agents tris(2-carboxyethyl)phosphine. We are in the process of isolating the component of YS which is responsible for slowing Shaker activation.

559 - Pos
S45-55 LINKER MUTATIONS IN A CLONED HUMAN K CHANNEL INCREASE 4-AP AND DECREASE SPOCIN TOXIN SENSITIVITY.
Changes in K current block by 4-AP (intracellular binding) and the α-k scorpion toxins (extracellular binding) from P. impiger (ITX-Ka) and T. serrulatus (TTX-Ka) were tested in closed, mutated human brain K1-4 K+ channels transiently transfected into HEK293 cells. Whole-cell patch clamping was used. Individual leucines (L) were replaced by phenylalanine (F) in the leucine heptad repeat region spanning the intracellular S45-55 linker. The L2F, L4F and L5F mutations increased 4-AP sensitivity (+400-fold). The voltage-dependence of activation and inactivation was shifted 30 mV (L2F & L5F) or 10 mV (L4F) in the depolarizing direction. L1F and L3F shifted activation ≈5 mV without affecting inactivation; 4-AP sensitivity was increased 7-fold (L1F) and 200-fold (L3F). ITX-Ka and TTX-Ka blocked L1K4 with IC50's = 5 nM, mutations L1F, L4F and L5F increased IC50's > 400-fold. Thus, mutations (F for L) in the leucine heptad repeat near the cytosolic end of K1-4 have opposite effects on 4-AP and α-k scorpion toxin sensitivity. These effects may arise from changes in gating that stabilize the channel closed state. The activation shifts, alone, may account for increased 4-AP sensitivity in L1F, L4F and L5F, while the effects of L3F (like L4F) may also enhance 4-AP binding.

560 - Pos
CHARGE IMMOLATION BY MTSET MODIFICATION OF SHAKER F370C CHANNELS.
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Baker et al. (Neuron, Vol. 20, 1203-1204, June, 1998) reported that residue F370C in Shaker potassium channel is accessible only when the channel is closed, and reaction with MTSET abolishes ionic current. From this result, we speculated that charge might be immobilized when MTSET reacts with 370C. The double mutant F370C/W454F of the Shaker potassium channel was expressed in Xenopus oocytes. The figure shows the gating currents at +40 mV from an inside-out patch exposed to 200 µM MTSET internal solution for 0 and 70 s; the total charge was decreased from 350 to 50 pC. Thus, most of the gating charge is immobilized by the modification.

561 - Pos
POTASSIUM-DEPENDENT CHANGES IN K+ CHANNEL PHARMACOLOGY.
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TEA block of the Kv2.1 potassium channel is potassium-dependent. In the presence of 100 mM internal K+ and 50 mM external K+, TEA blocked 100% of total current, with an IC50 of ~3 mM. Upon removal of external K+, the IC50 for TEA was unchanged but only ~90% of the total current could be blocked. In the presence of just 10 mM internal K+ and 0 external K+, TEA potency was unchanged, but block saturated with just 50% of the total current blocked. Inclusion of intracellular TEA to reduce K+ occupancy of the pore produced effects on internal TEA sensitivity similar to that of lowering of internal K+. In the presence of 100 mM internal K+ plus 20 mM internal TEA, external TEA potency was unchanged but maximal possible block was reduced from 90% to ~50%. Under all conditions, the reduction in TEA efficacy due to lowering K+ away from the cytoplasm was reversed by external K+ in a concentration-dependent manner, with an IC50 of ~10 mM. These results suggest that in a K+-conducting channel, external TEA potency is modulated by external K+ at physiological [K+]. Furthermore, these data suggest that the apparent affinity of the external blocking K+ site for K+, in Kv2.1 channels occupied by substituting [K+] is ~8-10 mM. Supported by NSF and AHA.

562 - Pos
POTASSIUM-DEPENDENT POTENTIATION OF K+ CHANNEL CURRENTS.
Michael J. Korn, Stephen J. Korn, University of Connecticut, Physiology and Neurobiology, 3107 Horsebarn Hill Rd, Storrs, CT 06269
The structural components that influence the interaction of K+ with sites in the potassium channel pore are still poorly understood. In most voltage-gated K+ channels, elevation of external [K+] potentiates outward current through the channel. We are using this potentiation to examine how structural components in the external vestibule of the channel influence the interaction of external K+ with the channel. The Kv2.1 K+ channel has lysine residues at positions 356 and 382 (equivalent to Shaker 425 and 451). Potentiation in Kv2.1 required [K+] in excess of 100 µM and potentiation peaked at 10 mM K+. Upon mutation of residues 356 and 382 to glycine and valine, respectively, currents were potentiated at [K+] less than 100 µM and potentiation peaked at 300 µM. The Kv1.5 channel contains a histidine at the position equivalent to K356 in Kv2.1. At pH 7.3 - 8.0, currents were potentiated by [K+] as low as 10 µM and potentiation peaked between 100 µM and 1 mM. At pH 6.6, current potentiation required [K+] in excess of 30 µM, and potentiation peaked at 3 mM. These data suggest that positively charged residues near the outer mouth of the external vestibule influence the [K+] dependence of functionally-relevant K+ binding sites in the pore. Supported by NSF and AHA.

563 - Pos
EXTERNAL Na+ AND K+ COMPETITION FOR A SITE OF THE PORRE OF SHAKER K CHANNELS.
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It is known that the K+ conductances (GK) collapses when the channels are closed in 0 K+ Na+ solutions, and that K+ protects with millimolar affinity. Also, preliminary results had suggested that not only the absence of K+ but also the presence of Na+ was required for GK to be stable (Gómez-Lagunas, F. 1997. J. Physiol. 499:3-15). Here it is reported that with NMG-containing, 0 Na+ and 0 K+ internal solutions: (1) 90% of the channels collapse with the delivery of 20 pulses in Na+ or NMG, in contrast only 19% collapse in zero Na+ (either in NMG/NMG) or in chloride, [NaMg](2). As the external [Na+] increases the collapse of Gk increases, following a Hill equation with n = 1.04 and Ed = 4.7 mV. The effect is not voltage-dependant. (3) External K+ competes with the binding of Na+. Then in 0 Na+, K+ binds with a KD of only 0.160 mM. After 20 pulses in NMG, most of the channels (> 80%) are not collapsed. Probably they are slow-inactivated. And if another 20 pulses are applied the amount of collapse does not increase; however if the next 20 pulses are applied in Na+ they collapse.
STUDYING SEQUENCE-FUNCTION RELATIONSHIPS IN THE K+ CHANNEL PERMEATION PATHWAY.

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We have determined the crystal structure of a potassium channel (KCa) from Streptomyces lividans to explore sequence-function relationships in K channels. A combination of dynamic programming and profiling techniques have been applied to construct a comprehensive alignment of the putative permeation pathways of 179 K-selective channels, including representatives from all major classes. Based on homology with the KCa channel, we have assigned segments of each sequence to specific structural domains.

The most variable segment is the turret region, which determines toxin affinity/resistance. The most conserved region consists of the selectivity filter and pore helix, suggesting that these structural motifs may be preserved throughout the entire K channel family. We have postulated that the functionally critical residues responsible for ion selectivity and conductance may lie along this highly conserved segment. To explore this further we have constructed two sets of sequence profiles for this region, one from 8 weakly-k-selective channels, and one from 94 highly-k-selective channels. Profile comparison has yielded a list of residues in low-selectivity channels that are uniquely different from residues at corresponding positions in high-selectivity channels. Structural analysis of KCa suggests that two of these residues are positioned to interact with the tyrosine in the K channel GYG signature sequence, and therefore may be critical determinants of selectivity in K channels.

564 - Pos

SIMULATION OF THE KCa CHANNEL, CONSIDERING THE POSSIBLE ROLE OF H+ TRANSFER.

Michael E. Green, City College of CUNY, 138th St. & Convent Ave., New York, NY 10031

The KCa bacterial K+ channel gates in response to a pH gradient. Presumably H+ transfer among the acidic and basic residues present in the channel protein is important in understanding the gating mechanism. Monte Carlo simulations of the channel, with explicit water, but no lipid outside the channel (no membrane) have been carried out. Appropriate charge states have been considered, local potentials near the groups that can be charged are used to determine which charge states are appropriate. An amino acid is assumed to be a candidate to have a non-standard charge state when, if an acid, [potential / 60 mV + δpK_a] > 0, or, if a base, [potential / 60 mV + δpK_b - pK_b] < 0 (using the pH of the conjugate acid form). The potential is then recalculated changing the amino acid for which the quantity in brackets is largest in absolute value. Potential distributions in the closed state, and the distribution of immobilized water, will be emphasized in discussion of the output. This information may be of importance in understanding gating as well; see Lu, Yin, and Green, (1999) Ferelectro, 220, 249-271; Yin and Green (1998), J. Phys. Chem. A 102, 7181-7190.

565 - Pos

IONIC SELECTIVITY IN K CHANNELS.

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Crystallography of the KCa channel (Doyle et al., 1998, Science 280:69) has shown that the pore holds about two cations in the region of the putative 'selectivity filter', which is lined by carbonyl oxygens of the peptide backbone. Four negatively charged sidechains belonging to K+ are embedded into the protein in positions facing the nitrogen of the pore lining peptide strands. The electrostatic attraction of cations by this configuration cannot generate specificity per se because the effective diameter of the structural 'anion' is very large. However, cations attracted by this nonspecific electrostatic force have to partition into the very narrow selectivity filter, where selective effects due to volume exclusion are very strong. The resulting selective effects can account for observed selectivities for K+, Rb+, NMe4+, selectivity against Na+ can be explained if this ion is assumed to have an effective diameter that involves incomplete dehydration. This selectivity by excluded volume effects is sensitive to ionic composition within the filter volume: the presence of a large diameter species increases repulsive effects, so a further increase of the fraction of large diameter ions becomes energetically even less favorable. Species of large diameter, like hydrated Na+, can displace K+ from the filter only when K+ concentrations in the baths are very small, thereby producing an anomalous mole fraction effect.

566 - Pos

SIMULATION OF THE KCa CHANNEL, CONSIDERING THE POSSIBLE ROLE OF H+ TRANSFER.

Michael E. Green, City College of CUNY, 138th St. & Convent Ave., New York, NY 10031

The KCa bacterial K+ channel gates in response to a pH gradient. Presumably H+ transfer among the acidic and basic residues present in the channel protein is important in understanding the gating mechanism. Monte Carlo simulations of the channel, with explicit water, but no lipid outside the channel (no membrane) have been carried out. Appropriate charge states have been considered, local potentials near the groups that can be charged are used to determine which charge states are appropriate. An amino acid is assumed to be a candidate to have a non-standard charge state when, if an acid, [potential / 60 mV + δpK_a] > 0, or, if a base, [potential / 60 mV + δpK_b - pK_b] < 0 (using the pH of the conjugate acid form). The potential is then recalculated changing the amino acid for which the quantity in brackets is largest in absolute value. Potential distributions in the closed state, and the distribution of immobilized water, will be emphasized in discussion of the output. This information may be of importance in understanding gating as well; see Lu, Yin, and Green, (1999) Ferelectro, 220, 249-271; Yin and Green (1998), J. Phys. Chem. A 102, 7181-7190.

567 - Pos

BLOCKADE BY EXTERNAL NICKEL OF HUMAN KV1.5 CHANNELS STABLY EXPRESSED IN CHO CELLS.

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Divalent cations are commonly used to block T- and L-type calcium channels and Na+/Ca2+ exchange from cardiac myocytes in studies focusing on potassium channels. It has been shown recently that external divalent cations could also interact with both, the native rapid component of the cardiac delayed rectifier current and HERG expressed in Xenopus laevis oocytes. We investigated the effects of Ni2+ on the human clone of KV1.5 channels expressed in a stable CHO cell line using a whole cell voltage-clamp method. The amplitude of the current was assessed during depolarizing steps to +40 mV from a holding potential of ~70 mV. Ni2+ dose-dependently blocked KV1.5 with a half-maximum inhibition (IC50) of 570 mV and in a voltage-independent manner. Activation voltage dependency was unaffected by 1 mM Ni2+ and the steady-state inactivation curve was not shifted after Ni2+ application. The present results suggest that KV1.5 channels are non-selectively blocked by Ni2+ from the external side. Since KV1.5 is known to underlie, at least in part, native potassium currents in cardiac and vascular myocytes, divalent cations and in particular Ni2+ might not be indicated to block Ca2+ channels in experiments studying these potassium currents.

568 - Pos

PROTONATION OF A HISTIDINE IN KALIOTOXIN (KTX) CAUSES pH-DEPENDENT KTX BLOCK.

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In order to investigate the pH-dependent KTX block of Kv1.1 we used the patch-clamp technique. Changing pH from 7.4 to 6.2 improved the KTX affinity to Kv1.1 by a factor of about 3.3. A similar, 2.4-fold increase in KTX block by lowering pH, was observed in the H404T mutant Kv1.3 channel. This enhancement in affinity was specific for KTX since CTX, NTX and KTX did not show this behaviour. KTX possesses a unique histidine at position 34 not present in CTX, NTX and KTX. To find out whether protonation of H44 is responsible for the pH-dependent KTX block we made Kv1.1 mutants H34K, R31A/H34K and H34E which exhibited a pH-dependent affinity to H404T mutant Kv1.3 channels. To determine which amino acids in the channel protein interact with H44 of KTX we tested some mutants of the Kv1.1 and the H404T/Kv1.3 channels. E33S in Kv1.1 seems to be one of the interacting residues since the E33S/H404T mutant Kv1.3 channels exhibited a reduced pH-dependent block of KTX and the pH-dependent block of Kv1.1 by a factor of about 3.4. Supported by grants from the BMBF (GZK Ulm, Project B1) and the DFG (Gr 8484-2).

569 - Pos

RHERETOPODAXIN AND DIVALENT CATION EFFECTS ON NATIVE A-CURRENT IN MURINE COLONIC MYOCYTES.

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We previously described a rapidly activating/inactivating delayed rectifier K+ current in murine colonic myocytes. Quantitative PCR amplification identified transcripts of the A-type K+ channel subunit Kv1.4, 1.2, and 1.3. Quantitative PCR has shown that Kv4.1 accounts for approximately 80% of the Kv4 transcripts. There are no specific inhibitors of Kv4.1 presently. Therefore, we examined the effects of the Kv4.2,Kv4.3 inhibitor hetretoxacin (HPTXII) as well as divalent cations (Ca2+, Zn2+, and Co2+) on native A-currents. HPTXII (100nM) increased peak current 10% without affecting sustained current. HPTXIII decreased macroscopic inactivation. The HPTXIII sensitive time constant of inactivation was 32ms, which is similar to that of the A-P sensitive current (36mA). Although HPTXIII did not change the V1/2 of activation and inactivation, half recovery time was increased. Ca2+ showed a dose-dependent decrease in peak current (EC50=535nM). Co2+ slowed inactivation and shifted the V1/2 of activation and inactivation to the right 16mV and 15mV, respectively. Surprisingly, recovery from inactivation was faster in the presence of Co2+. Zn2+ also showed dose-dependent decreases in peak current without changing sustained current (EC50=270nM). Other effects of Zn2+ were similar to Cd2+. Ca2+ was without effect up to 500µM. The pharmacological nature of native A-current matched well with that predicted by quantitative PCR. (Supported by NIDDK 41315).

K CHANNELS I

SUNDAY
578 - Pos

A PUTATIVE SALT BRIDGE BETWEEN GLUT AND LYS24 MAY EXPLAIN THE DIFFERENT BINDING KINETICS OF PI2 AND PI3 SCorpion TOXINS TO KV1.3
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The peptide toxins PI2 and PI3 purified from the whole venom of the scorpion Pandinus imperator differ only in a single amino acid residue, yet the binding affinity of PI2 for KV1.3 is 11 times greater than that of PI3 (dissociation constants: 44 pm and 500 pm respectively). The difference in the equilibrium dissociation constants can be attributed to the lower association rate of PI3. Based on the kinetics of current block and current recovery the K4 values for PI2 and PI3 are 2.87x10^11 (M^-1) and 1.28x10^10 (M^-1), respectively. However, the "off" rates of these blockers are in the same range (PI2: 0.0083 s^-1 and PI3: 0.0125 s^-1). PI3 has a glutamic acid in position 7 as opposed to a proline in PI2. A likely explanation for the difference in the "on" rates is based on NMR spectroscopic data: this Glu7 residue in PI3 forms a salt bridge with the positively charged Lys24. Once PI3 is near the channel protein, this salt bridge must be broken in order to allow Lys24 to enter the channel pore.

577 - Pos

TITYUSTOXIN BLOCK OF NATIVE AND CLONED SQUID POTASSIUM CHANNELS IS PH-DEPENDENT
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Molecular identification of Kv1 α-subunits expressed in squid giant fiber lobe (GFL) neurons and giant axons allows functional properties of the native and cloned channels to be compared. Tityustoxin-Kx (TtxK) is structurally related to these toxins and blocks K current in GFL neurons in a pH-dependent manner with an apparent pKa of 4.6-6.5 at 25° C. Our results suggest that protonation of H351 may render channels insensitive to TtxK. When expressed in oocytes, SxKv1A is blocked by TtxK with a similar pH-dependence. Mutating H351 to glycine does not greatly change the affinity of SxKv1A for TtxK (Kd is 10 nM) but essentially eliminates the pH-dependence described above. Substituting lysine for H351 results in nearly complete loss of toxin sensitivity. A similar effect was observed after treating oocytes expressing SxKv1A with 1 mM dithyl pyrocarbonate (DEPC), a modifier of histidine residues. These results are consistent with H351 mediating the pH-dependent TtxK block of SxKv1A channels and reinforce the identity of SxKv1A as the delayed rectifier K channel in GFL neurons and giant axons. Recombinant TtxK was a gift of M.P. Blumenthal, Univ. MD.

576 - Pos

IONIC STRUCTURE AFFECTS DIFFERENTIALLY THE BINDING OF κ-CONOTOXIN-PVIIA TO OPEN AND CLOSED SHAKER K-CHANNELS.
David Navas1, Consuelo Hernandez2, Esperanza Garcia2, Universidad Nacional Autonoma de Mexico, Circuito Exterior s/n, Ciudad Universitaria, Mexico DF 04510 Mexico, Universidad de Colorado, 1-κ-Conotoxin-pvii (κ-pvii) is a 27 residue peptide component of the venom of the hunting marine snail. This toxin blocks the pore of K-channels in a voltage dependent fashion with a biomolecular stoichiometry (Garcia et al, 1999. J. Gen. Physiol. 114:141). At neutral pH, this toxin has a +4 excess of charges. To understand the role that these charge excesses may play in the binding of κ-pvii to its receptor, we studied the effect of the ionic strength of its dissociation constant to Shaker (K)). 50-150 pm of CDNA encoding Shaker-IR under control of the CMV promoter was injected into the nucleus of Xenopus oocytes. Under TEVC, κ-pvii inhibition appears as time-dependent exponential relaxation from the value of inhibition at resting (closed channel) in a lower value at voltages positive to zero (open channels). We modified the extracellular ionic strength by replacing NaCl from ND96 with mannitol to preserve osmolarity. When ionic strength was varied from 60 to 160 mM we found that the open channel Kc measured at positive voltages, increased 5-fold, meanwhile, for closed channel Kc were measured at resting, increased 16-fold. A possible explanation for this differential effect is that channel openings connect intra- and extracellular environments, thus, buffering the ionic strength imposed experimentally.

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575 - Pos

FLUORESCENCE SCANNING OF THE EXTERNAL FACE OF THE SHAKER K+ CHANNEL
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K+ channels are comprised of four similar or identical subunits, each containing six transmembrane segments in a Shaker K+ channel. Each subunit was replaced with tryptophan and expressed in Xenopus oocytes. The equilibrium voltage activation curves and the activation and deactivation kinetics were compared with wild type to assess the type of tryptophan substitution at each position. Helical periodicity in the tryptophan tolerance was found through the entire S1 sequence. But only the intracellular 2/3 of S3 adheres to an o-helical pattern. Tryptophan tolerant residues are clustered approximately on half of each helical surface, indicating these residues are exposed to membrane lipid. With the previous study of S2 (Monks et al. 1999. J. Gen. Physiol. 113:415-423), the results suggest that S1, S2, and S3 are located on the periphery of the channel.

574 - Pos

HELICAL STRUCTURE AND PACKING ORIENTATION OF THE FOUR TRANSMEMBRANE SEGMENTS IN THE VOLTAGES-SENSING DOMAIN OF A K+ CHANNEL
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Voltage-gated K+ channels are tetramers with each subunit containing six (S1-6) putative membrane-spanning segments. The S5 through S6 region forms the central pore domain. A growing body of evidence suggests that S1-S4 comprise a domain-like voltage-sensing structure. While the topology of this region is reasonably well defined, the secondary and tertiary structures of these transmembrane segments are not. To explore the secondary structure of the voltage-sensing domain we used alanine-scanning mutagenesis through a region encompassing the first four transmembrane segments in the p47 voltage-gated K+ channel. 127 mutant channels were expressed in Xenopus oocytes and the mutation-induced perturbation in gating free energy (ΔDGp) determined from the voltage-activation relations. Residues with large ΔDGp values tend to cluster into four groups separated by stretches of relatively small ΔDGp values and the distribution of perturbation energy correlates nicely with the hydrophobicity profile. Analysis of the periodicity of ΔDGp for each of the four transmembrane segments using Fourier transform analysis suggests that S1-S3 are more o-helices. The segregation between protein-protein and protein-solvent interfaces for S1 and S2 is clearer than for S3 and S4, suggesting that the later two helices make more extensive protein contacts, possibly interfacing directly with the shell of the pore domain.

K+ channels are comprised of four similar or identical subunits, each containing six transmembrane segments, S1-6, and a P-region between S5 and S6. The S5-P-S6 domain lines the narrowest, most selective part of the pore, and contains elements of both an internal activation gate and an external slow inactivation gate, both of which must be open in order for the channel to conduct ions. Channels with only a pore domain have gates that open and close, but are intrinsically voltage-independent, leading to the idea that the S1-S4 voltage-sensing domains of the channel's four subunits wrap around and regulate the conformation of a pore domain core. While S4 has been shown to undergo voltage-driven rearrangements that move charged amino acids across the membrane, the behavior of S1-S3 is not clear. S2 and S3 contain acidic residues that appear to pair electrostatically with basic residues in S4. Neutralization of one acidic residue drastically reduces total gating charge, meaning that it may move across the membrane and/or influence either the movement of S4 or the shape of the electric field. To better understand the protein structure and motions in S1-S3, we carried out a fluorometric scanning of the entire externally exposed section of the protein in this region using voltage clamp fluorescence.

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576 - Pos
MODULATION OF KV1.1 K+ CHANNELS BY SIGMA RECEPTORS EXPRESSED IN XENOPUS OOCYTES.

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Sigma receptors modulate K+ current (IK) in various preparations, but because the sigma receptor is a receptor with little affinity to other ion channels, including K+ channels, the
modifications of current remain unexplored. In order to ascertain the molecular components required for ion channel modulation we attempted to reconstitute sigma receptors by heterologous expression in Xenopus oocytes. Sigma receptor expression was verified by constructing a fusion protein with the fluorescent label GFP. Oocytes injected with mRNA encoding this fusion protein became fluorescent. Confocal microscopy indicated that the fluorescence emanated from the plasma membrane. Sigma receptors have been shown to modulate transient IK in nerve terminals. Therefore, we selected the rat brain K+ channel Kv1.4, which has transient kinetics and is located in nerve terminals. When Kv1.4 was expressed in the absence of heterologous sigma receptor, IK was modulated by the sigma receptor ligands SKF10067 and DYT in 25% of tested cells. When sigma receptor antagonist olanzapine was cojected, modulation of Kv1.4 was eliminated. When both Kv1.4 and rat sigma receptor were expressed, sigma receptor ligands modulated IK in all tested assays. Thus, endogenous Xenopus sigma receptors modulate expressed IK in some instances, but full reconstitution of responses can be achieved by coexpressing the two proteins Kv1.4 and sigma receptor.

577 - Pos
CHANGES IN THE AFFINITY OF THE LOCK-IN SITE FOR Ba2+ IN THE CLOSED AND Ba2+ BLOCKED POTASSIUM CHANNELS IN SQUID AXONS.

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The potassium channel has three established sites for binding of K+ or Ca2+. To investigate the properties of these sites we have studied the Ba2+ block of the squid potassium channel and the shift of the Ba2+ lock-in site. Sigma receptors activate Ca2+ channel blocking. Therefore, 1) Occupasion of the lock-in site by Ba2+ slows both the rates of Ba2+ block (on rate) and the recovery from block (off rate) but with distinct Kd's. The Kd of Ba2+ for the on rate is approximately 5 mM and the Kd of Ba2+ for the off rate is less than 1 mM. Thus the affinity of the lock-in site for Ba2+ is higher when the channel is Ba2+ blocked, suggesting that Ba2+ occupancy may alter the confirmation of the selectivity filter. 2) There is little voltage dependence of the closed state recovery rate in TMA or ASW between -60 mV and -90 mV holding potential range. In 2B TMA, the closed state recovery is much more voltage dependent in the same voltage range: it is substantial at -60 mV and almost nonexistent at -90 mV. Such an increase in voltage dependence of the recovery can be attributed to an increase in the affinity of the lock-in site for Ba2+ at strongly hyperpolarized potentials. 3) The dwell time of Ba2+ in the lock-in site is dramatically increased if channels are kept at hyperpolarized potentials: a wash with 10 mM Ba2+ causes a 3X slowing of recovery at -90 mV for at least 500 s even though the exposure to Ba2+ is transient. At -70 mV the Ba2+ dwell time is much shorter. This shows that at -90 mV a single Ba2+ ion may remain in a Ba2+ blocked channel for more than 500 s.

578 - Pos
PULSED LOCAL FIELD FLUORESCENCE DETECTION ON INTACT MAMMALIAN HEARTS.

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Studies of Ca2+ homeostasis and changes in membrane potential in cardiac cell often involve conditions in which the cells are far from their physiological environment. Here we present a new experimental way to study cardiac myocytes at the whole heart level. The hearts, mounted on a Langendorff perusion system, were loaded either with the Ca2+ indicator Rhod-2 AM or with the potentiometric dye Di-8AesKa.A single multimode fiber optic positionned inside a macroascope pipette was used to propagate light pulses, at a wavelength of 532 nm, produced by a nano Nd:YAG laser. Very short (900 ps) and high amplitude (400 W) pulses were used to excite locally the tissue loaded with a fluorescent indicator each 80 microseconds. The fluorescence emitted by these dyes during the lifetime relaxation was collected through the same fiber and detected with an avalanche photodiode. Data was digitized and acquired at a very high sampling rate (up to 200 as per point) and the peak emitted fluorescence after each laser pulse was software detected as is shown in the figure. Simultaneous measurements of extracellular potentials, Ca2+ transients or optically recorded action potentials in several points at the same time were performed. This allow us not only to map the distribution of membrane potential and Ca2+ in the whole heart but in define delays between action potentials and Ca2+ release during a physiological cardiac cycle. (Supported by CONICIT S-9500093 to P.B, S-95000587 A.M and NIH AR41197 to M.F.)

579 - Pos
DEVELOPMENTAL CHANGES OF INTRACELLULAR Ca2+ TRANSIENTS IN BEATING RAT HEARTS.

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Contribution of sarcoplasmic Ca2+ entry and sarcoplasmic reticular (SR) Ca2+ release to the global intracellular Ca2+ transient was studied during postnatal heart development. To this end, Ca2+ transients were measured in beating hearts from newborn rats of different ages (1-21 days) using a novel local-field epifluorescence technique. Hearts were loaded with Rhod-2(AM)-CAm. Light was applied and collected through a small diameter (200 μm) multimode optic diameter held in place by a suction pipette. Brief flashes (<1 ms) of excitation light (532 nm) were applied every 90 μs by an Nd:YAG pulsed laser. Peak Rhod-2 fluorescence was measured for each flash. Pulsed illumination provided outstanding signal-to-noise and minimized photobleaching and cell damage. Different pharmacological probes (ryanodine, nifedipine and NiCl2) were applied by perfusion. The results showed that the relative contribution of SR Ca2+ release, L-type Ca2+ current, T-type Ca2+ current and Na-cAsa exchange to the global transient changes with development. At early stages, the sarcoplasmic Ca2+ flux predominates. As at later stages, Ca2+ induced Ca2+ release from the SR predominates. Thus, developmental changes in excitation-contraction coupling (previously evaluated mainly in cultured cells) were defined, here in intact beating hearts. Supported by CONICIT S-95000493, NIH HL75782 (MF) & AHA 9903082 (KMA).

580 - Pos
ROLE OF FKBP12.6 IN CARDIAC RVADYNOPLA CE RECEPTORS (RyR2) AS STUDIED IN FKBP12.6 GENE KNOCKOUT MICE (KO).

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The ryosode receptors (RyRs) of striated muscle are hetero-oligomers with structural formulae (RyRproteroms), FKBP, where RyRproteroms for skeletal muscle RyR1 and heart RyR2 have 2/3 sequence identity. FK506 Binding Protein (FKBP12.6) is associated with RyR1, whereas FKBP12.6 is a novel isoform associated with RyR2. In vitro studies show FKBP modifies RyRchannel activity but not RyR2. That is, removal of FKBP with FK506 activates RyR1 but not RyR2, albeit controversy exists regarding RyR2 modulation. In this study, channel properties of RyR2 from KO and wild type (WT) were studied in planar lipid bilayers. Both KO and WT RyR2 channels behaved similarly with respect to 1) high specific to cytosolic Ca2+ (0.1 micromole to 10 mM); 2) activation by 5μM caffeine but not by ATP (1-2 mM); 3) similar sensitivity to block by Mg2+ or ruthenium red; 4) identical current amplitude (from -60 to +60 mV); 5) subconductance states were rare; and 6) FKBP12.6 in the bathing solution did not change the channel characteristics. These studies show that RyR2 has similar single channel properties in the absence or presence of FKBP. (NIH-HL23711 and MDA).

581 - Pos
INTERPLAY BETWEEN CALCIUM BUFFERS AND FLUXES DETERMINES THE SHAPE OF THE DECA Y OF THE CALCIUM

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In cardiac muscle the time course of decay of intracellular Ca (Ca2+) is frequently used to study the cellular processes involved in relaxation. The aim of this work was to investigate, in greater detail, the kinetics of decay of Ca2+ in voltage-clamped single ventricular myocytes. Changes in cytoplasmic free Ca2+ were monitored using the fluorescent Ca2+ indicator Fura-3. We find that the decay of the caffeine-evoked Ca2+ transient is bi-exponential: showing an initial rapid phase followed by a slower monoeonic fall of Ca2+. Using a recently described method to measure intracellular Ca2+ buffering capacity (Trafford et al, 1999, Pfluegers Arch. 437:501-506), we have also measured the changes of total Ca2+. During the caffeine evoked Ca2+ transient the rate of fall of total Ca2+ towards saturation at high Ca2+, whereas the rate of fall of free Ca2+ is accelerated at high Ca2+. The discrepancy between free and total Ca2+ could be attributed to saturation of cytoplasmic Ca2+ buffers. In contrast, the decay of the systolic Ca2+ transient (even over the same range of Ca2+ as the caffeine-evoked one) can be reproduced by a single exponential. This single exponential behaviour can be explained either if the sk Ca2+ ATPase tends towards saturation (thereby compensating for the saturation of the buffers) or release or Ca2+ from the skt continues during the phase of decay of the Ca2+ transient. In summary the properties of Ca2+ buffering can influence the kinetics of Ca2+.
582 - Pos

EFFECTS OF Ca\(^{2+}\)/CALMODULIN DEPENDENT PROTEIN KINASE II ON PACEMAKER ACTIVITY IN THE SINO-ATRIAL NODE CELLS.


L-type calcium current (I\(_{\text{Ca,L}}\)) plays an important role in sino-atrial (SA) node pacemaking, contributing both to the action potential (AP) upstroke and pacemaker depolarization. Since Ca\(^{2+}\)/calmodulin dependent protein kinase II (CaMKII) regulates I\(_{\text{Ca,L}}\), we investigated its role in modulation of excitations of single rabbit SA node cells. Immunocytochemical staining showed a localized distribution of active CaMKII close to the sarcolemmal membrane. A specific CaMKII inhibitor, KN-93 (1 μM), but not its inactive analog, KN-92, robustly depressed the rate of spontaneous excitations from 162±17 to 61±4 beat/min (34°/6, P<0.01), AP amplitude from 90.3±3.6 to 62.3±1.7 mV (P<0.05) and maximum diastolic potential from -58.4±1.7 to -54.4±3.2 mV (P<0.01). KN-93 (3 μM) completely arrested SA node cells, indicating CaMKII is critical for pacemaking. In whole cell I\(_{\text{Ca,L}}\) recordings, KN-93 decreased I\(_{\text{Ca,L}}\) from 12±2 to 6±1 pA/pF without altering the current-voltage relationship. However, KN-93 shifted steady-state inactivation midpoint of I\(_{\text{Ca,L}}\) by 11±2 mV leftward (P<0.05), resulting in decreased window I\(_{\text{Ca,L}}\). Recovery of I\(_{\text{Ca,L}}\) from inactivation studied with a two-pulse protocol was significantly slowed by KN-93. Thus, the results show, that CaMKII plays a vital role in cardiac pacemaker activity via modulating property of I\(_{\text{Ca,L}}\) inactivation.

584 - Pos

SUBCELLULAR ORGANISATION AND FUNCTION OF Ryanodine RECEPTORS IN SINGLE CELLS ISOLATED FROM THE GUINEA-PIG SINO-ATRIAL NODE.

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To investigate the location of ryanodine receptors (RyR) in sino-atrial node (SAN) cells with respect to sarcolemmal proteins, isolated SAN myocytes were prepared for immunocytochemistry (using conventional techniques) and exposed to RyR\(_2\) α-actinin and myosin antibodies and Texas-red phalloidin. RyR\(_2\) labelling (detected using FITC conjugated secondary antibodies and confocal microscopy) was found to be distributed in distinct, transverse bands which penetrated deep into the cell but had a regular periodicity of ~2 μm. RyR\(_2\) was also frequently observed close to the sarcolemma. The banding pattern did not depend on the presence of transverse tubules since these were not detected in SAN cells by surface membrane labelling with di-8-ANEPPS. A comparison of the distribution of phalloidin and RyR\(_2\) α-actinin and myosin revealed that RyR\(_2\) was organised at the Z-line. To determine the functional role of RyR\(_2\), action potentials and calcium transients were recorded simultaneously from single SAN cells; in the presence of 2 μM ryanodine, frequency of beats and the amplitude of calcium transients were suppressed. These findings show the presence and organisation of ryanodine receptors in isolated SAN cells and provide further support for the importance of SR calcium release in regulating pacemaker activity of the SAN.

585 - Pos

EFFECT OF OUABAIN AND ACTODIGIN ON ACTION POTENTIALS AND CONTRACTILITY IN SINGLE CAT CARDIAC MYOCYTES.

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Electrophysiological and isotropic actions of cardiac glycosides have been studied extensively in multicellular preparations, but less is known about their effects in single cardiac cells - including potential pharmacodynamic differences between agents.

We measured changes in contractility and action potential (AP) characteristics (video edge-detection and high-resistance microelectrode recording) in isolated cat cardiac myocytes in response to isotropic concentrations of ouabain and actodigin. Analysis of concentration-effect curves for these agents revealed that 1) IC\(_50\) for ouabain and actodigin were 0.57 μM and 2.9 μM, respectively, and 2) AP shortening occurred at ouabain concentrations in the isotropic range, which did not occur with the semi-synthetic analogue actodigin. Voltage clamp studies indicated that ouabain did not cause AP shortening by alteration in I\(_{\text{Na}}\) or I\(_{\text{Ca,L}}\). These observations suggest that ouabain and actodigin have distinct differences in their action on cardiac cell function, including possible differences in isotropic mechanism of action.

586 - Pos

OUABAIN ENHANCES CONTRACTILITY OF ISOLATED CAT VENTRICULAR MYOCYTES IN SODIUM-FREE CONDITIONS.

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The isotropic and toxic effects of cardiac steroids are thought to result from Na,K-ATPase inhibition, with elevated Na\(^+\), causing increased Ca\(^{2+}\), via Na-Ca exchange. We investigated the effects of ouabain on contractility of isolated cat ventricular myocytes in Na\(^+\)-free conditions where the exchange is inhibited. We measured cell shortening in whole-cell voltage clamped myocytes exposed to Na\(^+\)-free external and internal solutions (LICI replacement). Membrane potential was held at -70mV and the test pulse was 0mV for 10ms (0.5Hz). Ouabain enhanced contractility by 118±38% in 1μM and 658±273% in 10μM (n=6, # n=5). When Ca\(^{2+}\), overload toxicity was indicated by spontaneous aftercontractions in control, ouabain decreased the cycle length. Changing the holding potential to -40mV (to inactivate Na\(^+\) current) did not alter the isotropic effects of ouabain (10μM). Rat ventricular myocytes did not have an isotropic response to ouabain (10μM). These results demonstrate that, in the absence of Na-Ca exchange, (1) ouabain retains its isotropic and toxic effects; (2) the isotropic effect shows typical species differences, including a low sensitivity in rat; and (3) the effects of ouabain do not require Na\(^+\) current. Thus, the cellular effects of ouabain in the heart may include actions independent of Na,K-ATPase inhibition and changes in Na\(^+\).
calcium loading maintenance is contraction, cells MM second normally 37 C at Ic.

In cardiac muscle, Ca\(^2+\) release from ryanodine receptors is locally controlled by Ca\(^2+\) inflow through L-type Ca\(^2+\) channels residing within the dyadic junctions. The unitary Ca\(^2+\) channel current, therefore, plays a pivotal role in controlling the efficacy of Ca\(^2+\)-induced-Ca\(^2+\)-release (CICR). Here we report the multiple conductance native L-Type Ca\(^2+\) channels in freshly isolated rat ventricular myocytes. With 100mM Ca\(^2+\) as the charge carrier, in presence of the Ca\(^2+\) channel agonist, FPL64176 (10 \(\mu\)M), depolarizing pulses from a holding potential of -40 mV to a test potential of 0 mV activated single Ca\(^2+\) channel openings with unitary currents of 0.25 pA and 0.15 pA. Current-voltage relations indicated single channel conductance of 6.9 pS and 3.0 pS respectively. The 6.9 pS is consistent with the known L-Type Ca\(^2+\) channel conductance. The 3.0 pS might correspond to a different conductance state of L-Type Ca\(^2+\) channel, because the channel open duration was elongated by FPL, similar to that of the 6.9 pS channel. It is unlikely to be a T-type Ca\(^2+\) channel, judging also from its frequent occurrence when the holding potential was -40 mV. The multiple conductance levels of Ca\(^2+\) channel may indicate heterogeneity of CICR at local sites. Modulation of the conductance levels, if it occurs, may alter excitation-contraction coupling in cardiac muscle.
MODULATION OF β-ADRENERGIC RECEPTOR RESPONSIVENESS OF L-TYPE Ca²⁺ CHANNELS BY TYROSINE PHOSPHORYLATION IN GUINEA PIG VENTRICULAR MYOCYTES

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Basal tyrosine kinase activity has been found to inhibit β-adrenergic regulation of cardiac ion channels. To investigate the mechanism by which tyrosine kinase activity exerts such actions, we studied the effects that the phosphorytrosine phosphatase inhibitors sodium vanadate, pervanadate, and SNP had on the CAMP-dependent regulation of the L-type Ca⁺ current in guinea pig ventricular myocytes (GPVM). None of the tyrosine phosphatase inhibitors employed altered the basal Ca⁺⁺ current, each were found to inhibit the Ca⁺⁺ current activated in the presence of the β-adrenergic receptor agonist isoprenaline. Conversely, no effect on CAMP-dependent responses elicited by activation of H₂ receptors by histamine or adenylyl cyclase with forskolin were observed with any phosphorytrosine phosphatase inhibitor. Direct measurements of β-adrenergic receptor responsiveness showed that attenuation of isoprenaline-stimulated CAMP production was also observed in the presence of sodium vanadate. Furthermore, the β-adrenergic receptor was demonstrated to be present in an immunoprecipitate of phosphorytrosine containing proteins from GPVM membranes by immunoblotting with β-adrenergic receptor antibodies. These results are consistent with the idea that basal tyrosine kinase activity exerts its inhibitory effect by phosphorylating tyrosine residues on the β-adrenergic receptor.

AMIODARONE BLOCKS I₉-A, AND I₉-B, BUT NOT K CURRENTS IN HUMAN ATRIAL MYOCYTES

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Studies suggest that amiodarone (A) exhibits antitachyarythmic activity through class I (block of I₉-A) and class III (AP prolongation) mechanisms. A is used for the treatment of tachyarythmias but effects on ion currents in human myocytes have not been defined. Myocytes were isolated from specimens of right atrial appendage from patients (age 50-79 yr) undergoing cardiac surgery. Specimens were transported in high-K solution, cut into pieces (1 mm), digested with collagenase and protease for 40 and stored in high K. Currents were measured using whole-cell voltage clamp. I₉-A was measured at 25 °C from Vh = -90 mV to TEA, cesium, and 10 mM Na⁺. K currents were measured at 37 °C in a Na⁺ and Ca⁺⁺-free bath solution Vh = -70 mV. I₉-B was measured at 37 °C in Tyrode (1 mM Ca⁺⁺) from Vh = -50 mV. A (10 mM) was added to bath. I₉-A was reduced 80% by A (21.0 ± 2.3 pA/pF Control, n=10 vs. -4.2 ± 0.2 pA/pF A, n=5, p<0.05). I₉-B was reduced 50% by A. I₉-A and I₉-B were unaffected by A. I₉-CA was not detected. Our results show that the acute effects of A on human atrial myocytes are due to decreases in I₉-A and I₉-B.

COMMERCIAL SAXITOXIN REDUCES L-TYPE CALCIUM CURRENT IN ADULT MOUSE VENTRICULAR MYOCYTES

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In studies of I₉-L, saxitoxin (STX) and tetradotoxin (TTX) are frequently used to selectively block sodium channels. In this study we provide evidence that commercial STX also inhibits L-type Ca²⁺ currents (Iₒ₉) in adult mouse ventricular myocytes. We measured inhibition of sodium currents (Iₒ₉) whole-cell voltage clamp technique: -80 to 0 mV, of Iₒ₉ (40 to 10 mV), and [Ca²⁺]²⁺ transients (fuo-3) in single mouse ventricular myocytes. STX or TTX was sharply applied before the test voltage pulse using a rapid solution switcher device. STX (10 μM, Calbiochem) and TTX (60 μM, Sigma) equally and completely blocked Iₒ₉. However, STX at 10 μM also reduced Iₒ₉ by 39% (p<0.0001; n=14). TTX at 40 μM had no effect on Iₒ₉. STX (10 μM) reduced the amplitude of the [Ca²⁺]²⁺ transients by 36% (p<0.0001; n=10). In contrast, TTX (60 μM) only reduced the amplitude of the [Ca²⁺]²⁺ by 5% (p=0.003; n=5). The Iₒ₉ was not altered by acetic acid (1 mM), the solvent which is used to prepare the commercial STX. STX (10 μM) obtained from Sigma Company also showed a similar inhibitory effect on Iₒ₉ (33%, p<0.0001; n=4). These results suggest that STX or a contaminant in commercially prepared STX solution partially blocks the L-type calcium channel in mouse ventricular myocytes.

ISOPRETERENOL POTENTIATES HALOTHANE DEPRESSION OF CARDIAC L-TYPE Ca²⁺ CHANNEL CURRENTS IN GUINEA PIG MYOCYTES

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We have recently shown that depression of ventricular L-type Ca⁺⁺ current (Iₒ₉) by isoflurane, an inhalation anesthetic, is enhanced by acetylcholine (AcH) but not by isoproterenol (ISO), and this effect was attenuated by atropine. The current study examines how ISO and AcH modulate the effects of the architectural volatile anesthetic halothane, on the atrial and ventricular Iₒ₉. Using the whole-cell patch clamp configuration on isolated guinea pig cardiomyocytes, Iₒ₉ was elicited during test pulses from a holding potential of -50 mV to +60 mV (10 mV increments). Halothane at clinically relevant concentrations of 0.28 and 0.50 mM significantly (p<0.05) attenuated atrial Iₒ₉, by 26.9±3.1 (n=10) and 42.5±16.8% (n=8), respectively, and significantly depressed ventricular Iₒ₉, by 28.2±3.2% (n=5) and 44.0±7.6% (n=5), respectively. Pretreatment of ventricular cells with AcH did not significantly alter halothane depression of Iₒ₉. In contrast, in the presence of ISO halothane at 0.28 and 0.50 mM, depressed ventricular Iₒ₉, by 34.9±1.8% (n=6) and 56.2±25.2% (n=5), respectively. This depression by halothane was significantly greater than those observed in the absence of ISO. In atrial cells, the effect of halothane at 0.28 mM was also significantly augmented in the presence of ISO, inhibiting Iₒ₉ by 51.0±12% (n=3). The results show that pretreatment of cells with ISO enhances the response of the Cav channels to halothane. In addition, the effect of ISO on the inhibition by halothane was greater in the atrial Iₒ₉ (1). (Amehesiology 1999; 1:1A413)
600 - Pos  
**EXPRESSION OF CARDIAC L-TYPE CALCIUM CHANNEL SUBUNITS IN THE EARLY EMBRYONIC CHICK HEART TUBE.**
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The cardiac L-type calcium channel is a multimeric protein composed of a pore-forming α1 subunit and regulatory α2δ- and β-subunits. The β-subunit, when coexpressed with the α1α subunit results in expression of a channel that is both voltage- and Ca2+-dependent. To determine if Ca2+ currents exist in the early heart tube, we have utilized a reverse genetics approach to study the expression of the α1α, α1β, and α1γ subunits in embryonic heart tissue extracts. We have observed a significant increase in the level of expression of the α1α subunit in the heart tube extracts compared to control extracts. This suggests that Ca2+ currents may exist in the early heart tube and could be of importance in the development of the cardiac pacemaker.
CHARACTERIZATION OF MAITOTOXIN-ACTIVATED NONSELECTIVE CATION CHANNELS IN RAT VENTRICULAR MYOCYTES.

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Maitotoxin (MTX) is a water-soluble membrane-impermeant polyisocyanic ether isolated from the marine dinoflagellate Gambierdiscus toxicus, and is one of the most potent marine toxins known. MTX has been shown to activate nonselective cation channels in cardiac, neuronal and pancreatic cells. We characterized the effects of MTX on action potential configuration, and whole-cell and single-channel currents in rat ventricular myocytes. Control myocytes (EGTA) 0.1 mM) had a resting membrane potential of -76 mV and an action potential duration at 50% (APD50) and 90% (APD90) repolarization of 134 ms and 664±11 ms (n=4), respectively. The effects of MTX (10-30 nM) could be divided into 3 distinct stages. There was an initial depolarizing effect of MTX by 3.5±1.3 mV (P < 0.05, n=4) and a significant prolongation of APD90 to 144±3 ms (P < 0.01) but not APD50 (126±3 ms) (stage 1). This was followed by a further membrane depolarization by 9.6±3.8 mV (P < 0.01), prolongation of APD90 to 241±6 ms (P < 0.01) and the appearance of triggered activity and membrane instability (stage 2). With longer exposures, RMP was severely depolarized to -22.9±2.1 mV (P < 0.01) and cells became excitable (stage 3). Although the arrhythmogenic effects were prevented when [Ca2+] was buffered with 5 mM EGTA, MTX was able to depolarize RMP by 3.5±1.3 mV (P < 0.05) and significantly prolonged APD90 (154±4 ms, P < 0.05) but not APD 50 (2±1 ms) (n=3). MTX (10-100 nM) activated a voltage-independent cation conductance which reversed at 0.32±0.77 mV (n=3) under biionic recording conditions ([Na+]j/([Cs+]j) 140/140 mV) and was impermeable to N-methylglucamine or chloride. Cell-attached single-channel analyses of MTX-activated channels revealed a slope conductance of 29±2 pS with Na+ as the charge carrier, and mean open and closed times of 16.7±3.2 ms and 0.44±0.01 ms (n=3), respectively. These results demonstrate that MTX activates nonselective cation channels in rat cardiac myocytes. Opening of these channels can enhance Na+ and Ca2+ influx, depolarize the resting membrane potential, and lead to the onset of arrhythmias and calcium overload.

SUNDAY
CARDIAC ELECTROPHYSIOLOGY: Ip, ICa,S, ICa,L-CHANNEL REGULATION

606 - Pos
DIRECT AUTOCORRELATION INHIBITION AND A-CAMP-DEPENDENT POTENTIATION OF SINGLE-L Type CATION CHANNELS IN BOVINE MYOCYTOPLASMIC CELLS

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L-channels inhibition by released neurotransmitters (autocrine modulation) is important for controlling neurosecretion in chromaffin cells. Using cell-attached recordings we focused on the negative coupling between single L-channels and secreted products (ATP and opioids) in order to assess whether this endogenous inhibition is membrane-delimited and interferes with the Ca2+-mediated Ca2+-regulation reported for other L-channels.

Our data show that, like N and P/Q-channels, the autocrine inhibition of the L-type is due to a direct action of PTX-sensitive G-protein, activated by opioids and peripheric autoreceptors. However, unlike N and P/Q-types, modulation of L-channels is markedly voltage-independent. Inhibited single L-channels have reduced Popen (0.2 vs 0.4 at +10mV), which mainly results from increased shut times. Mean open time, first latency and unitary conductance are unaffected.

In addition, we found that the autocrine direct inhibition is removed and L-channel activity strongly potentiated when PKA is stimulated by increased Ca2+ levels (8-CPT-CaMP or forskolin), as well as by H7 or H89. These drugs activate cAMP independently and may contribute to a wide-range control of neurosecretion, from marked inhibition to maximal potentiation.

607 - Pos
INACTIVATION OF CALCIUM CURRENT BY PERMEATING CALCIUM IONS IN SIMULATED CHANNEL CLUSTERS.

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Calcium currents through L-type calcium channels (DHPRs) display prominent calcium-induced inactivation under whole-cell conditions, while almost no inactivation is observed in single channels even when Ca2+ is the permeating ion. We used a channel simulation program (SCESim, iii21 Ltd., Slovakia) to investigate how Ca2+ cross-signaling between neighboring calcium channels might contribute to calcium current-dependent inactivation in calcium channel clusters. Calcium channels were described by a modified model of Iadh et al. (Biophys J 74;1149, 1998). It was assumed that formation and dissipation of Ca2+ gradients on channel opening and closure is instantaneous (Naraghi and Neher, J. New Rev 15;1997). Calcium channel clusters contain 1–25 channels positioned on a rectangular grid (spacing of 10–100 nm). Similar to experimental results, the dependence of inactivation on voltage was bell-shaped, and the rate of reactivation increased with hyperpolarization. Inactivation increased log-linearly with the number of channels per cluster and decreased hyperbolically with grid spacing. For a model with 25 channels/cluster with 75 nm spacing, the simulated calcium currents were in good agreement with calcium currents recorded in rat ventricular myocytes in the presence of apoCa2+. Our results suggest that Ca2+ current-dependent inactivation of DHPRs is a collective rather than individual channel phenomenon.

SUNDAY
PRECONDITIONING THE CAMP GENERATING SYSTEM VIA β2AR ENHANCES THE L-TYPE Ca2+ CURRENT IN CANINE VENTRICULAR MYOCYTES

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Earlier studies demonstrated no increase in L-type Ca2+ current (ICa,L) during β2 adrenergic (β2AR) stimulation in the presence of β2,AR inhibition with CGP20712A. We investigated whether β2,AR stimulation with zinterol (0.05μM) or epinephrine (0.01μM) modulates other effects on ICa,L acting via a-CAMP generating system. β2,AR was stimulated with norepinephrine (NE; 0.1μM) plus prazosin, muncaricin receptors (MR) with carbachol, adynylate cyclase (AC) with forskolin, and phosphodiesterase (PD) was inhibited with IBMX and theophyllin. Maximal peak ICa,L was determined using whole cell patch clamp at 37°C. β2,AR stimulation with CGP20712A abolished the effect of NE and isoproteenol (ISO). β2AR inhibition with ICI 118,551 reduced the Emax of NE and ISO by 60%. β2,AR stimulation enhanced the effect of a subsequent stimulation of β2,AR, AC, or inhibition of PD, but not of a preceding stimulation of β2,AR. Activation of MR reduced the effect of β2,AR on ICa,L by only 10%, but abolished the augmentation by β2,AR. When PDE was inhibited, β2,AR stimulation increased ICa,L. Inhibition of spontaneous activity β2,AR with CGP20712A also abolished the enhancing effect of β2,AR stimulation on ICa,L under these conditions. These observations suggest that β2,AR activation preconditions the CAMP generating system for forskolin and β2,AR stimulation, and activation of MR opposes this effect.

608 - Pos
REGULATION OF Ca2+ CHANNEL EXPRESSION IN SMOOTH MUSCLE BY CHRONICALLY INCREASED [Ca2+]i INVOLVES IP2, IP3 AND CALCIUM-SENSITIVE PROTEIN KINASES

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Culture of intestinal smooth muscle for 5 days in the presence of the growth stimulator fetal calf serum (FCS) reduces force and spontaneous activity. This correlates with decreased f-lye Ca2+ channel density measured in whole-cell patch clamp experiments (Gomez & Swerd 1997, J. Am. Physiol. 273: C171-20). The changes in channel expression are significant already after one day in culture. Culture with FCS reduces the total number of [HPN200-110 binding sites (from 520 fmol/mg to 200 fmol/mg) without changing the binding affinity (0.11 nM vs. 0.14 nM in controls), confirming reduced density of dihydropyridine receptors. The effects of FCS are reversed by the Ca2+ channel blocker verapamil and mimicked by the Ca2+ ionophore ionomycin, indicating an association with long-term changes in [Ca2+]i. Inclusion of 10 μM cyclopiazonic A in culture media prevents downregulation of Ca2+ channels by FCS, as studied in conventional whole-cell experiments, using Ba2+ as a charge carrier and including EGTA (2 mM) in the pipette solution. This suggests a role of Ca2+-modulin-dependent protein phosphatase type 2B (PP2B, calcineurin) in the altered channel expression. In addition to altering Ca2+ channel expression, culture with FCS reduces the capacity of the contractile system for force production, an effect that seems mediated by increased [Ca2+]i, during culture and may be associated with cellular damage as shown in vascular muscle (Lindqvist et al. 1999, Am. J. Physiol. 277: C64-73). Sustained increase in [Ca2+]i is known to cause activation of calcineurin and subsequent apoptosis in susceptible cells. In accordance with this, high molecular weight and the typical smallcysteine length DNA fragments (DNA laddering) were increased with extended time in culture with FCS.

103A
611 - Bosc

OVEREXPRESSION OF FRS2/ENG IN NG108-15 CELLS INCREASES LOW VOLTAGE-ACTIVATED CA CHANNEL ACTIVITY
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Frs2q (Angaut-Petit et al., 1993) is a recently identified member of the EF hand superfamily of calcium binding proteins. In D. melanogaster it was found to enhance frequency-dependent neurotransmitter release when overexpressed at neuromuscular junctions (Pongor et al., 1993). Mammalian homologues cause the direct activation of 3'-5'-cyclic nucleotide phosphodiesterase and calcineurin, and potentiate calcium-mediated activation of nitric oxide synthase. In several neuronal populations Frs2 (Q-1) is coexpressed with calcineurin or nitric oxide synthase (Shabad et al., 1996).

We transiently coexpressed the mouse frs2 in NG108-15 cells using a bicistronic expression vector (pcI neo/Rous/GFP, Trotter et al., 1997) and examined the whole-cell calcium currents. Overexpression of frs2 caused an increase in LVA calcium currents following differentiation in addition to a significant decrease in HVA calcium currents. The inactivation kinetics of the LVA currents and the fast component of the HVA currents were accelerated. To investigate whether these changes resulted from an enhancement of calcineurin activation we altered calcineurin expression. In cells with transiently lowered expression of calcineurin, the increase in current amplitude due to frs2 was reversed and the inactivation kinetics restored to values resembling controls.

Our results suggest that frs2 overexpression may enhance the calcium-mediated modulation of LVA and HVA calcium channels and thereby influence the control of synaptic efficacy.

613 - Bosc

REGULATION OF NEURONAL VOLTAGE-DEPENDENT Ca CHANNELS VIA A RAS/MAP KINASE PATHWAY
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The small G-protein Ras, a critical component in the signal transduction pathways regulating cell growth, is involved in the tonic up-regulation of voltage-dependent calcium channels (VDCCs) in rat sensory neurons. To investigate which effector(s) of Ras is involved in this process, a series of Ha-Ras mutant cDNAs (V12 constitutively active, V12/S185I inactive) downregulated downstream effectors, V12/S185I activates Raf-1, V12CA activates p110 α-subunit of phosphatidyl inositol 3-kinase, V12CA37 activates Raf/EDS) were expressed in primary cultured rat dorsal root ganglion neurons (DRGs). Comparison of whole cell Ca2+ currents revealed that V12Ras increased basal current compared with control GFP-transfected cells (41.40 %), whereas V12/S1851Ras reduced current (65.50 %). Expression of V12CA/Ras or V12CA37Ras had no effect but V12/S1853Ras also increased basal current (63.20 %) with a hyperpolarising shift in the current-voltage relationship, indicating that Raf-1 activation is required for Ras enhancement of Ca2+ current in these cells. Raf-1 activates MEK in the MAPK/kinase pathway and the MEK inhibitor, UO126 (10-20 μM), reduced current after 15-20 min (44.76 %), whereas inactive UO124 had no effect. Together these data suggest the involvement of a Ras/MAPK/kinase pathway in the regulation of VDCCs in DRGs. The timecourse for MEK inhibition suggests direct modulation of channels by Ras/MAPK/kinase although gene expression-modulated effects cannot be discounted. (Supported by Wellcome Trust)

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Comparing degrees of N-type channel inhibition by a spectrum of mutant Gα subunits has provided an initial map of effector interaction regions on Gα (1998, Science 280, 1271). However, the characterization of channel modulation was limited to a single voltage. Here we have developed a method to infer Gα binding ([Gα]a) and unbinding (kab) rates across a broad voltage range, with the goal of refining the picture of differential modulation by various mutations on the Gα, interaction surface. The time constants (τ) could then equal 1/(kab + [Gα]a), and the steady state fraction of unbound channels (Wun) would be Wun = (1 + [Gα]a) kub/(kab + [Gα]a). These functional forms made predictions of time constants for equilibration between inhibited and unbound forms of the channel, and for Wun at different voltages. The figure shows fits of these functions to data obtained in HEK 293 cells expressing N-type channels inhibited by mutant IBOA, which is believed to enhance Gα modulation. Explicit binding and unbinding rates at different voltages can be inferred from the fits, and comparison of these parameters among various Gα mutants may reveal understanding of their contribution to the interaction surface. (We thank SIBIA for N channel clone.)

616 - Bosc

SELECTIVE UP-REGULATION OF VOLTAGE-GATED Ca CHANNELS BY NEUROTROPHINS IN HIPPOCAMPAL NEURONS
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We have shown that 24 hours of application of BDNF on rat motoneurons produces a selective up-regulation of P/R-type currents, very likely due to the synthesis of newly available Ca channels (Baldelli et al., 1999, Eur. J. Neurosci. 11:1127). Here, we bring new evidence that in hippocampal neurons, BDNF, NGF and NT-3 have similar up-regulatory effects on the total Ca currents but their action is selective toward different Ca channels. NGF and NT-3 are more effective for L-type channels while BDNF preserves its selectivity toward non-L channels. The voltage-dependence of g(V), the time course of activation, inactivation and deactivation and the properties of unitary L-channels are not significantly affected by neurotrophins, suggesting that the up-regulation of Ca currents is likely due to an increased number of functioning channels. The effects are completely abolished by co-inubation of the neurons with either the tyrosine-kinase inhibitor K252a, the protein synthesis inhibitor anisomycin or the MAP-kinase inhibitor PD98059. Western-Blot analysis shows the efficacy of PD98059 to inhibit BDNF-induced MAP-kinase phosphorylation while immunoclochemistry assays displays the ability of the inhibitor to block BDNF-induced nuclear translocation of activated MAP-kinases.

Our results suggest that on a long time scale, neurotrophins selectively increase the synthesis of newly available Ca channels through the activation of tyrosine-kinase receptors and the involvement of MAP-kinase activity.

616 - Bosc

PHOSPHOLIPASES PARTICIPATE IN N-TYPE CA CURRENT INHIBITION BY A DIFFUSIBLE SECOND MESSENER PATHWAY
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The muscarinic receptor agonist oxotremorine-M (Oxo-M) inhibits N-type calcium currents in rat superior cervical ganglion (SCG) neurons by a membrane-delimited pathway and an unidentified diffusible second messenger pathway. We used standard patch clamp techniques to characterize this latter pathway using low internal divalent chelator, and barium (Ba2+) as the charge carrier. Neonatal rat SCG neurons were preincubated with 0.5 μM S-(+)-pertussis toxin to eliminate the membrane-delimited pathway and nimodipine (1 μM) was included in the bath to block L-type currents under these conditions, both application of 10 μM Oxo-M inhibited whole cell Ba2+ currents by 64.8 ± 2.9%. The presence of 2.5 μM U-73122, a selective inhibitor of phospholipase C (PLC), reduced this inhibition by 89.0 ± 6.1%. Similarly, when 25 μM oleoyloxyethyl phosphorylcholine, a highly specific phospholipase A2 (PLA2) inhibitor, was included in the bath solution, current inhibition also was reduced by 93.4 ± 6.6%. In addition, cells were unresponsive to Oxo-M when dialyzed with a G-protein antibody that recognizes the C-terminal of Gαq subunits. In cell-attached patch recordings, bath application of Oxo-M decreased N-type Ca channel activity. These data indicate that Oxo-M can modulate N-type Ca currents via Gαq, G-proteins, PLC, PLαs, and an unknown diffusible messenger. [Funded by grants from the Whitfield Foundation and the American Heart Association (9204222N)].
ARACHIDONIC ACID INHIBITS WHOLE CELL NEURONAL L- AND N-TYPE CALCIUM CURRENTS
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Arachidonic acid (AA) affects many ion channels, but its mechanism of action remains under investigation. In order to characterize how AA modulates neuronal calcium channel activity, we used whole cell patch clamp techniques to record calcium currents from neonatal rat superior cervical ganglion neurons, using barium as the charge carrier. In the presence of the L-type calcium channel antagonist PPL 64176 (1 μM), 5 μM AA inhibited the peak current (mostly made up of N-type current) and the slow component of the tail current (made up of L-type current) by 51 ± 32% and 42 ± 6%, respectively. Bovine serum albumin (1 mg/ml) reversed this inhibition. Two other fatty acids, oleic acid (5 μM) and myristic acid (5 μM) had no significant effect on currents, indicating some selectivity of action by AA. Inhibition of AA metabolism was without effect on the ability of AA to inhibit currents, suggesting a mechanism independent of AA metabolism. AA increased the activation kinetics selectively for N-type current but had no effect on the voltage-dependence of activation. However, AA did lead to an increase in holding potential-induced inactivation. Thus, AA inhibits whole cell currents by increasing holding potential-dependent inactivation, consistent with our single channel data and with previous results obtained from myocytes. [Funded by grants from the AHA.]

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EFFECTS OF PROTEIN KINASE C ACTIVATION ON UNITARY CALCIUM CURRENTS IN NEONATAL RAT SUPERIOR CERVICAL GANGLION NEURONS
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N-type calcium channels are phosphorylated by protein kinase C (PKC), leading to an increase in whole-cell current amplitude. This increase has been proposed to be via a relief of tonic G-protein-mediated inhibition. Previous studies have examined the effects of G-protein-mediated inhibition, and its relief, on unitary N-type calcium channel activity, but whether the effects of phosphorylation by PKC mimic this relief has not been examined at the single-channel level. We therefore recorded unitary N-type calcium channels in cell-attached patches and examined their biophysical properties following application of the PKC activator PMA (phorbol 12-myristate 13-acetate, 500 nM). Our experimental approach was to use 110 mM barium as the charge carrier, and apply voltage steps to +10 mV every four seconds from a holding potential of −90 mV. We found that phosphorylation by PMA led to an increase in N-type calcium channel activity. Specifically, opening frequency increased approximately 3-fold, and was reflected by a decrease in mean closed time. In addition, latency decreased by about 70%. No change in mean open time was observed, nor was the unitary current amplitude affected. These changes in unitary N-type calcium channel gating are consistent with those previously observed at the whole-cell level. [Funded by the NIH (NS34193)]

620 - Poo
FLUORESCENT MEASUREMENTS OF OXIDANT STRESS IN RABBIT VENTRICULAR MYOCYTES
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Reactive oxygen species (ROS) play important roles both as signalling molecules and as agents of cell injury. A prominent role for ROS is postulated in the pathophysiology of cardiac ischemia and heart failure, but the critical evaluation of this idea has been complicated by the inability to detect ROS reliably in living cells in real time. This study was designed to characterize a new fluorescent dye, 5-(and-6)-chloromethyl-2',7'-dichlorodihydro-fluorescein diacetate (CM-H2DCFDA, Molecular Probes) that has been modified to enhance retention in live cells. CM-H2DCFDA (40 μM) passively enters cells and when oxidized yields a fluorescent molecule that is trapped intracellularly. Fluorescence in isolated rabbit ventricular myocytes was measured over time on a confocal microscope at emission 529 nm. Under basal conditions, the time course of fluorescence in such cells remains stable over several hours, however, purposely repetitive laser scanning at short intervals resulted in a linear increase in fluorescence over time, which was significantly attenuated by the ROS scavenger with mercaptopyrroline (2 mM). We confirmed the ability to detect ROS production by adding 10 μM H2O2 to the cell suspension; this resulted in a brick, irreversible increase in fluorescence and, eventually, in cell death. The present method enables ROS detection in single cells in real time, under conditions amenable to simultaneous patch-clamp recordings.

621 - Poo
DO UNCOUPLING PROTEINS DECREASE CARDIAC EFFICIENCY IN THE HYPERTHYROID RAT HEART?
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The decrease in cardiac efficiency in the palmitate-perfused hypothyroid (TH3) rat heart is thought to be due to increased expression of uncoupling proteins (UCPs). Interaction of long chain fatty acids with these proteins reportedly dissipates the mitochondrial proton gradient. We have examined UCP expression and mitochondrial uncoupling in hearts from control and TH3 rats. UCP2 and UCP3 were detected by Western Blotting of mitochondrial preparations. Respiratory rates were determined in media containing: 1 mg/ml albumin (BSA); 1 mg/ml BSA + palmitate (0.4-0.9 mM) or 1 mg/ml BSA + hexanoate. State 4 respiration was measured in the presence of oligomycin (1 μg/ml) to study inner mitochondrial membrane uncoupling. Expression of UCP2 and UCP3 was significantly increased (p<0.05) in TH3 heart mitochondria. State 4 respiration increased in both control and TH3 mitochondria with increasing palmitate and was higher in TH3 mitochondria compared to control (significant at 0.8 and 0.9 mM). No uncoupling was observed in the presence of hexanoate. Thus, an increase in UCP expression in the TH3 rat heart leads to increased uncoupling in the presence of long chain fatty acids only, which may explain the decreased efficiency in TH3 hearts. This demonstrates a physiological effect of UCPs in tissue other than brown adipose tissue, both at the subcellular and whole organ level.
LITHIUM AND HEART ENERGETICS


The heat released by a heart muscle contraction can be decomposed into four components. Three of these (H1, H2, and H3) are independent of the pressure time integral (PII), and were proposed to be related to calcium binding processes (H1), ionic exchange (H2) and mitochondrial activity (H3) (Pilgrams Arch. 1995). The remaining component, H1, correlated with PII and was mainly attributable to the actomyosin ATPase activity. Lithium decreased PII from 14.2±0.9 to 8.2±0.9 mN/mm² s and increased the maximal rate of relaxation over Pressure ratio (R/P) from 3.3±0.1 to 4.9±0.3 s⁻¹, with no effects on either H1 or H2. While Li also decreased H1, H3/PII remained unaltered, indicating that the economy for pressure maintenance was unaffected. During an extra-stimulus applied 200 ms after the regular one, the presence of Li decreased PII and H3 without effects on the H3/PII ratio, but the H2 associated to the extrastimulus fell (0.04±0.08 mN/m² s). Li also decreased H4 from 15.2±4.0 to 1.9±0.6 mN/m² s which agrees with the stimulatory effect of Li on mitochondrial Ca efflux and subsequent decrease in the mitochondrial futile Ca cycle. The results suggest that the changes of cytosolic Ca induced by Li affects the overall contraction economy by affecting a mitochondrial component and the energy homeostasis.

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SENSITIVITY OF FORCE-INTERVAL RELATIONSHIPS IN DEVELOPING CHICK MYOCARDIUM TO Ca²⁺ CHANNEL blockade

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We used T-type and L-type Ca²⁺ channel blocking agents and the sarcoplasmic reticulum Ca²⁺ release blocker ryanodine, to determine the relative contribution of these pathways on contractile force. We used isolated, perfused embryonic cardiac muscle strips from stage 24 and stage 31 chick embryos to assess isometric twitch force amplitude and duration, steady-state force interval relations (FIR), post-isometric potentiation, and post-stimulus potentiation (PSP). Active force and all FIR increased with development. Nifedipine (1.0 nM) or 0.1 μM ryanodine reduced twitch force, PSP and PSP in all preparations. Similarly, 100 μM nifedipine or 0.5 μM mibefradil diminished twitch force, PSP and PSP. In addition, T-type Ca²⁺ channel blockers inhibited spontaneous contractile activity. Specimens displayed a positive staircase in normal buffer solutions which was diminished in the presence of the sarcoplasmic Ca²⁺ channel blockers. Thus, Ca²⁺ entry via T-type and L-type Ca²⁺ channels and intracellular Ca²⁺ storage influence active force generation and FIR during cardiac development.

This research was supported by (1) Civilian Research and Development Foundation #5270, and (2) National Institutes of Health, Individual NRSA, # F32 HL02020-01.

PERIPHERY TO CENTER CALCIUM GRADIENTS AND TRANSVERSE TUBULAR SYSTEM ABNORMALITIES IN VENTRICULAR MYOCYTES OF CRONICALLY PACED DOGS

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We investigated simultaneous defects in the transverse tubular network and subcellular Ca²⁺ gradients in canine failing myocytes using confocal microscopy. Dilated cardiomyopathy was produced in dogs by chronic rapid pacing. Enzymatically-associated myocytes from control and fast-paced hearts were stained with the non-permeating membrane dye Di-8 ANEPES to monitor the transverse-tubular system and loaded with fluo-4 AM to monitor cytosolic Ca²⁺. Arrays of highly-stained dots representing single tubules extending into the cell were observed in control and failing cells. In failing cells, the t-system was far less regular and was dramatically reduced in many fractions. The function of cells was occupied by the t-system was measured in single optical slices and was significantly reduced in cells compared to the intact system which was measured in single optical slices and was significantly reduced in cells compared to the intact system.

Ca²⁺ transients evoked by electrical stimulation revealed that in many failing cells, Ca²⁺ release started at the cell periphery and subsequently spread towards the center of the myocyte with an overall increase in the time to peak of the Ca²⁺ transient at the center of cells. Peripherally to center gradients were observed in far fewer control cells. An incomplete t-system in failing cells may lead to a loss in synchrony in myocyte activation which would explain the development of V-shaped spatial Ca²⁺ gradients similar to those observed in healthy atrial cells lacking a well-developed t-system.

CALCIUM SOURCES DURING BIGEMINIES: A MECHANICAL-ENERGETIC STUDY


The present study energetically investigates the sources of contractile Ca²⁺ during bigeminy (excess of post-extrasystolic contractions (ES) and post-extrasystole (PES) using a microtome technique (Pilgrams Arch. 429: 841-851, 1995). The extrasystolic interval (ESI) was decreased in steps, while the sum of ESI and post-ESI was held constant. Post-ESI potentiation was calculated as the difference (post-ESI - control PES). ES pressure-time integral (PII) was similar to the post-ESI potentiation for each interval tested. VER decreased ES PI (8.12±0.4 mN/mm² s vs. 4.6±0.41 mN/mm² s, p < 0.01, n=18), without effects on post-ESI potentiation. The ratio between the pressure-time integral and PES was consistent with the sum of the ESI and post-ESI, independently of the presence of VER or the variation of the ESI. These results suggest that during ES, Ca²⁺ contribution from the extracellular space is increased and, at least two fractions can be distinguished. One of them is affected by VER, and the VER-insensitive fraction would be responsible for the post-ESI potentiation. The unchanged VER/PII ratio suggests that, independently of the ES effects on the heart considered as a pump, in the rat heart muscle the bigemnic pattern does not determine an over-expertise of energy.

Supported by: CONICET PTFP 4567, UBA/RO1011 & FONCYT/PICT2264

CALCIUM TRANSIENTS IN GUINEA-PIG SINO-ATRIAL NODE CELLS IMAGED BY CONFOCAL MICROSCOPY

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Confocal microscopy was used to image spontaneous Ca²⁺ transients in guinea-pig sino-atrial node cells loaded with fluo-4 AM. An example of whole-cell Ca²⁺ transients (constructed from line scan images) is shown in the figure below: the rapid upstroke of the Ca²⁺ transient was preceded by a gradual rise in intracellular Ca²⁺. The initiation of the Ca²⁺ transient was asynchronous across the transverse axis of the cell, but was observed to begin at either edge of the cell and propagate towards the centre with a conduction velocity of 253±43 μm/s. Cyclopiazonic acid (a Ca²⁺ release inhibitor of the sarcoplasmic reticulum Ca²⁺ ATPase) reduced the amplitude and prolonged the time course of the Ca²⁺ transient. In addition to Ca²⁺ transients, localised 'spark-like' events were observed in several cells (peak 4.2±0.4 times resting level; rise time 114±4 ms; time constant of decay 80±11 ms; FWHM 1.9±0.2 μm), in contrast to guinea-pig ventricular myocytes in which sparks were rarely seen. The importance of these 'spark-like' phenomena to sino-atrial node function remains to be established.

REGIONAL DIFFERENCES IN EXPRESSION OF Ca²⁺ HANDLING PROTEINS IN SINOATRAL NODE

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Recently it has been suggested that intracellular Ca²⁺ plays an important role in pacemaking in the sinoatrial (SA) node. Because the SA node is heterogeneous, using immunocytochemistry we have examined, in different regions of the rabbit SA node, the expression of three different proteins involved in the regulation of intracellular Ca²⁺: Na⁺/Ca²⁺ exchanger, ryanodine receptor (sarcoplasmic reticulum, SR, Ca²⁺ release channel) and SERCA2a (SR Ca²⁺ pump). Single cells were isolated from the centre and periphery of the SA node: cells from the centre (length, 51±3 μm; width 24 ± 4 μm) were smaller than cells from the periphery (length, 88±3 μm; width 30 ± 5 μm). In 16 peripheral cells, labelling of the cell membrane by anti-Na⁺/Ca²⁺ exchange antibody was observed. In 16 central cells, there was little or no detectable labelling by the antibodies within the cytoplasm and punctate labelling of lower density next to the cell membrane. Data consistent with these findings were obtained from sections taken from the intact SA node. In summary, this study has provided evidence of a decrease in expression of Na⁺/Ca²⁺ exchanger, ryanodine receptor and SERCA2a from periphery to centre of the rabbit SA node. It is possible, therefore, that intracellular Ca²⁺ handling may vary regionally.
631 - Pos

GLYCOGEN AND THE MECHANISM OF OSMOTIC DAMAGE DURING MYOCARDIAL ISCHEMIA

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It has been reported that osmolarity alters glycogen metabolism in skeletal muscle (Low et al. J Physiol 493: 299, 1996). Recovery of contractile function after global ischemia depends on the pre-ischemic glycogen content of the heart. In this study, isolated ‘slow-twitch’ skeletal muscle perfused for 8 min with hyperosmotic (380 mM) buffer had 50% lower (p < 0.05) intracellular glycogen [90 ± 6 μmol(glycogen units/g protein)] than those perfused with hypotonic (220 mM) buffer [182 ± 32 μmol(glycogen units/g protein)]. The myocytes responded to recovery after 20 min total ischemia, in that hearts perfused with hypotonic buffer recovered 40% of contractile function, whereas those perfused with hyperosmotic buffer did not recover function and had greater loss of the energy metabolites ATP and PCr. To investigate whether pre-ischemic glycogen content was the factor that altered functional recovery, we perfused hearts with hypotonic buffer plus pyruvate, lactate and insulin to increase glycogen content to [186 ± 26 μmol(glycogen units/g protein)] before ischemia. The loss of ATP and PCr was decreased and contractile function recovered at the start of reperfusion but this was not maintained. Therefore, initial recovery from an ischemic insult correlated with the pre-ischemic glycogen content of the tissue, but long-term outcome was dependent on other factors that were modified by osmolarity.

634 - Pos

EFFECTS OF HALOTHANE ON CALCIUM BINDING KINETICS OF HUMAN RECOMBINANT CARDIAC TROPONIN C

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To determine whether halothane increases the dissociation of Ca2+ from troponin C as a mechanism for decreased myocardial Ca2+ sensitivity in cardiac muscle (Hquivos J 27:5546, 1990), we studied the kinetics of dissociation of Ca2+ from the single, regulatory site of cardiac troponin C by measuring the rate of Ca2+-mediated fluorescence changes in human recombinant cardiac troponin C labeled with IAANS in Cy-35 and Cy-48 (J Biol Chem 255:9635-9640, 1980) (Harcourt). Fluorescence (excitation 330 nm, emission >395 nm) of Haptropon C (100 μg/ml) in MOPS 50 mM, KCl 100 mM, pCa 3.4, pH 7.0, 20°C decreased after rapid (1.2 ms) max dissociation of Ca2+ and subsequently at a rate (kobs) of 8.7 μM/s (mean ± S.D., n = 8) (Fig. 2). Therefore, the inability of halothane to increase Ca2+ sensitivity in vivo may be due to the selective activation of different signaling pathways that may be specific for the nature and stage of cardiac disease. (F equal contribution)
635 - Pos
THE ANESTHETIC SEVOFLURANE DECREASES MYOFIBRILLAR Ca²⁺ SENSITIVITY IN MAMMALIAN MYOCARDIUM.
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The anesthetic effect of sevoflurane on the sarcolemma results from decreased intracellular Ca²⁺ availability with possible changes in myofibrillar Ca²⁺ sensitivity. To quantify the latter, intracellular Ca²⁺ transients and isometric force were measured in twitch contractions of ferret right ventricular papillary muscles microinjected with the photoactivatable Sevoflurane 2.7% (v/v) decreased both peak force and actinous length. Raising [Ca²⁺], with sevoflurane present to produce peak force equal to control increased intracellular Ca²⁺ transients higher than in control, this suggests that sevoflurane decreases myofibrillar Ca²⁺ sensitivity. Analysis of these Ca²⁺-back titration experiments with a mathematical model of intracellular Ca²⁺ buffers (troponin C, calmodulin, sarcoplasmic reticulum) suggests that sevoflurane 2.7% decreases Ca²⁺ availability to 86.8 ± 3.3 % (mean ± SE; n = 5) and myofibrillar Ca²⁺ sensitivity to 85.7 ± 6.9 % of control. Supported by NIH GM36385 and Mayo Foundation.

636 - Pos
PKA MODULATES LENGTH-DEPENDENT ACTIVATION IN MURINE MYOCARDIUM.
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cAMP-dependent protein kinase (PKA) is known to phosphorylate key myofilament proteins (troponin I and C-protein) resulting in a decrease of the Ca²⁺ sensitivity of tension. However, the impact of PKA treatment on length dependence of myofilament activation remains unclear. Accordingly, we determined the Ca²⁺ sensitivity of tension in "skinned" murine cardiac myocytes. Cell fragments were obtained by mechanical disruption in ice-cold standard relaxing solution followed by Triton X-100 (0.3 %, 6 min) treatment to remove all membranous structures. The relationship between free Ca²⁺ and tension was determined at two sarcomere lengths (SL), both prior to and after PKA treatment (3 μM), 30-45 min. Each curve was fit to a modified Hill equation. PKA treatment resulted in a Ca²⁺-tension relationship that was right-shifted at each SL (p<0.0001). Furthermore, the impact of SL on Ca²⁺ sensitivity as indexed by the Emax ([Ca²⁺]₅₀ at which tension is half-maximal) was increased following PKA treatment (see table; *p<0.0001). The Hill coefficient was not altered by PKA treatment or changes in cell length. These findings indicate that phosphorylation of key myofilament proteins by PKA induces a decrease in Ca²⁺ sensitivity of tension, while enhancing the length-dependent activation properties of the cardiac myofilament.

637 - Pos
EFFECTS OF α₁-ADRENERGIC STIMULATION ON STEADY STATE RELATIONSHIP BETWEEN [Ca²⁺], AND CELL LENGTH IN ISOLATED RAT VENTRICULAR MYOCYTES.
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We investigated the effects of α₁-adrenergic stimulation on the myofilament responsiveness to Ca²⁺ in intact cardiac myocytes. After the treatment with phenylephrine (0.2 μM), enzymatically isolated rat ventricular myocytes loaded with fura-2 AM (4 μM for 10 min), were stimulated at 10 Hz for 10 sec to produce transient and an instantaneous plot of [Ca²⁺], versus cell length ([Ca²⁺], trajectory) was constructed (22-24 °C). An α₁-agonist (phenylephrine, 1-100 μM) dose-dependently shifted the Ca-L trajectory to the left (sensitization of the myofilaments to Ca²⁺). An increase in [Ca²⁺], from 0.5 to 2 mM did not alter the phenylephrine-induced shift of the Ca-L trajectory. An α₁-agonist (prazosin, 1 μM) and a selective α₁-agonist (WB4101, 1 μM) reversed the effect of phenylephrine on the Ca-L trajectory. A Na/H exchange inhibitor (EIPA, 5 μM) reversed the phenylephrine-induced shift. We then measured pH; using the same protocol using the myocytes loaded with BCECF AM (a pH indicator) (8 μM for 15 min). Phenylephrine increased pH; which was reversed by phenylephrine-induced shift of the Ca-L trajectory. An α₁-agonist (prazosin, 1 μM) and a selective α₁-agonist (WB4101, 1 μM) reversed the effect of phenylephrine on the Ca-L trajectory. A Na/H exchange inhibitor (EIPA, 5 μM) reversed the phenylephrine-induced shift. These results suggest that α₁-adrenergic stimulation increases myofilament responsiveness to Ca²⁺, which is independent of [Ca²⁺], and could be related to intra-cellular alkalization mediated through the activation of the Na/H exchanger.

638 - Pos
ENDOCARDIAL-EPICARDIAL DIFFERENCES IN MYOCYTE CONTRACTILITY IN NORMAL AND FAILING RABBIT HEARTS.
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A coronary artery ligation model was used to induce heart failure (HF) in the rabbit. Myocytes were dissociated from sub-endoocardial and sub-epicardial regions of the left ventricle. Cell length measurements were made at 37°C. In normal hearts (sham operated), no obvious difference in fractional shortening was present at 0.3Hz stimulation (endo vs. epic. 7.1±0.5%; 6.9±0.7%). At 3 Hz, there was a trend towards greater fraction shortening in sub-endoocardial cells (endo vs. epic. 8.8±0.5%; 9.7±0.6%). HF sub-endoocardial cells showed a reduction in fractional shortening throughout 0.3-3Hz stimulation, while HF sub-epicardial cells showed reduced fractional shortening only at the lowest stimulus frequencies. At 3 Hz sub-endoocardial shortened less than sub-epicardial cells (endo vs. epic. 6.9±0.6%; 10.4±0.7%). This difference was not as pronounced at 0.3 Hz (endo vs. epic. 6.7±0.7%; 5.3±0.7%). These alterations result in reversal of the normal endo-epi contractile differences. Time to 50% relaxation showed no significant difference between both regions in sham and HF groups. Time to 50% peak shortening was increased in HF sub-endoocardial cells, particularly at higher stimulus frequencies (e.g. 3Hz, sham vs. HF; 35.9±2.32ms vs. 45±2.91ms). These results suggest that altered contractility in HF is differential across the left ventricular wall, resulting in the reversal of the endo-epi difference in cell shortening at 3Hz stimulation rate.

639 - Pos
APOPTOSIS IN CONTRACTING MYOCARDIAL PREPARATIONS PRELOAD- OR AFTERLOAD-DEPENDENT?
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Stretch-induced apoptosis is known to be involved in chronic heart failure. It is not known if myocyte apoptosis depends on increased pre- or afterload. To differentiate these, contracting multicellular cardiac preparations of the rabbit (avg. diam. 450 μm) were stimulated in a closed, sterile culture system under physiological conditions for 48 hours under different loading conditions. Continuous contractions of preparations of each heart (n=7) were performed under unloaded-isometric (no developed force (Fmax)) isotonic in absence of preload (no diastolic tension (Pdiast)) but Fmax and with pre- and afterload (Fmax stretched to 20-30% of Fmax). Standardized electrophoresis gels from myocardial DNA extracts were assessed for DNA fragmentation. Histological TUNEL-assay was performed to identify myocyte apoptosis. Stretched muscles (n=19) with initial Fmax of 9.1±1.2 mN/mm² at t=0 h increased to 22.4±2.1 mN/mm² at t=30 h, and declined to 10.2±1.5 mN/mm² at t=48 h, while Fmax did not change significantly (2.0±0.3 vs. 1.7±0.3 mN/mm² at t= 48 h). In the stretched preparations DNA fragmentation appeared earliest at t=24 h and reached a maximum of 0.40±0.08 LU (arbitrary DNA-Ladder Units per ng DNA) at t=48 h. Under unloaded-isometric conditions preparations showed 0.17±0.03 LU (p=0.05, n=8), and under isometric in the absence of preconditioning 0.2±0.03 LU. Therefore in a physiological range, increased loading conditions can induce apoptosis in contracting myocardium.

640 - Pos
CROSS-BRIDGE CYCLING RATE DURING THE DEVELOPMENT OF HEART FAILURE.
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Heart Failure (HF) is associated with depressed myofilament force generating capacity. Whether cross-bridge cycling is altered in HF, however, is largely unknown. Accordingly, we studied the economy of force maintenance (Eco) in skinned rat myocardium as well as beta myosin heavy chain (MHC-B) content during HF development. We employed the rat post myocardial infarction HF model, both in the early (12 weeks post MI) and late (28 weeks post MI) phase. Eco was estimated as force dependent ATPase activity. MHC-B mRNA was enhanced in both HF groups (109±27% & 164±27% Early & Late); likewise, MHC-B protein was also enhanced in both HF groups (29.3±3.3% & 38.1±4.1% Early & Late). Eco gradually decreased (cf. figure; solid circle line) as MHC-B increased in Sham animals by aging as well as by hypothyroidism (~100% MHC-B). 1/Eco, however, was significantly reduced from the MHC-B content (arrow). These findings suggest that reduced force dependent ATPase activity in HF is not due solely to a MHC shift. This phenomenon may play an important role in the depressed pump function that is seen in end-stage heart failure.
641 - Poss

POSITIVE INOTROPIC EFFECT OF XANTHINE OXIDASE INHIBITION IN A RAT MODEL OF CONGESTIVE HEART FAILURE

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Inhibition of xanthine oxidase (XO) activity enhances myocardial Ca\[^{2+}\] responsiveness in rat cardiac myocytes (Pérez et al., 1998, Circ. Res. 83, 423-430), and a positive inotropic action has also been demonstrated in vivo in a dog model of pacing-induced heart failure (Ekelund et al., 1999, Circ. Res. 85, 437-445). We examined the effect of the XO inhibitor oxypurinol on [Ca\[^{2+}\] ] in rat heart cells. Oxypurinol inhibited systolic [Ca\[^{2+}\] ] and force in right ventricular trabeculae of 18-20 month-old SHR rats which spontaneously develop congestive heart failure at that age. 100\mu M oxypurinol did not affect diastolic or systolic [Ca\[^{2+}\] ] during twitches elicited by 0.5-1 Hz field stimulation at various extracellular [Ca\[^{2+}\] ]. Oxopurinol inhibited systolic force by 78% (p<0.030) and 74% (p<0.039) at 1.0 and 1.5 mM extracellular [Ca\[^{2+}\] ], respectively. Oxypurinol did not affect the midpoint or cooperativity of the steady-state force-[Ca\[^{2+}\]] \_relation, but enhanced maximum Ca\[^{2+}\] -activated force by 53% (p<0.036) to values similar to those of SHR heart failure in the pre-failing state of hypertrophy. In conclusion, XO inhibitors act as potent positive inotropes in heart failure of diverse etiology.

643 - Poss

ORIGIN OF THE ALTERATION OF THE FRANK-STARKLING RELATIONSHIP IN HEART FAILURE

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The existence of the Frank-Starling relationship in pathological cardiac conditions is subject to controversy. We investigated this property in perfused whole heart from normal and post-myo-cardiac infarction (PMI) rats. A balloon connected to a pressure sensor was implanted in the left ventricle, and the intraventricular pressure was monitored following changes in volume. In the PMI rats, two groups of animal could be discriminated based on their passive and active properties. The first group with a very low level of remodeling had a preserved Frank-Starling relationship without any significant difference with the control. The second group of rats, in heart failure, was characterized by a tremendous fibrosis and ventricle dilatation, and presented an alteration of the Frank-Starling relationship. The passive and contractile properties of single skinned ventricular cells were measured as well as their stretch dependent Ca\[^{2+}\] sensitivity. Cells from failing hearts exhibited a lower stiffness and abnormal tension-pCa properties characterized by a decrease (from 0.4 pCaUnit in control to below 0.2) in the shift of pCa50 induced by a stretch from 1.9 \mu m to 2.3 \mu m. Biochemical analysis of contractile proteins were performed. Polycarylasides and electrophoresis (2.5-12%) revealed an altered form of titin in the pathological conditions. Based on a previous report showing that the digestion of titin by a mild trypsin digestion resulted in an alteration of the contractility of normal cells (Cazorla et al. 1999, IMF, 31, 1211-1227), we conclude that the inability of failing hearts to use the Frank-Starling relationship might be consecutive to titin alteration.
647 - Pos
The role of calpinin in regulating calcium-sensitization of smooth muscle (1) was investigated using mice with the b1 calpinin gene inactivated (2). In intact bladder smooth muscle, the amplitude of force developed by knockout (KO) smooth muscle was not significantly different from wild type (WT). Compared to WT, the amplitudes of carbocyl, phospho-ester, and GTPY mediated Ca$^{2+}$-sensitization, as well as the delay in onset and hysteresis of Ca$^{2+}$-sensitization initiated by flash photolysis of caged GTPY, were unchanged in permeabilized KO bladder smooth muscle. Relative to myosin heavy chain, actin and tropomyosin expression was reduced in KO bladder and was deficient smooth muscle and S-cadexin exhibited an upward shift in mobility using high-potency SDS-PAGE. Our results did not reveal a significant role for calpinin in signal transduction of Ca$^{2+}$-sensitization, but it is possible that compensatory mechanisms may have masked any potential effect of calpinin knockout. Supported by NIH (PO1-HL 19242 and PO1-HL 48807).


648 - Pos
CALONPIN INHIBITORS ENHANCE THE COOPERATIVE ACTIVATION OF UNPHOSPHORYLATED AND PHOSPHORYLATED SMOOTH MUSCLE MYOSIN BY REGULATED THIN FILAMENTS. See R. Basberie, University of Vermont, Physiology & Biophysics, Given Building C262, Burlington, VT 05405
We have previously reported that "turned-on" regulated thin filaments (actin-stabilized myofilaments) can be converted into "turned-off" filament by both unphosphorylated and phosphorylated smooth muscle myosin (Harder,J.R.; Muscle Res Cell Mot., 39, 1999). Calponin is suggested to augment Tm activation of thin filaments by phosphorylation, which can be achieved by either 1) treatment with NEM-S1, or 2) addition of 0.2 μM chickens' calpinin to the motility buffer. With turned-on thin filaments, unphosphorylated and phosphorylated smooth muscle myosin produced approximately equal force; this level of force was 4 to 5-fold greater than that produced by unphosphorylated actin filaments and phosphorylated myosin (see Fig). These results suggest that thin-filament regulation could be the predominant regulatory switch for activation of smooth muscle contraction, particularly in the presence of calponin. The role of phosphorylation may be to trigger the cooperative activation of thin filaments by increasing the on-rate for cross bridges.

649 - Pos
CALIBRATION OF MYOSIN LIGHT CHAIN PHOSPHORYLATION MEASUREMENTS. John S. Walker, L. A. Walker, R. L. Wardle, E. F. Ettor, R. A. Murphy, University of Virginia, Physiology, 1300 Jefferson Park Ave, Charlottesville, VA 22908
We examined the quantitation of myosin regulatory light chain phosphorylation (MLRCP) by immunoblotting. MLRCP was expressed as the ratio of the amount of phosphorylated light chain (Mph) to the sum of the phosphorylated and unphosphorylated (M) forms (i.e. R = Mph/M+Mph). We found that using the ratio of the signal intensity of Mph to the sum of the intensities of M and Mph gave an estimate of R that was heavily load dependent (see Table: Signal Ratio).

<table>
<thead>
<tr>
<th>Dilution</th>
<th>1/1</th>
<th>1/2</th>
<th>1/4</th>
<th>1/8</th>
<th>1/16</th>
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<td>34.2</td>
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<tr>
<td>Dilution Ratio</td>
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<td>28.6</td>
<td>26</td>
<td>25</td>
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</tr>
</tbody>
</table>

By using serial dilutions of each sample, we were able to provide a relative concentration calibration curve. Interpolation of phosphorylated values using this curve yielded values for R that were much less load dependent (see Table: Dilution Ratio). Further analysis with the explicit assumption that the antibody affinity was the same for both M and Mph yielded an estimate that was load independent. We propose that this method should be the method of choice when precise estimates of MLRCP are required. This work was supported by NIH grant PO1-HL 19242.

650 - Pos
CHANGES IN CYCLIC GMP AND MYOSIN PHOSPHATASE ACTIVITY DURING NITROVASODILATOR-INDUCED RELAXATION IN CAROTID ARTERY. Elaine F. Ettor, Richard A. Murphy, University of Virginia Health Sciences Center, Mol. Physiol. & Biophysics, 1300 Jefferson Park Ave, Charlottesville, VA 22908
Nitrovasodilators cause a rapid relaxation, in the presence of exocytotic stimuli, in smooth muscle that involves increases in intracellular [cGMP] and decreases in [Ca$^{2+}$] and myosin light chain (MLCP). This study was undertaken to see if changes in MLCP, phosphorylation activity also contribute to nitrovasodilator-induced relaxation. Several medial rings from a given swine carotid artery were stretched on force transducers, activated with 10 μM histamine, then relaxed with 10-100 μM sodium nitroprusside (SNP) in the continued presence of histamine. Individual rings were quick frozen (-80 °C) at different times during relaxation. [cGMP] was determined in tissue homogenates by radioimmunoassay. SNP increased [cGMP] and decreased force in a concentration-dependent manner. [cGMP] peaked within 5 minutes, at the time maximal relaxation was observed, then gradually declined to a suprasellar level. Tissue homogenates were assayed for myosin phosphatase activity using 7Pi-phosphorylated native smooth muscle myosin as substrate. Myosin phosphatase activity increased above resting levels and the levels in activated tissues during the 5 minute SNP-induced relaxation. Thus, direct measurements suggest that myosin phosphatase activity in intact vascular tissue is modulated during nitrovasodilator-induced relaxation in association with increases in [cGMP]. Supported by NIH PO1-HL 19242.

651 - Pos
Myosin light chain phosphatase (MLCP) is composed of three subunits, PP1 (catalytic subunit), M10 (regulatory subunit), and M20. Among them, the function of M20 is unknown. Previously, we reported that M20 localizes with microtubules (Biophys. J. 76, A296). We further studied the role of M20 in organization of microtubules. GFP tagged M20 was expressed in Cos7 cells and microtubules were formed by anti-tubulin antibody staining. Microtubules in cells expressing GFP-M20 were not elongated compared to those in control cells and often showed hair-like structures, suggesting a change in microtubule polymerization-depolymerization.

To examine whether or not M20 expression alters microtubule assembly, rhodamine-phalloidin was microinjected into cells expressing GFP-M20 and to observe its incorporation into microtubules. Rhodamine-phalloidin in M20-GFP expressing cells was incorporated into microtubules significantly faster than in control cells. The results suggest that M20 may play a role in modulating microtubule organization in vivo. While other subunits of MLCP were not localized to microtubules by themselves, they become co-localized with microtubules in M20 expressing cells. Therefore, it is plausible that MLCP holoenzyme might be responsible for this change in microtubule organization, while M20 functions as a microtubule targeting subunit. Supported by NIH grants HL04426 and HL08031.

652 - Pos
EFFECTS OF PROTEIN KINASE C INHIBITORS ON MYOSIN PHOSPHATASE ACTIVITY AND FORCE DEVELOPMENT IN RAT TAIL ARTERIAL SMOOTH MUSCLE. L. S. Weber, L. S. Weber, Y. Sasaki, K. Sasaki, Michael P. Walsh*, University of Calgary, 3Aash Chemical Industry Co. Ltd., Fiji, Sinovel, 416-0934, Japan
Myosin light chain phosphorylation is the primary switch for smooth muscle contraction and occurs principally at 519 of the 20 kDa light chain (LC20). In some circumstances, T18 phosphorylation may also occur. PKC can regulate LC20 phosphorylation indirectly via signaling pathways leading to inhibition of myosin light chain phosphatase (MLCP). The goal of this study was to determine the relative importance of myosin light chain kinase (MLCK) and PKC in basal and stimulated LC20 phosphorylation in rat tail arterial smooth muscle strips (RTA). Two MLCK inhibitors (ML-9 and wortmannin) and two PKC inhibitors (chelerythrine and calphostin C) that have different mechanisms of action were used. Results showed the following: (1) baseline LC20 phosphorylation in intact RTA is due to MLCK; (2) α,δ-adrenergic stimulation increases LC20 phosphorylation via MLCK and a chelerythrine-sensitive, but calphostin C-insensitive, kinase; (3) Ca$^{2+}$-induced LC20 phosphorylation in skinned RTA is effected by MLCK; (4) very little LC20 phosphorylation occurs in intact or skinned RTA at rest or in response to contracile stimuli; (5) the level of LC20 phosphorylation correlates with the degree of relaxation in intact and skinned RTA, although the steady-state tension-LC20 phosphorylation relationship is markedly different between the two preparations such that the basal level of LC20 phosphorylation in intact muscles is sufficient to generate maximal force in skinned preparations. This may be due, in part, to differences in phosphatase-kinase activity ratio, resulting from the reversal of a signaling pathway leading to MLCP inhibition following detergent treatment.
Phosphorylation of myosin light chain (MLC) is the primary mechanism of smooth muscle contraction. The phosphorylation level is determined by the balance between MLC kinase and phosphatase (MLCP). C2⁺, a smooth muscle-specific MLCP inhibitor protein, has been demonstrated to be preferentially phosphorylated by PKC in vitro, thereby increasing C₂⁺ sensitivity of MLC phosphorylation and contraction (J. Physiol. 308, 871, 1980). C2⁺ phosphorylation at Thr-18 appears to be required for reconstitution of PKC-induced contractile Ca²⁺ sensitization in Tritons X-100-denatured smooth muscle (J. Physiol. 520, 139, 1999). In this study, we investigated using anti-phospho-C2⁺ antibody if in situ C2⁺ can be indeed phosphorylated by PKC activator, excitatory amines and GTP-γS. Using Western blotting, the specificity of the antibody was demonstrated as the density of unphosphorylated C2⁺ was only 1% of phosphorylated form whereas equal applied, PKDs, phospholipase, endothelin and histamine but not high K⁺ significantly increased phosphorylation levels of C²⁺ in intact artery. In the oxytocin-permeabilized preparations, GTP-γS phosphorylated C2⁺ at constant Ca²⁺ more than phospholipase and histamine. GDPγS potently and Rho-kinase inhibitor Y27632 partially inhibited the histamine-induced C²⁺ phosphorylation and contraction. These results suggest an existence of proposed PKC-C2⁺-MLCP pathway and another novel signaling pathway of G-protein-C2⁺-MLCP (excluding PKC) leading to the Ca²⁺ sensitization of smooth muscle contraction. Supported by NIH HL51824.

CGRP EFFECTS ON MURINE MYOMETRIAL INTRACELLULAR [CA²⁺] AND FORCE
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Calcitonin Gene Related Peptide (CGRP) is a natural relaxant of the uterus. Its inhibitory actions on myometrial contractility are well established, however it is not known how CGRP does affect the smooth muscle signal transduction mechanisms of contraction and relaxation. We examined the effects of CGRP (1 μM, 10 μM) on intracellular calcium concentration, indicated by the ratio of the FURA-2 AM 340/380nm fluorescence, under different experimental paradigms. When the myometrial strips were incubated in CGRP for 1 minute prior to excitation, the Ca²⁺ concentration and the isometric force induced by short pulses of 50mM K⁺, 20mM K⁺ and 10mM ACh, were significantly reduced. Their ability to contract recovered on average in 10 minutes, indicating that CGRP intracellular signaling is slow and outlasts the ligand removal. When applied during a prolonged contraction, CGRP relaxes the contracted muscle, but does not appear to affect the intracellular Ca²⁺ concentration. This suggests that CGRP can inhibit contraction at least at two steps leading to cross-bridges cycling: an early stage of contraction, impeding the excitation-Ca²⁺ release coupling, and a subsequent stage, directly affecting the acto-myosin interactions in a Ca²⁺- independent manner.

ALTERED CALCIUM SENSITIVITY OF FORCE IN HYPERTRrophied SMOOTH MUSCLE.
Ulfr Malmberg, J. Bonniercoeur, R. Sjøve, A. Amer, Lund University, Sweden.
Effects of smooth muscle hypertrophy on excitation-contraction coupling were investigated in the rat portal vein. Hypertrophy was induced in vivo by an increase in transmural pressure for 7 days. Basal intracellular calcium ([Ca²⁺]) was similar in controls and hypertrophied veins, 75 ± 2 and 95 ± 13 nM. The relation between [Ca²⁺] and force, during activation with high-K⁺, was shifted towards higher [Ca²⁺] in the hypertrophied veins compared to the controls. The increase in [Ca²⁺] at a given extracellular [Ca²⁺] was larger in the hypertrophied veins compared to the controls, suggesting a net increase in, or altered properties of, the L-type calcium channels. The increase in [Ca²⁺] after neuronal (NA) activation was lower in the hypertrophied veins compared to the controls. This could reflect alterations in Ca²⁺ release/elminination mechanisms in the sarcoplasmic reticulum or in receptor associated activation of Ca²⁺ influx. Interestingly, the NA induced force of the hypertrophied veins was lower than expected from the free [Ca²⁺], which could suggest alterations in the receptor mediated sensitisation to Ca²⁺. In conclusion, hypertrophy of smooth muscle is associated with alterations in both electromechanical and pharmacomechanical coupling.

PHOSPHOLAMIN (PLB) REGULATION OF BLADDER CONTRACTILITY: EVIDENCE FROM GENE TARGETED (PLB-KO) AND SMOOTH MUSCLE SPECIFIC EXPRESSION (PLB-SMOE) OF PLB ENZYMES.
PLB modulates contractility in cardiac and aortic muscle but its functional significance in bladder is unknown. We investigated this by measuring the force[Ca²⁺] relations using carbachol (CCh, 0.1-30 μM) in bladder from PLB-KO and PLB-SMOE mice, generated with the Smpl-kd-actin promoter, have approximately 4-12 fold overexpression of PLB by Western blot analysis (Sutliff et al., Biophys. J., 76 (1) 131, 1999). CCh increased [Ca²⁺] and force in time- and dose-dependent manners in the wild type (WT) bladder (EC₅₀ = 2.75 ± 2.26 μM, respectively). In the PL-KO, the maximum increase in [Ca²⁺] and force were significantly decreased (41.6 ± 21.5% of WT; EC₅₀ values = 1.93 ± 1.34 μM) and CCh was significantly increased (EC₅₀ values were 0.63 ± 0.11 μM). CPA did not affect the CCh-induced increase in [Ca²⁺] and force in PLB-SMOE bladder. These results are consistent with PLB modulation of [Ca²⁺] via SERCA inhibition. Importantly, our data show that PLB can play a major role in bladder function. Supported by NIH HL07881 (LR5408123B) & HL54620 (JP6).

OBSTRUCTION INDUCED CHANGES IN SMOOTH MUSCLE OF THE RABBIT URETHRAL BLADDER.
Siu Xiaolong, R., S. Morehead, Dept. Pharmacology and Physiology, MCP Hahnemann University, Philadelphia, PA 19129
Obstruction of the urethra results in numerous alterations in the urinary bladder that impair both its storage and emptying properties. The goal of this project was to determine if the functional changes that occur in the urinary bladder following outlet obstruction are associated with changes at the level of the contractile apparatus. Rabbit urinary bladders were obstructed for 14 days by a ligature tied around a catheterized urethra (8 French catheter). Small strips of bladder smooth muscle were mounted for measurement of isometric force, isotonic shortening velocity, and quantification of myosin light chain (MLC) phosphorylation levels. Muscle strips from obstructed bladders showed increased spontaneous phasic activity, increased sensitivity and higher levels of stress in response to KCl or carbachol stimulation as compared to control. Muscle strips from obstructed bladder had significantly elevated basal MLC phosphorylation levels and stimulation with either KCl or carbachol produced only small but significant increases in MLC as compared to control. In contrast to the positive alterations noted on force generation, Vmax during KCl stimulation of muscle strips from obstructed bladders was 16-fold lower than control (0.078 ± control vs 0.170 ± obstructed). Our results suggest that the remodeling known to occur following bladder outlet obstruction produces a muscle cell that develops higher levels of force but with greatly reduced contractile crossbridge cycling rates. The higher levels of stress at reduced force suggest that the material properties of the bladder smooth muscle may play a greater role in excitation-contraction coupling. This project was supported, in part, through grants from RO1 DK57525 and University of Pennsylvania Urology Center Grant P50 DK52620.
659 - Pos AIRWAY SMOOTH MUSCLE DYNAMICS: PERTURBED MYOSIN BINDING AND LARGE SCALE PHENOMENA
Srebiljah M. Mijalskevich, J. J. Fredberg, Harvard School of Public Health, 665 Huntington Avenue, Boston, MA 02115

We have recently shown that cross-bridge dynamics can account for depression of airway smooth muscle force and stiffness with increases of tidal stretch, including the threshold dependence of smooth muscle lengthening with increasing tidal force amplitude (Am. J. Respir. Crit. Care Med., 159:999-1007, 1999). The predictions of this model match fairly well with experimental data obtained in bovine tracheal smooth muscle. For instance, at tidal volumes of 100 ml/kg, the predicted reduction in force (26%) and stiffness (50%) agree with experimental data obtained in guinea pig tracheal smooth muscle. In addition, the model predicts that the magnitude of the force depression is proportional to the tidal volume, as observed experimentally. These predictions also agree with the predictions of the model for changes in airway smooth muscle stiffness with changes in inspiratory pressure and tidal volume. Thus, the model provides a useful framework for understanding the effects of tidal volume on airway smooth muscle mechanics.

660 - Pos EXPRESSION OF THE MHC SM1/SM2 AND MLC1α/β ISOFORMS AND UNLOADED SHORTENING VELOCITY OF ISOLATED ARTERIAL CELLS
Jesffer J. Sherwood, T. J. Eddinger, Marquette University, 530 N. 15th St., Milwaukee, WI 53233

This study was designed to examine SM1/SM2 and MLC1α/β expression and unloaded shortening velocity in single cells from different regions of the rabbit arterial system. Expression of the SM1/SM2 and MLC1α/β isoforms showed no significant difference between cells isolated from the intimal (19.5±10.3% SM2 and 26.9±9.0% MLC1α (mean±SD), n=27) versus adventitial (25.0±12.3% SM2 and 34.5±6.7% MLC1α, n=28) layers of the carotid media. However, SM1/SM2 and MLC1α/β isoform expression was significantly different (p<0.001) in smooth muscle cells (SMCs) isolated from the saphenous artery (52±12.4% SM2 and 14.5±12.5% MLC1α, n=6) as compared to SMCs isolated from the carotid (22±11.7% SM2 and 30.8±9.1% MLC1α, n=5) and femoral arteries (22±7.8% SM2 and 25±8.3% MLC1α, n=10). The unloaded shortening velocity of SMCs isolated from the saphenous artery (8.8±0.6 mm/s, n=4) was significantly faster (p<0.05) than the shortening velocity of carotid SMCs (4.2±0.5 mm/s, n=6). In all of the arterial cells examined, there was no correlation between the expression of the SM1/SM2 and the MLC1α/β isoforms. The preliminary results of this study suggest that: 1) different regions of the arterial system heterogeneously express the SM1/SM2 and MLC1α/β isoforms and that 2) expression of SM1/SM2 and MLC1α/β is not coordinately regulated.

661 - Pos THE ROLE OF SARCOPLasMIC RETICULUM DURING SHORTENING-INDUCED ATTENUATION OF [Ca²⁺]s AND MYOSIN LIGHT CHAIN PHOSPHORYLATION IN AIRWAY SMOOTH MUSCLE
Chi-Ming Hai, Steven S. Brown, University Box G-B3, Providence, RI 02912

Intracellular [Ca²⁺] ([Ca²⁺]s) is tightly regulated by sarcoplasmic reticulum (SR) and plasmelmmal mechanisms. Precedings, we have and others have shown that agonist-induced [Ca²⁺]s increases during airway smooth muscle shortening. In this study, we tested the hypothesis that SR Ca²⁺ uptake is the mechanism responsible for shortening-induced attenuation of [Ca²⁺]s. We used cyclopiazonic acid (CPA) to inhibit SR-Ca²⁺-ATPase and measured [Ca²⁺]s and force during isometric contraction as well as isotonic shortening. To determine (CPA) for maximal inhibition of SR Ca²⁺ uptake, smooth muscle was first Ca²⁺-depleted by repeated carbachol-activations in Ca²⁺-free medium. Smooth muscle was then treated with various (CPA), and CPA was reinsered. As measured by carbachol-dependent transient contractions in Ca²⁺-free medium, 10 µM CPA maximally inhibited SR Ca²⁺ uptake. In Ca²⁺-containing medium, 10 µM CPA increased resting [Ca²⁺]s, and has force; however, 10 µM CPA had insignificant effect on carbachol-induced steady-state [Ca²⁺], myosin phosphorylation, and active force during isometric contraction. During isotonic shortening, 10 µM CPA also had insignificant effect on [Ca²⁺]s, and myosin phosphorylation. These data indicate that SR Ca²⁺ uptake is not the primary mechanism responsible for shortening-induced attenuation of [Ca²⁺]s, thus, suggesting the involvement of Ca²⁺ extrusion mechanisms.

662 - Pos INTERNAL FORCE BALANCE IN HIGHLY SHORTENED SMOOTH MUSCLE TISSUES.
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When a smooth muscle tissue undergoes extreme shortening at very low afterload, mechanical interactions between cells and the connective tissue matrix form a 'ingenuity structure,' in which crossbridge forces causing shortening are balanced against tension in radial connective tissue that resists lateral expansion. If either of these force-bearing elements is disrupted, the dimensions of the active tissue will adjust accordingly. Previous work used collagenase to disrupt the connective tissue component. This study, again on electrically-activated canine tracheal smooth muscle, employed brief (1-second) mechanical perturbations (large amplitude vibrations) to cause temporary disruption of stressed crossbridges. Following the vibration episode, fully unloaded muscles, while still active, receded to a longer length or exerted a pushing force, depending on the external mechanical conditions. This showed that smooth muscle, when highly shortened, adopts mechanical properties not present at greater lengths. The new mechanical state, which represents a type of insensitivity structure, is characterized by an internal balance between forces supported by radial connective tissue elements and active crossbridges.

663 - Pos THIOPHOSPHORYLATION-INDUCED INCREASE IN Ca²⁺-SENSITIVITY OF SMOOTH MUSCLE CONTRACTION IS INHIBITED BY STAUROSPORINE BUT NOT BY THE RHODIN-INKINER B-27632
Gabriele Pfister, Dagmar K.U. Sonntag, University of Cologne, Robert-Koch-Str.39, D-50931 Köln, Germany

Incubation of 8-oxo-permeabilized longitudinal smooth muscle from the guinea pig with ATP, GTP and ML-9 (300 µM) at pH 7.4 induced an increase in Ca²⁺-sensitivity of contraction, i.e. force of 6.2 prior to incubation with ATP/GTP was 8.16% and afterwards 44.2% of the force at pH 7.4 before incubation with ATP/GTP. However, while carbachol-induced Ca²⁺-sensitization is completely inhibited by ecosysten and C, Y-27632 (10 µM), staurosporine (2 µM), and partially by genistein (100mM), the ATPs-induced Ca²⁺-sensitization is only inhibited by staurosporine. Force after incubation with ATP/GTP and Y-27632 (100 µM) or staurosporine (2 µM) at pH 6.2 was 37% and 13% of force, respectively. The protein kinase C inhibitor peptide 19-31 neither inhibited carbachol- nor the ATPs-induced Ca²⁺-sensitization at a concentration of 30 µM which completely inhibited the FOSs-induced sensitization in nonmice muscle. The ATPs-induced sensitization is associated with phosphorylation of several proteins, one of which is the 150 kDa subunit of myosin phosphate (MYPT1). Staurosporine, but not Y-27632 inhibits this phosphorylation. The ATPs-induced Ca²⁺-sensitization may be due to phosphorylation of MYPT1 (Trinkle-Mulcahy, JBC 270:18191,1995) whereby the kinase is inhibited but a stimulatory effect of the phosphorylation is not inhibited. This suggests that Ca²⁺-sensitization is associated with a protein kinase C inhibitor peptide and that this peptide does not depend on changes in [Ca²⁺].

664 - Pos REGULATION OF CALCIUM SENSITIVITY IN AIRWAY SMOOTH MUSCLE BY MECHANISMS INDEPENDENT OF CHANGES IN MYOSIN LIGHT CHAIN PHOSPHORYLATION

We sought for evidence that increases in force for a given intracellular [Ca²⁺] (calcium sensitization) can occur. A tracheal smooth muscle (CSTM) by mechanisms independent of changes in regulate, in myosin light chain phosphorylation (MLC). When applied to -toxin-permeabilized CSTM stimulated with increasing [Ca²⁺], the receptor agonist acetylcholine (ACH, 100 µM) and endothelin (1) ET-1, 100 nM) decreased ECa for [Ca²⁺] without significantly affecting maximal force. Acute stimulation of G-proteins with AIF, (2 mM) or GTPßS had similar effects. However, chronic stimulation of AIF, (2 mM in low [Ca²⁺] over 1 hour) significantly increased maximal force developed to high [Ca²⁺] (145±0.6% of control), changing the MLC-P-force relationship so that more force was developed for a given MLC-P. In contrast, maximal force was not increased after chronic stimulation with ACH, ET-1, or GTPßS. Downregulation of G-proteins by prolonged GTPßS exposure demonstrated that increases in maximal force produced by chronic exposure to AIF, were indeed mediated by G-proteins. We conclude that chronic activation of heterotrimeric G proteins by AIF, but not receptor agonists, increases calcium sensitivity in part by mechanisms that do not depend on changes in MLC-P. Supported by HL-45532 and HL-54757.
665 - Pos
EICOSAPENTAENOIC ACID (EPA) INDUCES Ca²⁺-INDEPENDENT ACTIVATION AND TRANSLOCATION OF ENDOTHELIAL NO SYNTHASE.
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In order to examine the role of polyunsaturated fatty acids in the regulation of endothelial functions, changes in cytosolic Ca²⁺ concentration ([Ca²⁺]i) and NO production were measured in endothelial cells in situ in the bovine aortic valves, by a fluorometry of fura-2 and 2,3-diaminophloxine, respectively. EPA (60μM), but not its metabolites (docosapentaenoic acid and docosahexaenoic acid), increased NO production in endothelial cells in situ and induced endothelium-dependent relaxation of bovine coronary arteries preconstricted with U46619, whereas it induced little increase in [Ca²⁺]i in endothelial cells in vitro. EPA induced greater production of NO, but much smaller elevation of [Ca²⁺]i, than a Ca²⁺ ionophore (ionomycin) did.

The EPA-induced vasorelaxation was inhibited by L-NAME, a NO synthase (NOS) blocker. Immunoblotting revealed that endothelial constitutive NOS (eNOS), but not inducible NOS, was present in endothelial cells in situ. An immunomonitoring analysis of eNOS and caveolin-1 in cultured endothelial cells on the conflonal microscope revealed that eNOS was colocalized with caveolin in the cell membrane, while EPA (60μM) stimulated the translocation of eNOS to the cytosol with the extent being comparable to that of the eNOS translocation induced by a [Ca²⁺]i-elevating agonist (1 μM bradykinin). These results suggest that EPA may play a pivotal role in Ca²⁺-independent activation and translocation of endothelial NO synthase in endothelial cells in situ.

666 - Pos
INTEGRIN-DEPENDENT MODULATION OF VASCULAR SMOOTH MUSCLE FUNCTION.
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Originally identified as mediators of cellular attachment to the extracellular matrix, integrins have emerged as important mediators for cell signaling. Using integrin binding peptides known to specifically engage particular integrins, we examined: 1) extracellular signal-regulated kinase (ERK1/2) activity, and 2) contractile force, in porcine carotid arteries. ERK1/2 activity was measured in arterial strips held at slack length, and following the application of a mechanical load. Preincubation of loaded arterial strips with the cyclic peptide eRGDdFV and cRGDFdFV produced a time- and concentration-dependence reduction in basal ERK1/2 activity up to 43±2% and 42±2%, respectively. Corresponding decreases in phosphorylation of the high and low molecular weight isoforms of caldesmon (CaD), known to be substrates for ERK1/2, were not statistically significant, suggestive of a dissociation between ERK1/2 activity and CaD phosphorylation. eRGDdFV significantly attenuated the mechanical load-induced increase in ERK1/2 activity in arterial strips preconstricted at a slack length, supporting a role for integrins in mechanotransduction. eRGDdFV and cRGDFdFV (1 mM) also caused relaxation of arterial strips preconstricted with ET-1 (50 nm) by 59±3% and 32±3%, respectively. Maximal relaxation occurred within 5 minutes, and could be accounted for by a reduction in L-NAME phosphorylation; no effect on ERK1/2 activity was detected. Moreover, prolonged incubation (2 hours) with eRGDdFV or cRGDFdFV attenuated the development of ET-1 (10 μM) stimulated force (eRGDdFV: 47%, cRGDFdFV: 51%). These data demonstrate that integrin force by vascular smooth muscle is in part modulated by integrin engagement. This modulation may arise from either the structural requirement for cellular adhesion or the activation of a signaling pathway that can influence force.

667 - Pos
EFFECTS OF STRETCH ON ERK 1/2 PHOSPHORYLATION, GROWTH AND CONTRACTILITY OF VASCULAR SMOOTH MUSCLE IN ORGAN CULTURE.
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The spontaneously active smooth muscle of rat portal vein hypertrophies at elevated transmural pressure. We mimicked this response in vitro by culturing strips of rat portal vein under load. In medium containing 10 % fetal calf serum (FCS) wet weight increased in three days by 56 %, whereas that of undistended control veins increased by 24 %. Stretch increased rates of [H]-thymidine and [H]-thymidine incorporation in cultured veins. In medium without FCS no weight increase was observed. The stretch-induced increase in wet weight was decreased by PDB, but this effect as well as protein contents relative to dry weight was unaffected by stretch. SDS-PAGE gels showed no obvious effects of either FCS or stretch on the relative pattern of contractile protein expression. The contractile force after culture was greater whether than that of unrestretched veins, with a shift of the active length-force relation towards greater length in stretched veins. Contractility was diminished after culture with FCS and reciprocal to the level of growth. Culture with A23187 entry blocker (1 μM verapamil), which diminished active tone, did not alter the effects of FCS on growth and contractility. Angiotensin II (10 μM) caused no growth during culture in the absence of FCS. Stretch was associated with transient ERK 1/2 activation, subsiding to basal level within 24 h. The tyrosine kinase inhibitor herbimycin A (0.5 μM) partially (9 % growth) and the MEK kinase inhibitor PD 98059 (10 μM) totally inhibited the growth response and these effects correlated with the relative levels of inhibition of ERK 1/2 phosphorylation by these agents. We conclude that stretch produces growth dependent on ERK 1/2 activation via tyrosine kinase receptors but not directly dependent on increased Ca²⁺ influx or active contraction associated with the myogenic response to stretch.

668 - Pos
SPHINGOSYLPHOSPHORYLCHOLINE (SPC) AS A MEDIATOR FOR Ca²⁺ SENSITIZATION OF CONTRACTILE APPARATUS IN HUMAN AND PORCINE VASCULAR SMOOTH MUSCLE: INVOLVEMENT OF RHO-KINASE AND HYPERLIPIDEMIA.
Noriyasu Morikage, Natsuko Todoroki-Ikeda, Masafumi Sato, Kimiko Mogami, Yoichi Mizunaki, Kenkutsu Esato, Sei Kobayashi, Yamaguchi University, Ube, Yamaguchi 755-8505 Japan
The aim of this study was to clarify the regulatory mechanisms for the Ca²⁺-independent contraction presumably mediated by the activation of rho-kinase in human and porcine arterial smooth muscles. Fura-2 fluorescence revealed that SPC induced contraction and slightly increased the cytosolic Ca²⁺ concentration ([Ca²⁺]i), resulting in an increase in the force/[Ca²⁺]i ratio. In mechanical relaxation-stimulated smooth muscle, SPC induced contraction at constant [Ca²⁺]i (0.5 μM) in the absence of GTP, being compatible with its role as an intracellular messenger, but not as extracellular agonist for the receptor. In human arteries obtained from the patients undergoing vascular and gastrointestinal surgeries, SPC induced a large and slight contraction of the arteries obtained from the patients with high and normal serum cholesterol levels, respectively. The SPC-induced contractions were inhibited by a rho-kinase blocker. These results suggest that SPC may be a novel mediator for Ca²⁺ sensitization of vascular smooth muscle contraction mediated by rho-kinase, and that SPC may play a causal role in abnormal vascular contractility associated with hypercholesterolemia.

669 - Pos
DECREASED CONTRACTILITY OF DETERUSOR SMOOTH MUSCLE FROM DIABETIC RABBITS IS ASSOCIATED WITH TRANSLLOCATION OF PROTEIN KINASE C B II.
Diabetes was induced in New Zealand white rabbits by intravenous injection of alloxan (150 mg/kg body weight) into an ear vein under anaesthesia. Approximately 50% of the injected animals became diabetic, as determined from blood glucose concentration (>300 mg/dl 1 week after alloxan and at sacrifice). Normal rabbits and rabbits receiving 5% sucrose water served as controls. At the end of the experiments, rabbits were sacrificed, bladder smooth muscle strips were removed and analyzed for (1) force production, (2) diacylglycerol content, and (3) the translocation of protein kinase c (PKC). The force produced by muscle strips from diabetic rabbits was 30-40% decreased. The composition of diacylglycerol was also decreased in the bladder smooth muscles, whereas it was increased in aortic smooth muscles from diabetic animals. Using specific antibodies, we determined the expression of various PKC isoforms in the cytosolic and membrane-associated fractions. Our data indicates that PKC B II in the soluble fraction is decreased in response to diabetes, whereas the PKC is not altered, as determined by Western blot analysis. Data from this study show that treatment of diabetic rabbits with zopolrestat reverses the diabetic-induced alterations in the detrusor smooth muscle contractibility, expression of myosin light chain kinase, and translocation of PKC B II. Supported by NIH grants.

670 - Pos
CAN GREATER CREATINE KINASE ACTIVITY BE RESPONSIBLE FOR HYPERTENSION OR ALTERED VASCULAR CONTRACTILITY IN BLACK PEOPLE OF SUB-SAHARAN AFRICAN DESCENT?
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There is growing evidence that energy metabolism in vascular smooth muscle is an integral component of vascular function. ADP is important for generating the latch state in vessels and is also a potent inhibitor of relaxation. Thus changes in ADP concentration at or near the contractile proteins will impact on contractility. It has been shown that, of black people of sub-Saharan African descent contain higher levels of creatine kinase. Such an increase in the activity of creatine kinase could lead to significant differences in the ADP concentration changes close to the contractile proteins.

At the contractile proteins, phosphate transfer from PCr could have a rate anywhere up to the maximum ATPase rate, which, on the basis of kinetic values (J. Theoretical Biol. 173:207-211, 1995), would imply that free [ADP] is around 50 μM (2.7 times higher than its equilibrium value). Calculations show that doubling the CK activity, would decrease [ADP] by about 40%, to 30 μM, moving it closer to the equilibrium value (1.8 times equilibrium). Furthermore, the increased creatine kinase concentration will increase the ratio of bound to free ADP and thus exacerbate the above changes. This may alter the timing and capacity for the vessels to enter the latch state and/or alter contractility. Another consequence of this change may be a lowered ability of the level of ADP to modulate myosin ATPase activity, and hence an increase the velocity of contraction. This paper presents a hypothesis to account for some of the cardiovascular changes seen in black people of Sub-Saharan African descent using reported differences in muscle creatine kinase.
SMOOTH MUSCLE

671 - Pos DEVELOPMENTAL CHANGES IN SMOOTH MUSCLE ACTIVITY IN THE NEONATAL RAT UTERINE BLADDER.
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The aim of our studies was to perform a frequency analysis of spontaneous and evoked contractions of smooth muscle of the urinary bladder from 1-5 week-old rats. Muscle strips were prepared from the base and dome of the bladder. Developmental changes in activity of muscle strips were studied using a Fast Fourier Transformation (FFT) algorithm. Generally, there was no detectable peak in the power spectrum curves from 1- week-old rats, but in 2-week-old animals spontaneous activity was observed and one FFT peak was detected. Activity in the bladder base was significantly faster (0.205±0.034 Hz) than in the dome (0.082±0.014 Hz, p<0.05). Strips from bladder base exhibited a second peak approximately 0.5 Hz at 3-4 weeks of age. This second peak was also observed in dome strips from 5-week-old rats. At this time the magnitude of the first peak was reduced and the second peak became dominant. In presence of the musaracine agonist carbacol (1μM) spontaneous activity was stimulated and the second peak in the FFT curves was masked in strips from 1-2 week-old rats. Electrical field stimulation (EFS, 2 Hz and 600 shocks) elicited neurally-mediated contractions which were blocked by TTX. Activity at the same frequency of EFS could be detected in FFT curves for 5-6 min after the end of stimulation, indicating prolonged entrainment of spontaneous activity. These data suggest that the maturation of voiding function in the neonatal rat is associated with marked changes in the intrinsic properties of the bladder smooth muscle.

672 - Pos IN VITRO RECONSTRUCTION OF 'CATCH' STATE OF MOLLUSCAN SMOOTH MUSCLE.

"Catch", the state where muscles maintain high passive tension with little energy expenditure is observed in some molluscan smooth muscles. Induction and release from catch involves respectively, Ca2+-calmodulin-dependent phosphatase and cAMP-dependent protein kinase. To investigate the molecular mechanism, we constructed an in vitro assay using proteins from Mytilus byssus retractor muscles: native thick filaments were attached to glass surfaces, then fluorescently-labeled thin filaments were added. In the presence of MgATP and EGTA, thick filaments did not bind to the thick filaments prepared from cAMP-treated homogenate of the muscles. After pre-incubation of these thick filaments with Ca2+ and soluble proteins from byssus retractor muscles, thin filaments bound tightly in the presence of MgATP and EGTA, but released on addition of cAMP in the presence of MgATP, EGTA and soluble proteins. Binding forces between thick and thin filaments were measured by a glass needle: they were at least 100-300pN per μm of thin filament, similar to catch tension in vivo. These results suggest that reversible binding tight between thick and thin filaments observed in vitro corresponds to 'catch' in vivo so that this assay provides a useful tool for elucidating components essential for controlling 'catch'.

673 - Pos FUNCTIONAL PROPERTIES OF A SMOOTH MUSCLE CATION CHANNEL (TRP6) EXPRESSED IN SFP INSECT CELLS.
Ruben C. Ansting, Greg J. Walker, Helen A. Maneo, James L. Kanyo, Burton Horowitz, University of Nevada, Reno, Reno, Nevada 89557.

Nonselective cation channels (NSCC) are a target of excitatory agonists in smooth muscle. While modes of activation often vary, the pathway of excitatory neurotransmitter includes activation of a NSCC resulting in Na+ influx, which depolarizes the cell membrane and triggers the activation of voltage-dependent calcium channels. There is evidence for the role of a NSCC current in muscular responses of colonic smooth muscle. The molecular identification of NSCC in smooth muscle is not clear; however, the transient receptor potential (TRP) channels have similar characteristics to NSCC. We have previously shown evidence for the existence of a TRP6 transcript within murine colonic smooth muscle cells (Biophys. J., 76(1): A292, 1999). TRP6 was cloned from smooth muscle and expressed in SFP cells, which allow for high levels of expression of the deduced protein. Expression of TRP6 results in an inwardly rectifying cation current in symmetrical NaCl solutions using the whole cell patch clamp technique. Current measured at -100mV was 264±45 pA, compared to untransfected cells which are 33±5±4 pA. To examine ionic selectivity, Na+ was replaced in the bath with increasing concentrations of mannitol, resulting in a leftward shift in the reversal potential by ~30mV. 500 μM Ca2+ inhibits the current by 50%. Future studies involve investigating the single channel properties of TRP6. Supported by NIH DK41315.

674 - Pos VOLTAGE-INDEPENDENT InsP3, SENSITIVE Ca2+ STORE REFILLING IN SMOOTH MUSCLE.
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InsP3-mediated Ca2+ signaling is an important component of the response to many hormones, neurotransmitters and growth factors and so the store content must be maintained in the face of changing cellular circumstances. Although store-operated Ca2+ release (SOCE) is a well understood process, there is some controversy with regards to the energy state of Ca2+ stores in different cell types, with reports of ATP, MgATP and ADP being stored in the same or different stores. We have investigated this question using single smooth muscle cells, where we have observed large Ca2+ store refilling which is voltage independent and is sensitive to InsP3. We have shown that store refilling is increased by ATP and MgATP, consistent with store refilling being a voltage independent event. While the presence of MgATP is consistent with its role as an ATP scavenger, these results suggest that MgATP may have a role in store refilling. Further studies are needed to determine the role of MgATP in store refilling and to determine the mechanism of MgATP induced store refilling.

675 - Pos NO' (BUT NOT NO) RELAXES AIRWAY SMOOTH MUSCLE VIA GMP- INDEPENDENT RELEASE OF Ca2'.
L. J. Janssen, H. Lu-Chao, M. Premji, McMaster University, Hamilton, Ontario L8N 4A6 Canada.

Nitric oxide is generally envisioned as a free radical (NO), and several mechanisms have been identified whereby this electrically-neutral molecule relaxes smooth muscle. Nitric oxide can also exist in cationic (NO+) and anionic forms (NO2-) and this mechanism has been suggested to play a role in allergic asthma, where NO2- may be released at low pH. However, the role of NO2- in airway smooth muscle is not clear. In the present study we examined the role of NO in relaxations of human bronchial smooth muscle strips to NO. Relaxations were observed with NO from 2 to 100 μM, but not NO2-. We conclude that NO releases internal Ca2+ in a GMP-independent fashion, leading to activation of Ca2+- dependent myofilament Ca2+ release, while NO releases the airways through a GMP-dependent, Ca2+-independent pathway. Supported by the Medical Research Council of Canada.

676 - Pos REGULATION OF THE SR CALCIUM CONTENT IN SINGLE SMOOTH MUSCLE CELLS: SIMULTANEOUS MONITORING OF INTRALUMINAL AND CYTOSOLIC Ca2'.
Anthony Shmygol, Narayan wayan, D. A Eisner, UNIVERSITY OF LIVERPOOL, University of Manchester, Physiological Laboratory, University of Liverpool, UK.

Sr Ca2+ release and re-uptake are required for normal function of smooth muscle (Ca2+) homostasis in a vast variety of cells, including smooth muscle. Until recently, Sr Ca2+ release and re-uptake were investigated through measurement of cytosolic [Ca2+], using high affinity calcium indicators or intramural Ca2+ (Ca2+ intracellular stores). However, it is clear that the Sr Ca2+ stores are a composite of different stores. To arrive at a more complete picture of the Sr Ca2+ stores, we used a comprehensive Ca2+ imaging strategy and the Sr Ca2+ release and re-uptake were monitored simultaneously in a single cell. In this study, we have used a combination of two different signaling pathways (InsP3, rising 340, 380 (for Fura-PE3 excitation) and 495 nm (for mag-Fluo-4 excitation)) and 37°C for 35 min and consecutively with 3μM mag-Fura-PE3/AM at room temperature for 15 min. Subsequently, the cells were washed with free solution and incubated at room temperature for at least 30 min to allow de-estification of the indicators. The cells were illuminated at 340, 380 (for Fura-PE3 excitation) and 495 nm (for mag-Fluo-4 excitation) using a fast monochromator (Cairn Optoscan). The frequency of wavelength alternation was set to 25 Hz which was fast enough to resolve rapid changes in Ca2+ with acceptable signal to noise ratio. The fluorescence ratio increase was calculated from the ratio of the corrected Sr Ca2+ signal using a monochromator and subtracting the background signal. A complete restoration of the Sr Ca2+ content was achieved only in the presence of extracellular calcium. These data indicate that extracellular calcium is vitally important for the restoration of the Sr Ca2+ content, decreased by the agonist application.
677 - Poo

EPR SPECTROSCOPIC AND STRUCTURAL DYNAMICS OF FHC-MUTANT MYOSIN REGULATORY LIGHT CHAINS IN MUSCLE FIBERS.

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Several different point mutations in the myosin regulatory light chain (RLC) cause the human heart disease, familial hypertrophic cardiomyopathy (FHC). Our goal is to understand the biophysical and biochemical basis of the mutant phenotypes by performing site-directed spin labeling (SDSL) and electron paramagnetic resonance (EPR) spectroscopy on FHC-mutant RLC in muscle fibers. We have expressed rat ventricular RLC (VRLC) in E. Coli and purified the VRLC using anion-exchange chromatography. To facilitate SDSL, an N-terminal microsequence was created, because the wildtype VRLC does not contain any cysteine. This served as the background for the ProMAg FHC-mutant VRLC. For each of these mutants, the FDNA spin label was attached to Cys92 and the labeled VRLC was exchanged into rat muscle fibers, so that the structural perturbation caused by the FHC mutation was assessed by EPR spectroscopy, while simultaneously monitoring the isometric force during contraction. We used the molecular modeling program Insight II to construct energy-minimized models for the LC domain containing these rat VRLC mutations. This allowed us to visualize probable structures and to perform molecular dynamics simulations that were compared directly with EPR spectroscopic data. Our preliminary results suggest that the FHC mutation perturbs the flexibility of the region that connects the two principal lobes of the RLC.

679 - Poo

CHEMICALLY MODIFIED RESIDUES IN MYOSIN ACT AS STRUCTURAL PERTURBANTS AND SIGNAL DONORS TO DETECT CONFORMATION DURING TRANSITION.

Katalla Ajjal, Sungjo Park, Thomas P. Burghardt, Mayo Foundation

Structural changes in myosin occurring during ATP hydrolysis appear to involve two conserved glycine residues in the peptide backbone that pivot to facilitate the swinging movement of the lever arm relative to the catalytic domain of the molecule. Lever arm movement seemingly occurs in synchrony with the myosin ATPase intermediates during chemomechanical energy transduction (Moralez & Born, 1979, PNAS USA 76, 3857). The modification of key residues at either of two lever arm/catalytic domain interfaces dramatically accelerates ATPase indicating the significance of these regions in transformation. Spectroscopic probes directed to these special residues act as both structural perturbants and signal donors that detect the conformation changes at the interfaces during ATPase and correlate local structure with particular myosin ATPase intermediates. The modified residues are equivalent to site specific mutations with intrinsic sensitivity to local structure. The current work compares the structure/dynamics of these regions in skeletal and cardiac myosin. Supported by NIH (RO1 AR39288) and the Mayo Foundation.

680 - Poo

SPECTROSCOPIC ISOLATION OF PUTATIVE ATP-SENSITIVE TRYPTOPHAN(S) IN MYOSIN SUBFRAGMENT I.

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Fluorescence quenching was used to isolate tryptophan 131 (Try131), and the putative ATP-sensitive tryptophan residue(s) in myosin subfragment 1 (SI). Try131 was selectively and specifically modified by DHNBS, and the difference spectra between unmodified and modified SI in the presence of nucleotide and nucleotide analogs were collected as a function of fluorescence quencher concentration. The observed Stern-Volmer quenching constant of Try131 is not affected by nucleotide and nucleotide analogs (P<0.05), suggesting that the vicinity of Try131 does not change its conformation during ATPase cycle. The Stern-Volmer quenching constants of the putative ATP-sensitive tryptophan residue(s) in SI were also isolated by taking a difference fluorescence spectrum between SI in the presence and absence of ATP with neutral and anion quenchers. The observed acrylamide quenching constant for ATP-sensitive Try is 3.51, whereas KI quenching which is greatly affected to microenvironment of tryptophan because of charge interactions, is close to zero for ATP-sensitive Trp. These findings suggest that the ATP-sensitive tryptophan(s) is in negatively charged surroundings. Taken together with previous observations (Park et al. (1996) Biochem. Biophys. Acta 1296, 1-4), the putative ATP-sensitive tryptophan is Try130 and one or more of the remaining tryptophan residues excluding Trp131. Supported by NIH (RO1 AR39288) and the Mayo Foundation.

681 - Poo

FUNCTIONAL DETERIORATION OF THE DIAPHRAGM MUSCLE OF α-SARCOCYLAN-NULl MICE WITH AGE.


In male mice, limb muscles adapt to the ongoing degeneration and even hypertrophy, but with age the diaphragm (DPM) muscle shows progressive fiber necrosis and an increasingly large deficit in force (Lynch et al., Am. J. Physiol. 272-C2063, 1997). Compared with dystrophin-null mice α-sarcoglycan null mice in α-sarcoglycan (α-Sca)-null mice display a more severe necrosis and surprisingly a greater hypertrophy of at least the small hind-limb muscles. We hypothesized that with greater muscle fiber necrosis, DPM muscles of young α-Sca-null mice would develop a greater force deficit than DPM muscles of young μd null mice and the rate of deterioration with age would be more pronounced. Indeed DPM muscles were removed from deeply anesthetized mice 6, 12 and 18 months of age. Strips were cut parallel to the fibers with the attachments to ribs and tendon left intact. Maximum isometric tetanic force (Pt) and power during isometrically shortening contractions were measured in vitro at 22°C. Pt was normalized by fiber cross-sectional area and power by muscle mass. Force had values ranging from 33% to 53% of control SI, and 35% to 45 W/kg for power, with no differences (P>0.05). Expressed as a percentage of the control value, DPM muscles for young and older α-Sca-null mice are greater than μd null mice by 25-33%, and 35-45% respectively, for normalized power. The degree of functional impairment in the DPM muscle of α-Sca-null mice appears to be comparable to that observed for dystrophin-null μd null mice. Supported by AG-15434, AG-13283, and MDA.

682 - Poo

DRAMATIC HYPERTROPHY OF SMALL LIMB MUSCLES IN SCD4 NULL MICE IS MAINTAINED WITH AGING.


The mouse digiterum longus (EDL) and soleus muscles of α-sarcoglycan (Scg4) null mice show progressive dystrophic symptoms through 6 months of age. Apparently as an adaptation to muscle damage, muscles of 6 month old mice show a dramatic 50% hypertrophy which maintains absolute force at or above control values. The purpose was to investigate the effects of the Scg4 deficiency with aging. We hypothesized that maintaining the 50% hypertrophy depletes the capacity of the muscles for regeneration and with aging the muscles would atrophy and become weaker. EDL and soleus muscles from 12- and 18-month-old male Scg4 null and wild type control mice were placed in a 25°C bath and tied to a servosensor and force transducer. Maximum isometric tetanic force and power during isovelocity shortening contractions were determined. Compared with muscles from age-matched control mice, the masses of EDL and soleus muscles of Scg4 null mice were 50% greater. Absolute forces of EDL muscles from 18-month-old Scg4 null mice were greater than those of wild type animals, but forces normalized by muscle fiber cross-sectional area and powers normalized by muscle mass were lower due to the large muscle masses. No difference was observed in absolute forces of soleus muscles of the 18-month-old Scg4 null and control mice, resulting in lower normalized forces and powers. We conclude that the EDL and soleus muscles of Scg4 null mice maintain absolute force through 18 months of age with no evidence of decreased regenerative capacity. Supported by PO1-AJ-15434, P30-AG-13283 and Muscular Dystrophy Association.
683 - POS
RESTRICTION OF CELL BULGING DURING ISOMETRIC CONTRACTION REDUCES FORCE GENERATION IN SKELETAL MUSCLE
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In skeletal muscle cells, isometric contraction is accompanied by bulging at the midportion of the cell, between the tendon ends (Biophy. J. 59:926-932, 1991). This effect is not produced by cell stretching, but is due to either water redistribution within the cell or to translation of the cell against the collagenous material driven net gain of water. At present, it is unknown whether contraction-induced cell bulging plays a physiological role. Experiments were designed to assess the effect of bulging on isometric force generation, and the interaction of intracellular pressure using barnacle skeletal muscle cells. The experimental protocol consisted of assessing the effect of extracellular stimulation on isometric force, cell diameter (measured as cross sectional area) at the midportion of the cell, intracellular pressure (measured from the basal end of the cell), and intracellular membrane potential (Vm) under conditions in which cells were either allowed to bulge freely or when bulging was mechanically restricted. This manipulation consisted of placing a ring of perforated plastic tubing (with a diameter larger than the cell's diameter at rest but smaller than the one reached during bulging) around the midportion of the cells. Control experiments demonstrated that: i) measurements of force generation and intracellular pressure are independent from each other; and ii) cell bulging during isometric contraction is not due to cell shortening resulting from compliance of the tendon and/or the force transducer. Isometric force was highly correlated (correlation coefficient = 0.85) with the generation of an intracellular negative pressure. Bulging restriction did not affect the extracellular electrical stimulation-induced changes in Vm, but reduced the generation of force and negative intracellular pressure by 79-90%. Thus, restriction of cell bulging during contraction inhibits force generation.

685 - POS
SKELETAL MUSCLE FUNCTION AND WATER PERMEABILITY IN AQUAPORIN-4 DEFICIENT MICE
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It has been proposed that aquaporin-4 (AQP4), a water channel expressed at the plasma membranes of skeletal muscle cells, is important in muscle physiology and in the pathophysiology of Duchenne's muscular dystrophy. To test this hypothesis, muscle water permeability and function were compared in wildtype and AQP4 knockout mice. Immunohistochemistry and freeze-fracture electron microscopy showed AQP4 protein expression in plasma membranes of fast-twitch skeletal muscle fibers of wildtype mice. Osmotic water permeability was calculated from measured osmotic conductivity measured from isolated muscle fibers. Osmotic water permeability was lower in field stimulated mouse quadriceps muscle fibers from AQP4 knockout mice compared to wildtype mice. Water permeability at 600 mOsmole was 2 fold higher at 150 mOsmole in AQP4 knockout mice. The data suggest that AQP4 knockout mice have reduced osmotic water permeability.

686 - POS
EFFECTS OF SPINDLE AND ENDURANCE TRAINING ON STRESS-STRAIN CURVES OF FAST AND SLOW TWITCH RAT SKELETAL MUSCLE
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We investigated the effects of endurance and sprint training on passive mechanical properties of Plantaris and Soleus skeletal muscle. Eight-week-old male Wistar rats (n=18) were divided into three groups: control, sprint-trained and endurance-trained. The trained animals exercised for 10 weeks on a treadmill. Under anesthesia, Plantaris and Soleus muscles were lengthened cyclically to 20% of resting length (1.12*0.02, 1.85*0.11, 1.12*0.02 in intermediate, fast and slow muscle groups respectively) n=5 and was observed between 30% and 40% of maximum strain. The muscle was fixed for histological section; 6 muscular sections were analyzed. Differences were significant between training groups in stiffness and force. The exercise increased stiffness and MDE in either muscle.
609 - Poo RESOLUTION OF MILLISECOND CHANGES IN THE ORIENTATION OF THE SPIN-LABELED MYOSIN LIGHT-CHAIN DOMAIN IN MUSCLE
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Using electron paramagnetic resonance (EPR) of spin-labeled muscle fibers, we resolved two distinct orientations of the myosin light chain domain (LCD) in muscle (Baker et al., Proc. Natl. Acad. Sci. U.S.A. 95, 2944 (1998)). The distribution between these two distinct orientations has been monitored previously in steady-state relaxation and contraction. In this study, millisecond changes in this distribution have been detected after flash photolysis of caged ATP, to initiate either relaxation (caged ATP) or contraction (caged ATP + Ca2+) from scalarium fibers starting in rigor. Transients acquired upon relaxation reveal the rate at which scallop myosin detaches from actin and assumes a bimodal orientation distribution. Transients acquired upon contraction reflect the rate at which this distribution shifts, and the light-chain domain rotates to generate force in an active muscle fiber. Analysis of these rates gives insight into how the rate of structural changes within myosin relate to biochemical rates determined from previous studies of scallop myosin kinetics.

609 - Poo A NOVEL METHOD FOR EXPLORING THE STRUCTURE OF THE MYOSIN DIMER IN MUSCLE.
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Biochemical and mechanical studies on muscle indicate that cooperative interaction between the two heads of the myosin dimer is important for optimum motor performance, yet little is known about the structural changes that accompany these interactions. EPR and EM studies suggest that there are two distinct conformations of the myosin molecule. This model can be tested and refined using site-directed labeling and Resonance Energy Transfer. We have developed a method for reliably labeling equivalent sites on each RLC within the myosin dimer, so that each head in a muscle fiber contains a donor probe on one RLC and an acceptor probe on the other. This technique results in donor/acceptor labeled myosin molecules within functional (calcium-sensitive) muscle fibers. With this system, energy transfer in the static limit and the rapid diffusion limit can be used to observe the intramolecular distance and relative motion of the two myosin heads under different physiological conditions. Performing these experiments within the native environment of the muscle fiber allows for interactions with actin and stabilization of the myosin heads by the thick filament. Correlation of the results with previous results from orientational probes supports a model of muscle contraction in which the myosin head is flexible and interacts dynamically with actin and with the thick filament backbone.

691 - Poo EXPRESSION OF SMOOTH MUSCLE MYOSIN SM-B ISOMORPH IN DIFFERENT LAYERS OF RABBIT URETHRA CORRELATES WITH SHORTENING VELOCITY.
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The longitudinal and circular smooth muscle layers of the urethra have distinct contractile properties that may be related to their roles during micturition (Aner et al., 1998, Urol. Res. 26:423). Preliminary work by our lab demonstrated that smooth muscle myosin heavy chain mRNA transcripts in the male rabbit urethra consist of about 20% SM-A and 80% SM-B isoforms. The SM-B transcripts encode a 7-amino acid insert near the ATP binding region that is associated with increased ATPase activity (Kelly et al., 1993, J. Biol. Chem. 268:12848). The goals of the present study were to determine the level of SM-B expression in the individual longitudinal and circular smooth muscle layers and whether the level correlated with their shortening properties. RT-PCR analysis, revealed 95 versus 55% (n=3) SM-B content in longitudinal versus circular smooth muscle layers. This SM-B expression pattern correlated with a higher maximum shortening velocity in the longitudinal (0.116 ± 0.004 Lois/96) compared to the circular layer, (0.049 ± 0.004 Lois/96) in Ca2+ activated (pCa 4.5, 0.5mM calmodulin, pH 7.1, 1mM-200mM, at 23°C), chemically-skinned preparations. Thus, the relative level of SM-B myosin isoform expression may account for the different contractile properties of the longitudinal and circular smooth muscle layers of the urethra. Supported by NIH DK52620 and DK52752.

691 - Poo KINETICS OF RELAXATION IN SKELETAL MUSCLE IN THE PRESENCE OF A TNC MUTANT WITH INCREASED CA2+ AFFINITY.
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Experiments were designed to test the idea that Ca2+ the dissociation rate from TnC can modulate the rate of relaxation in skeletal muscle. Relaxation was induced in skinned rabbit psoas fibers at 15°C by photolysis of diazo-2 in the presence of wild-type TnC or a TnC mutant M82Q. We have shown that compared to wild-type TnC, M82Q TnC exhibits an increased Ca2+ affinity and a decreased Ca2+ dissociation rate; and that this results in a faster relaxation rate versus Ca2+ relationship in fibers (Rennie et al, Biophys. J. 72: A282, 1997). Fibers which contain wild-type TnC relax with a half-time (57 ± 5 ms, N = 5) which is similar to that observed in fibers which contain endogenous TnC. In contrast, fibers which contain M82Q TnC relax 2.2 times more slowly (128 ± 11 ms, N = 5) at the same relative force (0.5 P/Force) and at similar diazo-2 buffering capacity, i.e., free [diazo-2]/free [Ca2+], 1840 ± 3238. This 2.2 fold increase in relaxation half-time in fibers compared to the 4.9 fold decrease in Ca2+ dissociation rate from M82Q TnC in solution. In contrast M82Q TnC has an insignificant effect on rate of contraction of fibers (30 ± 7 s-1, N = 2) when compared to wild-type TnC (33 ± 5 s-1, N = 4). These results provide direct evidence that the affinity of TnC for Ca2+ can dramatically influence kinetics of skeletal muscle relaxation. (Support from NIH AR20792.)

693 - Poo PROTEIN-PROTEIN INTERACTIONS BETWEEN SKELETAL MUSCLE PROTEINS AND THE CIC-1 CHLORIDE CHANNEL.
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The role of cytoplasmic domains of the voltage-gated chloride channel CIC-1 is unknown. In other channels, these regions are involved in regulating channel activity and in anchoring of the channel via protein-protein interactions (PPIs). The CIC-1 carboxyl terminus is of interest because mutations in this region are associated with loss of function and myotonia, and because it is proline-rich. Proline residues are often involved in PPIs. We performed a yeast two-hybrid screen of a human skeletal muscle cDNA library with the CIC-1 carboxyl terminus (aa 874-988) as bait. Interaction between the bait and prey proteins results in expression of reporter genes. By assaying for transcriptional activity of the reporter genes, we identified five candidate interacting proteins involved in muscle contractility: TITIN; myosin heavy chain Ca2+-ATPase; tropomyosin C; and desmin. We are utilizing multiple biochemical techniques to examine the physiological relevance of these interactions.

Why would contractile proteins interact with membrane ion channels? The interactions could serve to maintain proper spatial orientation of the proteins. It is also conceivable that a contraction-excitation feedback mechanism might exist to increase chloride conductances (GCI) during vigorous contraction. This mechanism would depend upon direct PPIs between muscle contractile proteins and CIC-1. We are interested in testing this hypothesis.

694 - Poo EFFECTS OF pH AND PI ON RAT MYOCARDIUM CROSSBRIDGE KINETICS STUDIED BY SINEUSOIDAL ANALYSIS.
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In ischemic cardiac muscle, pH falls Pi rises, altering myofilament Ca-sensitivity and force. Effects on myocardial work or power are less well defined. We study crossbridge (XB) kinetics using sinusoidal analysis in Triton-permeabilised, ventricular trabeculae. Dynamic stiffness (±20.5% length change at pCa4, 20°C) is measured at several frequencies. Others report that the frequency fmax where dynamic stiffness is lowest, corresponds to XB rate, presumably mean rate. At pH 7.6, fmax (mean±sem) was 1.73±0.37Hz. Maximum stiffness alters with pH, in line with Ca2+- activated force. The stiffness-frequency plot shifts to higher frequencies between pH 7.5 and 6.5. However, at pH 6.5 or lower, the plot lacks a clear fmax trough, probably indicating a wider range of XB cycling in acidosis. The tension-longitudinal phase relationship also has a marked frequency dependence (max phase lead, 37.3±6.9, 45.9±6.4 and 28.5±4.4 degrees at pH 7.5, 7.0 and 6.5). At pH7, 10mmol L Pi increases fmax (+ from 1.50±0.26 to 5.04±0.65Hz), but depresses stiffness and phase shifts; lower values signify reduced oscillatory work. Although average XB rate decreases in acidosis (from APase measurements, Eban et al, 1994, J Physiol 476, 501-516), the range is appreciable. A larger scatter of XB rates combined with raised Pi will contribute to lower muscle force and work output in ischemia.
695 - Pos

CHANGES IN FORCE DURING EARLY POSTNATAL DEVELOPMENT IN RAT DIAPHRAGM MUSCLE DEPENDS ON MYOSIN HEAVY CHAIN ISOFORM EXPRESSION

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In previous studies, we found that specific force of rat diaphragm muscle doubles from birth to 84 days (adult). We hypothesized that 1) force per cross bridge varies with myosin heavy chain (MHC) isoform expression with greater force per cross bridge in fibers expressing fast MHC isoforms compared to slow and neonatal isoforms. 2) Specific force depends on the number of cross bridges in parallel, or MHC content per half-sarcomere. Studies were performed on single Triton-X permeabilized fibers activated at a pCa of 4.0. MHC isoform expression was determined by SDS-PAGE. MHC content per half-sarcomere was determined by densitometric analysis and comparison to a standard curve of known MHC concentrations. Fiber cross-sectional area and volume were determined by confocal microscopic analysis. Fast MHC isoform expression increased from 0 to 84 days resulting in an increase in specific force of ~50%. When normalized for MHC content, fibers expressing MHCslow and co-expressing MHCmed had lower force than fibers expressing fast MHC isoforms. This suggests a lower force per cross bridge in fibers expressing MHCslow and MHCmed.

696 - Pos

DERENATION REDUCES MAXIMUM SPECIFIC FORCE IN RAT DIAPHRAGM FIBERS EXPRESSING FAST MHC ISOFORMS

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Previously, we found that unilateral denervation (DNY) drastically reduced maximum specific force in the rat diaphragm muscle (DAM). We hypothesized that 1) the effect of DNY is more pronounced in fibers expressing fast MHC isoforms and 2) the effect of DNY on specific force reflects a reductions in myosin heavy chain (MHC) content per half-sarcomere, or the number of cross bridges in parallel. Studies were performed on single Triton-X permeabilized fibers activated at a pCa of 4.0. MHC isoform expression was determined by SDS-PAGE. MHC content per half-sarcomere was determined by densitometric analysis and comparison to a standard curve of known MHC concentrations. Fiber cross-sectional area and volume were determined by confocal microscopic analysis. After two-week DNY, the specific force of fibers expressing MHCfast was reduced by ~60% (MHCfast expression was absent) while the specific force of fibers expressing MHCmed and MHCslow was unaffected. DNY also reduced the MHC content in fibers expressing MHCmed with no effect on fibers expressing MHCfast and MHCslow. When normalized for MHC content per half-sarcomere, force generated by DNY fibers expressing MHCfast was lower than control fibers. These results suggest the force per cross bridge is also affected by DNY.

697 - Pos

KETTIN: A LINKER BETWEEN ACTIN AND MYOSIN IN DROSOPHILA INDIRECT FLIGHT MUSCLES

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Kettin is a ~500-700 kDa protein of insect striated muscle known to bind to actin in the Z-disk region of the sarcomere. To investigate a possible functional role of this protein, we performed stretch experiments on single myofibrils isolated from Drosophila indirect flight muscle (IFM). A sensitive force measurement method was employed along with fluorescence/immunofluorescence microscopy techniques. Stretching myofibrils from their slack sarcomere length (SL) of ~3.6 μm to 23.8 μm in relaxing solution led to substantial SL inhomogeneity; A-bands appeared damaged. Analysis of phase-contrast and fluorescence images of myofibrils stained with rhodamine-phalloidin suggested that on high stretch, actin filaments break in the I-band. Immunofluorescence images of myofibrils extended to SLs between 3.6 and 4.5 μm and labelled with fluoresec/r-male-anti-keratin antibodies showed strong Z-disk staining. In another set of experiments, a calcium-independent gelsolin fragment was used to extract the actin filaments. Actin removal was demonstrated by rhodamine-phalloidin staining. Upon stretch of gelsolin-treated myofibrils, SLs remained remarkably homogeneous, even when sarcomeres were extended to ~4.5 μm SL. Anti-keratin antibodies stained not only the Z-disks but also a distinct epitope in each half-sarcomere, which localized to the ends of the A-bands. Before and after gelsolin treatment, myofibrillar stiffness was determined from the force response to 20 Hz sinusoidal oscillations imposed by a micrometer. Following gelsolin application, we observed an immediate drop in myofibrillar stiffness to ~30% of the initial value; after 30 minutes of gelsolin treatment only ~5% of the initial stiffness remained. From these results, it appears likely that kettin functions as a stiff linker protein connecting actin and myosin filaments. Kettin could thus be responsible for the high stiffness of Drosophila IFM necessary for stretch activation.

699 - Pos

THE EFFECT OF DEUTERIUM OXIDE ON THE CONTRACTION CHARACTERISTICS OF GLYCEROL-EXTRACTED SKELETAL MUSCLE FIBERS

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To give information about the involvement of water in the characteristics of muscle contraction coupled with ATP hydrolysis, we studied the effect of partial or complete replacement of H2O in the experimental solution with D2O on the contraction characteristics of glycerol-extracted rabbit psoas fibers fully activated with CaCl2 (pCa 4). Muscle fiber stiffness was measured during both development and relaxation of isometric force by applying sinusoidal vibrations (0.1 Hz, 0.1% of LT) to the servo-motor. The force-velocity curve covering load range from 0 to 50% was obtained by applying a parabolic decrease in fiber length with the servo-motor. The Ca2+- activated isometric force increased by ~10% by replacing 25-50% of H2O with D2O, while it decreased with increased D2O concentration above 70%, being ~50% of the control value after complete replacement of H2O with D2O. The stiffness-isometric force relation remained unchanged in all the conditions examined. The maximum shortening velocity was reduced by D2O nearly in parallel with the Ca2+-activated isometric force. The curvature of the force-velocity curve was measured much less pronounced when both the Ca2+-activated isometric force and the maximum shortening velocity were reduced by ~50% with D2O, suggesting the modification of D2O of efficiency of chemo-mechanical energy conversion in muscle fibers.

700 - Pos

EFFECT OF EXTRACELLULAR CA ON THE PHOSPHORYLATION OF MYOSIN-BINDING PROTEIN (MYBP-C) IN CARDIAC MUSCLE

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Measurements of maximum Ca-activated force (Fmax), structure of thick filaments within the intact lattice and structure of isolated thick filaments all indicate Ca regulation of the contraction by change in a thick filament protein, presumably by phosphorylation. Because MyBP-C and regulatory light chain of myosin (RLC) are phosphorylated by Ca-regulated kinase (CAMK), we measured phosphorylation patterns of MyBP-C under 3 different conditions: 1) quiescent in 2.5 mM Ca KCl/solution (Ca-K); 2) quiescent in 1.25 mM Ca KCl; and 3) quiescent in 1.25 mM Ca KCl followed by quiescent for 10 min in 7.5 mM Ca-K. Quiescence alone will decrease intracellular Ca concentration by 60% (Bassani et al. Biolphys J.1995). Phosphorylation was measured by examining samples of MyBP-C and isoelectric focusing to separate each form. Phosphorylation is lower in 1.25 mM Ca-K than in 2.5 mM, and it is increased by 10 min in 7.5 mM Ca-K. Patterns of phosphorylation are different among the 3 preparations. Lowering Ca appears to block addition of the first phosphate. These results suggest that Ca-regulated kinase (CAMK) modulate the structure of the thick filament and the contractility of the cell. Supported by NIH grant to SW
781 - PoS

**EFFECT OF EXTRACELLULAR CA ON THE STRUCTURE OF THICK FILAMENTS IN CARDIAC MUSCLE**

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Thick filaments contain (MyBP-C) every 43 nm along the thick filament within the C zone. In heart, MyBP-C contains 3 phosphorylated by Ca-calcium regulated kinase (CAMK) and PKE. One site must be phosphorylated first, and this site may be preferred by phosphorylation by CAMK. To determine whether Ca-regulated phosphorylation of MyBP-C modifies contractility, quiescent cardiac cells were soaked in normal, reduced, and concentration of reduced followed by elevated Ca-KReps solution (Ca-K). This site was not phosphorylated by CAMK. In this study thick filaments were separated from identified cells and their structure examined electron microscopically in negatively-stained samples. Thick filaments structure is changed in 1.25 mM Ca-K. Orientation and of crossbridges change. Ten min in 7.5 mM Ca-K produces a second change. Apparently Ca regulated change in thick filaments can modulate contraction. Because phosphorylation of RLC increases Ca sensitivity without changing Fmax (Sweeney & Stall JIP 1986), phosphorylation of MyBP-C is the likely regulatory receptor (9 reported by NSH grant to SW)

782 - PoS

**THE EFFECTS OF TEMPERATURE AND P, ON SINGLE MUSCLE FIBER CONTRACTILE PROPERTIES.**

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Recent evidence has shown that the effect of a high [H+] on force (P), in single skinned mammalian muscles is significantly reduced at high temperatures (28-32°C). However, it is not yet known whether the effect of an increased intracellular phosphate (P) is attenuated at higher temperatures. The purpose of this study was to compare the effects of 30mM P, on the contractile properties of skinned single rat soleus type I muscle fibers at 15°C and 30°C. The contractile properties measured included P, maximum velocity (Vmax) and fiber power. Each fiber was subjected to 2 levels of P, (0 and 30mM P) and two temperatures (15 and 30°C). A 2-way repeated measures ANOVA revealed that the fiber power was reduced in P, associated with 30mM P, was significantly greater at 15°C (58%) vs. 30°C (22%) (Shown in Figures). Vmax was unaffected by P, at 15°C, and showed a significant increase of 24% at 30°C. Relative power was reduced by 41% at 15°C and 17% at 30°C (p<0.05). In addition, P, caused peak power to occur at a lower force level at both 15°C and 30°C. The reduced inhibitory effect of P, on contractile function at 30°C vs.15°C is a novel finding and implies that the inhibition of P, would be reduced as temperature increases.

783 - PoS

**TEMPERATURE CHANGE DOES NOT AFFECT THE FORCE MEASURED BETWEEN SINGLE ACTIN FILAMENTS AND HEAVY MEROYOMYSIN MOLECULES PREPARED FROM RABBIT SKELETAL MUSCLES.**

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The temperature dependence of sliding force, velocity, and unbinding force was studied on actin filaments when they were placed on heavy meromyosin (HMM) attached to a glass surface. A fluorescently labeled actin filament was attached to the glass-coated surface of a 1 μm poly- nystrene bead. The bead was trapped by optical tweezers, and HMM-actin interaction was performed at 20-35°C to examine whether or not force is altered by the temperature change. Our experiments showed that sliding force increased with temperature (Q10=1.4±0.4, n=10) and that the velocity increased more (Q10=2.9±1.1, n=11). The slight increase in force is caused by the increase in the number of available cross-bridges for actin interaction, because the cross-bridge number similarly increased with temperature (Q10=5.1±0.6, n=5) when measured during rigor induction. We further found that unbinding force measured during the rigor condition did not differ with temperature. These results indicate that the amount of force each cross-bridge generates in fixed and it does not change with temperature. We found that the above generalization was not modified in the presence of 1 mM MgATP or 8 mM phosphate.

784 - PoS

**FORCE RESPONSE TO FAST RAMP STRETCHES OF ACTIVATED FROG MUSCLE FIBRES IN PRESENCE OF METHANOL.**

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The experiments reported here were made to investigate the force response to ramp stretches of single frog muscle fibres in which the active tension generation was abolished by trapping the troposin-tropomyosin-actin complex in an off-state using methanol. Fibres were mounted between a fast force transducer and a moving coil motor using aluminium clips to reduce tendon compliance. Sarcomere length (L0) was measured with a striation follower device. A series of ramp stretches of various amplitude (<1% ±1%) and velocity (5-20 s-1) was applied to the fibres in normal Ringer and in presence of added methanol. Methanol (5%) completely and reversibly abolished both twitch and tetanic tension. However, in agreement with previous results with HMM, the stretch applied to the stimulated fibres (developing zero tension), produced a force transient significantly greater than the passive force response, indicating an increase in fibre stiffness. Following a single stimulus fibre stiffness increased before tension and decreased back to the resting level before the peak of the twitch. Following a tetanic stimulation fibre stiffness remained elevated for about the stimulation time. This agrees with the idea that stiffness increase follows the time course of intracellular Ca²⁺ concentration.

785 - PoS

**EFFECT OF TEMPERATURE ON THE CROSS-BRIDGE KINETICS IN RABBIT SOLEUS SLOW-TWITCH MUSCLE FIBERS.**

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Elementary steps of the cross-bridge cycle in slow-twitch fibers from rabbit soleus were studied in the temperature range 5°C - 37°C by sinusoidal analysis. Effects of MgATP and phosphate were studied, and the temperature dependence of the kinetic constants was deduced. The MgATP association constant decreased when temperature was increased. The association and backward rate constants of ATP-actinization and cross-bridge detachment steps had Q10 of about 3, hence their equilibrium constants changed little with temperature. Q10 of the force generation step was 6.2 and 0.8 of its reversal step was 2.5, hence its equilibrium constant increased significantly with temperature. This temperature effect was fitted to the modified Van't Hoff Equation to deduce ΔΗ° (standard enthalpy change, 72±20 kJ/mol), ΔS° (standard entropy change, 250±70 J/K/mol), ΔG° (free energy change, 125±5 kJ/K/mol). These results indicate that the force generation step is an entropy driven, endothermic reaction that accompanies burial of large surface area. These observations are consistent with the hypothesis that the hydrophilic interaction between residues of actin and myosin and between residues of myosin head underlies the mechanism of force generation. The phosphate association constant changed little with temperature. A decrease in isometric tension with temperature is explained by the increased number of cross-bridges in tension generating states.

786 - PoS

**Ca²⁺-RIGOR AND Ca²⁺-FREE RIGOR: A DIFFERENCE IN DISTRIBUTION OF CROSS BRIDGE LENGTH.**

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The tension development in rigor depends among different other factors on the presence or absence of Ca²⁺. This difference can be explained by a difference of the distribution function of the length of pulling cross bridges. To investigate we measured the relation between stiffness, both in- and out-of-phase, the developed tension and strain put on the fiber and so on the cross bridge of skinned frog single muscle fibers. The single fiber was brought in rigor by removal of ATP. [Ca²⁺] was 0 or 10^5 M. The strain was put on the fiber by changing its length. Initial sarcomere length was set at 2.15 μm. Stiffness in steady state was measured by means of a 0.05% step three minutes after the conditioning length settings between -0.8 and 1.5% of the initial length. In-phase steady stiffness proved to be dependent on [Ca²⁺]. The relation between in-phase stiffness and strain was 2.3 nm/half sarcomere shifted leftward, when [Ca²⁺] was increased from 0 to 10^5 M. The stiffness curved horizontally at 2 nm/half sarcomere at [Ca²⁺]=0, while at [Ca²⁺]=10^-5 M this already occurred at the first stretch. These results proved to be independent of frequency. When the fiber was slowly stretched up to 12 nm sarcomere shortening occurred. This stiffness did, however, not relax as fast at a slower rate.

Further stretching causes both detachment of cross bridges at large length and replenishment of pulling cross bridges at the low end of the distribution.
707 - Pos EFFECTS OF TEMPERATURE AND IONIC STRENGTH ON MYOSIN REGULATORY LIGHT CHAIN (RLC) ORIENTATION IN SINGLE SKELETAL MUSCLE FIBERS.
Pairs of cysteine residues were inserted in chicken gizzard RLC at positions 100-108, 100-113, 104-115, or 108-113 and cross-linked by bifunctional rhodamine (BR; Corrie et al., Nature, 400, 423-430, 1999). The BR-RLCs were then used to immobilise single fibres from rabbit psoas muscle. RLC orientation was measured by fluorescence polarisation as the tilt ($\beta$) between the fibre axis and the lever axis of the RLC region and the twist ($\gamma$) of the RLC region around the lever axis (ibid). Orientational disorder was modelled using Gaussian distributions of $\beta$ and $\gamma$. In relaxed fibres the orientation parameters were independent of temperature in the range 5-20°C, where the helical order of myosin filaments is expected to change. When ionic strength was lowered from 150 to 20 mM in relaxation (2°C), $\beta$ did not change but $\gamma$ increased towards its rigor value; this increase was dependent on overlap between myosin and actin filaments. When temperature was increased in ~1°C from 5 to 20°C during active contraction, $\gamma$ again increased reversibly without significant change in $\beta$. This orientation change is distinct from that produced by imposing rapid shortening steps during active contraction (ibid.). Supported by MRC, Welcome Trust and BBSRC (UK).

708 - Pos CONTRACTILE PROPERTIES OF ISOLATED MUSCLE SPINDLES FROM FROG.
Frog intact muscle spindles were mounted in an organ bath (1-3°C), and force and shortening velocity were recorded both from the muscle spindle as a whole and from short marked segments along the spindle using force-clamp and segment length-clamp techniques. The rate of rise of force during tetanus was less than 1/3 of that recorded in ordinary fibers of the same muscle species. Maximum tetanic force produced by the spindle fibers was likewise smaller (98 ± 10 kN m², mean ± SEM, n = 11) than in regular fibers (280 ± 10 kN m², n = 20). The force-velocity relation had the same biphasic shape as previously observed in ordinary muscle fibers, the transition between the two phases being close to 80% of maximum tetanic force (cf. Edman, J. Physiol. 1988, 404, 301-321). However, the speed of shortening of the spindle fibers, measured at any given load, was lower than that in ordinary fibers. $V_{max}$, the maximum speed of shortening, measured in isometric- and capsule-free segments was 0.96 ± 0.07 ML s⁻¹ (n = 6), which is merely half the value of $V_{max}$ recorded in ordinary fibers at the same temperature (Edman, 1988, see above). The intracellular Ca⁺⁺, sent, based on measurements with fluo-3, did not differ markedly from that in ordinary muscle fibers.

709 - Pos EFFECTS OF RAPID SHORTENING ON ATP HYDROLYSIS BY ACTIVE SKELETAL MUSCLE FIBERS.
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The ATPase of single skinned muscle fibers immersed in silicone oil was measured continuously using an NADH-linked fluorescence assay (excitation 365 nm; emission 425-475 nm). Artefacts due to fiber movement in the excitation beam were eliminated using carboxy-xanthydramine fluorescence at 570-650 nm. Active isometric ATPase rate was 0.18 mM s⁻¹ in rabbit psoas fibers at sarcomere length 2.70 µm, 10°C. During shortening at maximal velocity (1.2 fibre lengths s⁻¹; measured by stack test) the ATPase increased to 1.7 mM s⁻¹. Force redevelopment at sarcomere length 2.43 µm after shortening was fitted by a double exponential with rate constants ~18 and ~3 s⁻¹. There was no evidence for extra ATP hydrolysis accompanying force redevelopment, and the steady rate of post-shortening ATPase was 0.20 mM s⁻¹. Implosion of a rapid stretch to sarcomere length 2.70 µm at the end of shortening also had little effect on ATP hydrolysis. Repeated shortening-restretch cycles separated by a variable interval of isotonic contraction were used to study the kinetics of the re-establishment of the isotonic distribution of ATPase intermediates after shortening. This process appears to be substantially slower than the rate of isotonic force redevelopment. Supported by Wellcome Trust UK.

710 - Pos TEMPERATURE DEPENDENCE AND EFFECTS OF PEG STRONGLY CONSTRAN CROSS BRIDGE MODELS.
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Fully activated rabbit psoas fibers were tested for isometric force ($F_i$), stiffness by small steps ($k_s$), and stretch force ($F_s$) by constant velocity ramp stretches (5% $k_s$ at 2 kHz), at temperatures (T) of 5, 10, 20 or 30 degrees, in the presence or absence of 5% PEG 4000. Stiffness was independent of temperature in the absence of PEG, but declined with temperature in the presence of PEG. $F_s$ increased with temperature, both in the absence and presence of PEG. $F_s$ increased with temperature in the absence of PEG, but decreased with temperature in the presence of PEG. Both $k_s$ and $F_s$ were greatly enhanced by PEG, but $F_s$ was enhanced only mildly. The $k_s$ and $F_s$ data are consistent with models including at least two attached states, in which an increase in $T$ causes a population of more forceful, strongly-bound cross bridges to displace a population of less forceful, weakly-bound cross bridges. The $F_i$ data are consistent with models in which $P_i$ is produced primarily by the weakly-bound cross bridges. The simplest model consistent with all the data has a strongly-bound force-producing state that is strengthened by increasing $T$, but is relatively unaffected by PEG, and a weakly-bound stiffness-producing state that becomes stiffer with PEG, and is relatively unaffected by $T$.

711 - Pos POLARIZED FLUORESCENCE DEPLETION REPORTS ORIENTATION DISTRIBUTION AND ROTATIONAL DYNAMICS OF MUSCLE CROSS-BRIDGES.
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A novel method has been developed to obtain high resolution orientational distributions and dynamics from fluorescence polarization (FP) experiments in muscle fibers. Gizzard myosin regulatory light chain (RLC) was labeled with the 6-isomer of IATR (Ling et al., Biophys. J. 70:1836, 1996) and exchanged into rabbit psoas muscle fibers. FP data from single probes were limited to the 2nd- and 4th-rank order parameters, $P_i$(cos $\theta$) and $P_i$(cos 4$\theta$), of the probe angular distribution ($\theta$) relative to the fiber axis and $(P_s)$, a coefficient describing extent of rapid probe motions (Dale et al., Biophys. J. 76:1606, 1999). We applied intense 12 µs photoexcitation pulses to transiently populate the probe triplet state and measured polarization of the ground state depletion using a weak interrogation beam. Transient photobleaching provides $P_i$(cos 4$\theta$) as well as $P_i$(cos $\theta$) and $(P_s)$, which describe the extent that $(P_i)$ and $(P_s)$ are reduced due to motions on timescales longer than the fluorescence lifetime but shorter than the photoexcitation pulse. In active contraction RLC motions were intermediate between relaxed and rigor $(P_i)$ and $(P_s)$ values ± s.d. in Table. Due to the extensive mobility present in active and relaxed fibers, $(P_i)(0.14 ± 0.08)$ was available only in rigor. These results indicate that the previously observed disorder of the light chain region in contraction can largely be accounted to dynamic motions. Supported by NIH, MRC and MDA.

120A
712 - Pos INTERHEAD DISTANCE MEASUREMENTS IN DIMERIC NCD BY FRET
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Kinesin and nod are motor proteins which walk's along the microtubule lattice. Although several X-ray structures of these proteins have recently been solved, no experimental evidence is available concerning changes in the head-to-head distances in relation to the nucleotide intermediate. We have modified E. coli-expressed dimeric noc comprising as 250-700 and measured the interhead distance by FRET. The labeling stoichiometry with either the donor (monobromokhmalate, MBM) or the acceptor (DABM) was 1:1 (40:1) per head. Several lines of evidence suggest that the modified residue is C670. For FRET measurements noc was initially labeled with MBM (5-10%) and the remaining thyros were saturated with DABEM. From a large FRET efficiency (ca. 50%) observed the distance between Cys residues was estimated to be about 30-35 Å, in good agreement with the X-ray derived separation. A small but discernible effect (6%) of ATP and AMP-PNP on the fluorescence intensity of the donor was seen. The influence of these 2 nucleotides on the interhead separation was examined and found to be negligible. The data are consistent with a model of kinesin movement in which structural alterations in the motor domains leading to a change of the distance between the heads occur only upon formation of a ternary complex with microtubules.

713 - Pos CLOSING THE NUCLEOTIDE TRIPHOSPHATE BINDING DOMAIN IN NCD Todd Melchardt*, Roger Cooke', E. Paté', Peter Kollman', UCSC, WSU, Pullman, WA 99164

We have simulated a conformational change in the kinesin family motors using molecular dynamics simulations (MDS). In all myosin crystal structures, the substrate triphosphates are enclosed in a narrow tunnel-like structure, the "Pi-tube." The catalytic domains of myosin and kinesin-family motors have considerable structural homology. A significant difference is at the triphosphate binding domain, where switch 1 has pulled away from the triphosphates in all kinesin-family structures. This opens one side of the Pi-tube, exposing the triphosphates to solvent. We have previously shown that the closed, myosin Pi-tube must open during the hydrolysis cycle, a state missed by the crystal structures. The reverse question is whether the Pi-tube can close in kinesin-family motors? We have addressed this question using simulated annealing and MDS. Using Ds1 as a template, the coordinates of switch 1 of noc were modified manually to close the Pi-tube. Four molecules, noc with the open and closed structures with either bound MgADP or MgATP were hydrated and energy minimized. AMBER MDS (500 psec, 300C) showed that for the noc, closed Pi-tube, there are abundant interactions between triphosphates and protein, homologous to those seen for myosin. In contrast, for the open Pi-tube, the interactions between protein and nucleotide remain pathetic. A clear trend was seen in the free energies of the various configurations. The closed Pi-tube structures were more stabilized compared to the open structures, and with ATP more stable than ADP. These simulations provide structural and energetic support for the hypothesis that the switch 1 region in kinesin-family motors moves over the substrate, forming a Pi-tube, similar to that seen for myosin.

714 - Pos MECHANISM OF FORCE PRODUCTION BY SINGLE KINESIN MOLECULES
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How do mechano-enzymes such as kinesin, myosin, and polymers convert biochemical energy into mechanical work? Prior theoretical models for kinesin, which has two ATPase motor domains and moves along microtubules (MTs) in discrete 8-nm steps, are "loosely-coupled" and predict that the number of ATP hydrolyses per step increases with applied load. Here, we show that a simple "tightly-coupled" mechanism, in which this coupling constant stays fixed at 1:1, offers several advantages. The mechanism: (i) pinpoints the primary effect of load to a conformational change immediately following ATP binding; (ii) naturally leads to tight-coupling, as found experimentally, because hydrolysis occurs only after a motor domain undergoes this conformational change; (iii) provides a quantitative account of velocity data over a wide range of loads and ATP levels; (iv) makes new predictions that are testable with current experimental methods. Like transcription by RNA polymerase (RNAP), the kinesin mechanism involves thermally activated transitions within a free energy landscape. Similar considerations explain our observations of kinesin proactivity: The mean distance traveled before detachment obeys a Michaelis-Menten equation with load-dependent parameters. Thus, free energy landscape models may help unify understanding of seemingly disparate motor proteins.

715 - Pos ATPase KINETIC CHARACTERIZATION OF MUTANT HUMAN KINESIN MOTORS DEPENDENT UPON NUCLEOSIDE TRIPHOSPHATE BINDING.

The 3D structures of conventional kinesin motor domain have been obtained, but the structural bases of how MTs stimulate kinesin ATPase as well as how ATP turnover is coupled to force-production are still unknown. Woehlke et al. (Cell 90: 207, 1997) generated 35 alanine mutations in kinesin motor domain residues that identify three ATPase domains (Y138A, loop 1 (L248A/D249A), and E250A). Here, we examined three mutants: Y138A, loop 1 triple mutant (L248A/D249A/E250A), and E311A that exhibited 3-fold reductions in both MT gliding velocity and ATP-stimulated ATPase activity. Here, we examined the underlying defects in these mutants by steady-state kinetic analysis. Mutants exhibited wild type second-order ATP binding kinetics. The Y138A and loop 1 triple mutants exhibited defects in nucleotide hydrolysis (>5-fold decrease in rate), even though the mutated residues are not located within the active site. These mutants also exhibited 2-4 fold reduction in MT-stimulated ADP release rate. Suggesting that Y138 and loop 1 may participate in the allosteric pathway in which MT binding leads to accelerated product release from the active site. Finally, with the E311A mutant, we found that neither the rate of nucleotide hydrolysis nor ADP release can account for its slow ATP turnover and gliding velocity. A step subsequent to nucleotide hydrolysis but before ADP release appears to be rate-limiting. We speculate that E311A, a highly conserved residue in the kinesin superfamily, plays an important role in force production.

716 - Pos CROSSLINKING OF THE KINESIN NECK COILED-COIL: EFFECT ON PROGRESSIVE MOVEMENT ALONG MICROTUBULES.
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Kinesin can move along a microtubule for several micrometers without dissociating. Two heads of a dimer are thought to move processively in a head-over-head manner; however, the mechanism of such cooperationality remains unknown. A point of controversy concerns whether the neck-coiled-coil adjacent to the catalytic domain needs to extensively unwind during the mechanochemical cycle to allow one kinesin head to reach a new tubulin binding site. To test this model, we constructed a kinesin mutant in which the neck coiled-coil can be covalently stabilized by a disulfide bond. We used a cysteine-like 560-amino acid kinesin fused to GFP with residue 344 replaced with a cysteine residue. Under reducing conditions, single GFP-tagged Y344C molecules moved along axonemes with a similar velocity and run length to wild type kinesin (21 mm/min and 1.1 mm, respectively). After oxidation, ~70% of kinesin dimers became crosslinked. This treatment decreased the run length by 30%, but did not alter the velocity. Subsequent treatment with a reducing reagent completely recovered the run length. We are working to observe motility using kinesin crosslinked with higher efficiency and at other points along the coiled-coil. Our present results suggest that the neck coiled-coil does not need to unwind for processive movement of the kinesin dimer along a microtubule.

717 - Pos STATISTICAL ANALYSIS OF KINESIN KINETICS BY APPLYING METHODS FROM SINGLE CHANNEL RECORDINGS.
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We measured the motility of kinesin-coated beads at saturated ATP concentrations and low loads with three-dimensional optical trapping interferometry. Under these conditions high thermal fluctuations of the marker bead occurred and represented a limiting factor to spatial resolution. In order to overcome this problem, we analyzed the three-dimensional displacement traces by using a suit of programs designed to ease analysis of Single Channel records. This method allowed us to resolve the discrete stepping of kinesin and the stepping rate constants at high ATP concentration and load. We could detect also backward steps at low load. At high loads of more than 6 pN and a bandwidth of 50 kHz a periodic clustering of 8 run steps could be observed. We compared the dynamic behavior of a wild type kinesin constructs with the one of a single-headed mutant. The truncation of one motor head led to a dramatic decrease of the force generation of kinesin from about 6 pN (for the wild type) to less than 0.25 pN for the chimera.
THE RISING PHASE OF KINESIN'S 8NM STEP

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Using laser-trapping nanometry, it has been shown that single kinesin molecules slide along microtubule with 8nm steps. To understand the mechanism of this sliding movement, details of the steps must be scrutinized with high space and temporal resolution. We improved the resolution by using a small bead of 0.2um in diameter, which has lower viscous drag than beads of 0.5-1um previously used. This small bead was diagonally illuminated by a focused laser beam, and the light scattered by the bead was projected on a quadrant photodiode as a dark-field image. This system allowed us to measure displacement of single kinesin molecules with a temporal resolution of 10ps at a spatial resolution of 3nm. This temporal resolution was 20-fold higher than that of previous apparatuses. The step size of kinesin molecules was 8nm. The rising phase velocity of 8nm step, which was defined as a slope at the rising phase, was ~100nm/s, regardless of ATP concentration. This velocity is about 100-fold faster than the average sliding velocity of kinesin, indicating that the duty ratio is 1% of the overall ATPase cycle time.

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MOION OF A SINGLE MOLECULE OF CATALYTIC CORE DOMAIN OF KINESIN ALONG A MICROTOUBULE IN THE PRESENCE OF ADP

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In order to study the roles of the two-headed structure and the neck region of kinesin in movement, we have examined the motility of Drosophila kinesin fragments truncated at 411, 351, 340, 327 amino acid residues. Movements of single molecules of kinesin fragments labeled with Cy3-C-terminus or Cy2-C-terminus on their C-terminus (411-Cy3, 351-Cy3, 340-Cy3, 327-Cy3), were observed on a microtubule (MT) with low-background total internal reflection fluorescence microscopy (Vale et al., 1996). Fluorescence intensities showed that 351-Cy3, 340-Cy3 and 327-Cy3 were monomers, while 410-Cy3 dimer, in agreement with the sedimentation assays in solution. Single one-headed kinesin, 351-Cy3 and 340-Cy3 progressively moved along a microtubule although the travel distance was short (~0.1um) as presented (Inoue et al., ’3rd Annual Meeting), indicating that the two-headed structure is not essential for processive movement. In this study, we examined the motility of 327-Cy3, which contains catalytic core domain and lacks the neck region. In the presence of ATP, 327-Cy3 rigidly attached to a MT and detectable movement was not observed. In the presence of ADP, where kinesin is known to weakly bind to a MT (Lomborg et al.,1993), we found that 327-Cy3 molecules continuously moved along a MT. The maximum travel distance without detaching from a MT was 1.1um. However, the movement was not unidirectional but random in both directions, that is, thermal. These results suggest that movement of kinesin is originally thermal motion and the neck region plays an important role to bias the thermal motion for unidirectional movement.

The conventional kinesin heavy chain (KHC) forms a homodimer that moves on microtubule (MT) tracks by the force generated from ATP hydrolysis. Using mutants in the unc-116 gene in Caenorhabditis elegans, defective in the KHC, we have examined genetic interaction between the KHC and genes encoding other kinesin like proteins. Here we propose a model in which an atypical kinesin like protein VAB-8, may serve as a link between the MT based KHC movement and the actin based motility. VAB-8 has a motor domain located in the N-terminal that has some but not all residues critical for the formation of the ATP binding pocket and its carboxy terminal tail which in delink the actin based motility. Genetic interaction data suggest that coexpression of unc-116 and vab-8 is essential for the viability and growth of animals. Similarly, using West-Wester blotting approach, we have shown that VAB-8 binds directly with actin as well as tubulin, an actin monomer. Genetic interaction data provide a critical link between the microtubule and actin-myosin based intracellular transport.

THE SINGLE-MOLECULE INTERACTION OF MONOMERIC KINESIN WITH MICROTOUBULES

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Conventional kinesin is a processive enzyme, able to execute hundreds of rounds of ATP hydrolysis and movement without detaching from a microtubule. The prevailing model asserts that kinesin uses its two motor domains in an alternating, hand-over-hand fashion. Accordingly, truncated kinesins that lack the dimerization domain(s) are non-processive in motility experiments. However, kinetic determinations of the number of ATP's hydrolyzed per microtubule-kinesin interaction consistently give numbers larger than 1, and some monomers appear as "processive" as dimers under the kinetic conditions with hydrolysis of ~18-28 ATP's per interaction (Jiang et al., J. Biol. Chem. 272 (1997) p. 7626, Moyer et al., Biochem. 37 (1998) p. 800). How should the chemical processivity of monomers be interpreted, and what does it tell us about the mechanism? One interpretation with recent support is that the second motor domain is required to pull the first off the microtubule (Hancock et al., J Cell Biol 140 (1998) p. 1395). Hence, monomeric kinesins are defective in releasing from the microtubule, and execute multiple rounds of ATP hydrolysis at a single binding site. However, ATP hydrolysis events separated by 1-dimensional diffusion of the kinesin along the microtubule would be equally consistent with the kinetic data.

To determine the processivity of kinesin monomers using an independent observable and gain insight into the corresponding mechanical behavior, I have imaged the interaction of monomeric kinesin-GFP molecules with anomalous microtubules. The residence time an individual motors spends within a diffraction-limited area decreases with increasing [ATP], and corresponds to 2-4 ATP's for the human kinesin construct (K339-GFP) employed. Further details will be presented.
SUNDAY

KINESIN & KINESIN-FAMILY PROTEINS

724 - Pos RIBOSE-MODIFIED ATP ANALOGS (EDA-ATP AND 2'3'-O-Cy3-EDA-ATP) ARE SUBSTRATES FOR INNER-ARM DYNEIN SUBSPECIES-C OF CHLAMYDOMONAS

FLAGELLUM, A PROCESSIVE SINGLE-HEADED-MOTOR.

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Inner-arm dynein sub-species-c from Chlamydomonas flagellum is a single-headed dynein motor that moves processively along microtubules [Sakakibara, et al., Nature, 1999, 400, 586-590]. To characterize its enzymatic properties and investigate further its processivity, we tested as substrates the ribose-modified ATP analogs, 2'3'-O-[4-(2-aminomethyl)carboxamido]yl)-ATP (EDA-ATP) and 2'3'-O-Cy3-EDA-ATP with kinetics similar to ATP, and the latter, conjugated with the strong Cy3 fluorophore, have been used in single molecule kinetic studies [Eccleston et al., 2016, Biophys. J. 70, A159; Oiwa et al. ibid]. Under steady-state conditions, dynein sub-motors hydrolyzed 2'3'-O-Cy3-EDA-ATP with turnover rate 0.036 s⁻¹ but the 3'-O-Cy3-EDA-ATP isomer at 0.016 s⁻¹, both much slower than for ATP, 0.68 s⁻¹. Both isomers of Cy3-EDA-ATP were poor substrates for the movement of microtubules in vitro by dynein sub-species c. In contrast, EDA-ATP, the precursor of Cy3-EDA-ATP, was a good substrate for such movement: the velocity of taxol-stabilized microtubules gliding on dynein-coated surfaces was 1.4 µm s⁻¹ at 1 mM EDA-ATP, comparable to that with 1 mM ATP. These results suggest that 2'-O- and 3'-O-ethylendiamine-linked ATP derivatives are potential substrates for dynein sub-species-c with the 2'-O-isomer being preferred over the 3'-O-isomer.

SUNDAY

MODIFIERS OF RYR FUNCTIONS

725 - Pos METHANETHIOISULFONATE DERIVATIVES INHIBIT THE CARDIAC AND SKELETAL CA²⁺ RELEASE CHANNELS.

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The effects of the methanethiosulfonate derivatives MMTS, MTSES and MTSEA⁺ on the cardiac and skeletal muscle Ca²⁺ release channels (ryanodine receptors) were determined in single channel measurements. SR vesicles or purified release channels were incorporated into planar lipid bilayers. The three MTS compounds caused a time-dependent and voltage-independent inactivation of channel activity in both channel isoforms when added to the cis (cytosolic) side of the bilayer. Channel current amplitude was not significantly changed, at variance with a previous report (J. Gen. Physiol. 1997, 255-264). Lumenal MMTS and MTSEA⁺ were less effective in inactivating the channels, and lumenal MTSES was without effect. Cytosolic thiold scavengers eliminated the effect of lumenal MMTS, MTSES and MTSEA⁺, did not prevent inactivation of channel activity by cytosolic MTS compound. We suggest that MTS compounds inactivate the cardiac and skeletal muscle release channels by reacting with cytosolic sulfhydryl groups of RyR. Supported by NIH.

726 - Pos IDENTIFICATION OF CYSTEINE 3635 OF RYR1 AS A TARGET OF OXIDANTS AND NITRIC OXIDE.

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RYR1 is modulated by oxidants and nitric oxide (NO). Oxidants increase channel activity and produce inter-subunit disulfide bonds. NO blocks the effects of oxidants on the channel. Recently, we demonstrated that oxidation blocks calmodulin (CaM) binding to RYR1 and, conversely, that CaM prevents oxidation of RYR1. In contrast, NO inhibits the binding of Ca²⁺ free calmodulin (Apocalmodulin) but not Ca²⁺CaM. Triglyceride digestion of RYR1 in the presence of CaM, followed by N-terminal sequencing reveals that bound CaM protects against cleavages at amino acids 3630 and 3637. The amino acid sequence between these cleavage sites is AVVACFIR. In this study we asked whether the cysteine at position 3635 is one of the cysteines altered by oxidation and nitrosylation. Nitrosylation or alkylation prevents formation of inter-subunit disulfide bonds. Treatment of SR membranes with CaM prior to alkylation with N-ethylmaleimide (NEM), however, protects both cysteines needed for inter-subunit disulfide bond formation. Using Edman degradation we demonstrate that cysteine 3635 is rapidly alkylated by [3H]NEM under conditions that label less than 5% of the total sulfhydryls on RYR1 and that bound CaM protects this cysteine from alkylation. We propose that cysteine 3635 is a target of both oxidants and NO and is located near or within the CaM binding site.

727 - Pos HYPERREACTIVE SULFHYDRL МоЕTIES CONSTITUTE A REDOX GRADIENT SENSOR OF RYANODINE RECEPTOR TYPE 1 COMPLEX.

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Sulfhydryl modifying reagents alter the activity of ryanodine receptors (RyR1). A few highly reactive, functionally important sulfhydryl moieties within the RyR complex were previously identified with CPM (7-diethyl amino-3-(4-maleimidophenyl)-4-methylcoumarin). To elucidate their possible physiological significance, we measured the reconstituted RyR channel activity in planar lipid bilayer (BLM) in the presence of transmembrane redox potential gradient formed by reduced glutathione (GSH) and glutathione disulfide (GSSG). The redox potential on either side of the BLM alone induces no significant change in channel open probability (Po). However, only when the redox potentials were set across BLM, did channel Po begin to closely follow the transmembrane redox gradient. With a fixed lumenal redox potential (within the physiological range of +160 to +185 mV, Hwang et al. 2004), sequential elevation of the cytoplasmic potential from -220 mV to -160 mV significantly enhances channel Po. Po of RYR channels responded to change in transmembrane redox potential gradient. This suggests that hyperreactive thiols lost their response to transmembrane redox potential gradients without exhibiting overt changes in channel gating characteristics. Similarly, this loss of redox control could be demonstrated by exposing native channels reconstituted in BLM to 40 nM CPM for 5 min in the cis chamber. These results reveal that hyperreactive thiols in the RyR1 complex constitute an essential component of a redox gradient sensor. Sponsored by NIH.

728 - Pos REDOX STATE OF PURIFIED SKELETAL MUSCLE RYANODINE RECEPTOR ALTER CHANNEL RESPONSE TO MODULATORS.

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We have recently identified two classes of RyR1 channel activity with distinct open probabilities (termed high-Po and low-Po) upon exposure to Ca²⁺ concentrations in rabbit skeletal muscle. Effects of redox reagents and channel modulators on the high-Po channel and the low-Po channel were compared to characterize the two channels in detail. When KCN was used as a current carrier, the channel conductance and mean open time were similar between channels. Addition of DTT converted the high-Po channel to a state similar to the intact low-Po channel. The high-Po channel responded to caffeine, and adenine nucleotides. The low-Po channel was activated by an oxidant, pCMPS, dose-dependently. The Ca²⁺ dependence of the oxidized low-Po channel was similar to that of the intact high-Po channel. The low-Po channel was insensitive to caffeine or adenine nucleotides, but could still respond to pCMPS. All channels were locked at subconductance open state after exposure to ryanodine, and closed by subsequent addition of ruthenium red. These results suggest that redox states of the channel alter the response to some channel activators such as Ca²⁺, caffeine and adenine nucleotides, as well as the channel gating.
Recent studies indicate that calcium release channel (RyR) is thought to be a candidate target for certain therapeutic interventions. The RyR, a member of the ryanodine receptor family, is expressed in skeletal muscle, heart, and neuronal tissues. It is a non-selective cation channel that is responsible for initiating calcium release from intracellular stores. RyRs are involved in various physiological processes, including muscle contraction, neurotransmitter release, and cardiac arrhythmias.

In skeletal muscle, RyRs play a crucial role in regulating calcium release during excitation-contraction coupling. The RyR channels are tetrameric proteins composed of four identical subunits. Each subunit consists of a large cytoplasmic domain, an intramembrane domain, and an extracellular domain.

The RyR channels are sensitive to various modulators, including inorganic ions, neurotransmitters, and second messengers. One of the key modulators is calcium itself, which can reversibly regulate RyR function. Calcium binding to RyRs is a critical mechanism for modulating their activity and is thought to be involved in the gating of RyR channels.

In addition to calcium, other ions such as magnesium and potassium also modulate RyR function. For example, magnesium ions can shift the calcium release curve to the left, indicating a higher affinity of RyR for calcium. Similarly, potassium ions can shift the calcium release curve to the right, indicating a lower affinity for calcium.

Furthermore, RyR function is regulated by a variety of post-translational modifications, including phosphorylation, nitrosylation, and ubiquitination. These modifications can alter the gating properties of RyR and modulate its activity.

In conclusion, RyR channels are complex ion-conducting proteins with multiple regulatory mechanisms. Understanding the molecular basis of RyR modulation is essential for developing new therapeutic strategies to treat various disease conditions associated with dysregulated RyR function.
734 - Pos
DIRECT MEASUREMENT OF TENSION DEVELOPMENT IN THE MEMBRANE OF THE CONTRACTILE VACUOLE OF PARAMECIUM
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The contractile vacuole of Paramecium multimicronucleatum shows periodic rounding—slacking cycles even after it has been isolated from the cell. This cycle is thought to reflect the periodic change of the tension in the contractile vacuole membrane, and/or a surrounding structure. We employed a novel technique to measure the tension development of the isolated contractile vacuole membrane. A cantilever was made from a long elastic carbon fiber (8 μm) with a piece of short glass rod (2 μm) glued perpendicularly to the tip of the fiber. The contractile vacuole, isolated with the cell with a small amount of cytosol, was suspended in mineral oil with the aid of a suction pipette. The membrane of the contractile vacuole was pushed against the glass tip so that the cantilever was bent away from the vacuole. Displacement of the cantilever tip was measured by the use of a position sensor. The obtained results indicate that during the rounding phase, the tension of contractile vacuole membrane reaches 5 mN m⁻¹, which is more than 10-fold higher than that of the slackening membrane but is within the limits of maximum tension, without breakage, which has been reported for stretched biological membranes. The mechanisms of tension development will be discussed in terms of membrane tabulation. Supported by NSF MCB 8909929.

735 - Pos
A DNA PUMP ISOLATED IN ITS INTERMEDIATE STATES.
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Bacteriophage T7 pumps DNA into a protein capsid, during its morphogenesis in an infected cell. To characterize intermediate states of the T7 DNA packaging, T7 capsids that have incompletely packaged DNA (ipDNA-capsids) have been isolated and characterized. Aggregation of ipDNA-capsids had thwarted their complete purification, in previous studies. Purification of ipDNA-capsids has been achieved here by revising our previously-used procedure of centrifugation in a cesium chloride density gradient, followed by buoyant density centrifugation in a metrizamide density gradient. DNA-free capsids are in two categories, metrizamide high density (MHD) and metrizamide low density (MLD). A permeability barrier present in MLD capsids causes the low density via a high hydration. Some ipDNA-capsids are found here to be in the MLD category. These ipDNA-MLD capsids have a permeability barrier that is possibly a gate (valve) that is part of the pumping cycle. The ipDNA of ipDNA-MLD capsids varies in length. The ipDNA-capsids are fractionated by ipDNA length during buoyant density centrifugation in a cesium chloride density gradient. The ipDNA-MLD capsids are unusually stable; 80°-85°C is required to expel ipDNA from an MLD capsid. The ipDNA-capsids have a variable mobility during non-denaturing agarose gel electrophoresis. The variable mobility also appears to be a consequence of change in state during the pumping cycle. This work was supported by a grant from NIH (GM24365).

736 - Pos
MECHANICAL PROPERTIES OF A SINGLE-HEADED PROGRESSIVE MOTOR: INNER-ARM Dynein SUBSPECIES C OF CHLAMYDOMONAS FLAGELLA STUDIED AT THE SINGLE MOLECULE LEVEL.
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Inner-arm dynein subspecies c from Chlamydomonas flagella is a single-headed dynein which is capable of generating progressive movement of microtubules [Sakakibara, et al., Nature, 1999, 400, 586-590]. To characterize its mechanical properties and investigate further its processivity, we measured force and movement developed by a single molecule of the dynein subspecies c with the optical trap nanometry. A polyacrylamide latex bead coated with a small number of dynein molecules was captured with the optical trap and brought into contact with a microtubule fixed on surface of a coverslip. The movements of the beads were measured by the quadrant photodiode sensor with nanometer- and millisecond-resolution. We showed that the beads carrying a single subspecies c molecule move progressively along the microtubule in 8-ns steps but slip backwards under high loads. The force-velocity relation of single subspecies c molecules was measured and was almost linear: the velocity decreased linearly with increase in loads but decreased more steeply near the maximal load. These results indicate that dynein subspecies c functions in a very different way from conventional motor proteins, and has properties that could produce self-oscillation in vivo.

737 - Pos
THERMODYNAMICS OF A MOTOR ENSEMBLE
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We present a thermodynamic model for an ensemble of biomolecular motors that step along tracks with motor-track (e.g., myosin-actin) binding and drive tracks in the opposite direction with motor-track detachment. While a motor-track binding equilibrium generates no net movement, chemical perturbations of this equilibrium bias net movement in a direction that opposes the direction of the chemical perturbation (i.e., the irreversible biochemical step). Based on this model, we derive an expression for the velocity at which an ensemble of motors (a sufficient number for constant velocity) can move a track. For reversible track binding, velocity is a function of the motor-track binding energy (a linear function of the ensemble force), whereas for irreversible track binding, velocity is a strict function of the kinetics of the chemical perturbation. The model predicts that biased movement stalls (i.e., velocity is zero) when the motor-track binding energy is zero and 50% of the motors (i.e., heads) are attached to the track. Using electron paramagnetic resonance of spin-labeled myosin, we measure a force-dependent equilibrium constant for actin-myosin binding that provides experimental support for the basic assumptions of this model.

738 - Pos
PROLONGED CULTURE AND SURVIVAL OF ADULT MOUSE SKELETAL MUSCLE FIBERS USING SERUM-FREE MEDIA

It has been shown that adult skeletal muscle fibers isolated from rats can be maintained in culture for up to a week in media containing serum (Budoff and Bell, J. Physiol. Lond., 271:557-567, 1977) but, by 4-5 days in culture, satellite cells migrate and previously undamaged fibers start to sprout and de-differentiate (Bischoff, Devl. Biol. 115:129-139, 1988). When proliferating cells are killed by the addition of cytosine arabinoside (araC) to media containing serum, fibers can be maintained with normal morphology for up to 8 days (Hinterberger and Barad, Devl. 109:139-148). Our current studies examine the maintenance of isolated adult mouse FDB fibers in a serum-free culture, in addition to the above conditions used previously. Preliminary results of these studies suggest that satellite cells can be kept in a quiescent state and muscle fibers can be maintained for long periods of time using a serum-free culture. After seven days in serum-containing media, almost none of the starting fibers were identifiable. The fibers exhibited sprouting, lost their striated appearance, and were fused with the satellite cell layer in the dish. When 10 μg/ml araC was added to the serum-containing media, some sprouting was observed three and five days. After seven days, sprouting fibers were no longer present and 22% of starting fibers were viable with normal morphology. Fibers cultured in serum-free media could be maintained for longer periods without disruption of fiber morphology. After seven days in serum-free media, 65% of the fibers remained viable. These cultures did not contain any sprouting fibers at any time, and very few fibers or satellite cells were attached to the disk. We have currently been able to maintain fibers in serum-free media for as long as 15 days. Thus, use of serum-free media may provide a means of studying isolated adult muscle fibers during prolonged culture. Supported by grant NS33578
741 - Pos
FEMTOSECOND FLUORESCENCE SPECTROSCOPY OF TRYPTOPHAN MUTANTS AS MEASURED BY AN UPCONVERSION TECHNIQUE
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An ultraviolet fluorescence upconversion spectrometer was developed using a sub-ps Ti:sapphire regenerative amplifier. After frequency tripling, excitation wavelength was continuously tunable from 290-300nm, and polarization was under automatic control with a servo-driven half-wave plate. Ultraviolet fluorescence and a (servo-stage) delayed laser fundamental pulse were mixed in a thin BBO crystal. Non-collinear mixing lead to a background-free upconverted signal, and time resolution of the instrument was <700fs/PFWHM. The initial anisotropy of p-terphenyl in CH2Cl2 was found to achieve the theoretical maximum, 0.40.

Total fluorescence decay and anisotropy decay curves of tryptophan and related compounds were measured in various non-vicious solvents. We found that solvent-dependent relaxation and 1LB-1LA conversion occur in the first few picoseconds. The initial anisotropy, ρ0, depends strongly on solvent and excitation wavelength. In contrast with previous reports, the initial anisotropy of tryptophan above 340nm in water was consistently found to be well below 0.40. Models for the solvent relaxation and internal conversion will be discussed, along with preliminary results in proteins.

742 - Pos
DECAY-ASSOCIATED SPECTRA AND MUTAGENESIS RESOLVE TRYPTOPHAN FLUORESCENCE IN APOMYOGLOBIN.
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Time-resolved fluorescence experiments were done on apomyoglobin containing one or two tryptophan (Trp) residues. This included sperm whale and horse globins containing two Trp residues (positions 7 and 14), and tuna myoglobin with Trp only at position 14. The engineered proteins were sperm whale mutants with only Trp7 or only Trp14 (the other Trp is replaced by phenylalanine). Two-component decay-associated spectra, DAS, were used to adequately describe the fluorescence of one- and two-Trp proteins. In unfolded globins, all DAS were found to be indistinguishable (i.e., the DAS for all lifetimes superimposed). In compact states (native and molten globule), DAS components for Trp14 also superposed, although DAS from Trp7 were well separated by 5 nm in both states. Importantly, in spite of their spectral superposition, Trp14's from proteins of different origin displayed very different DAS amplitudes. These likely reflect species-dependent differences in interactions of Trp14 with the neighboring cluster, e.g. the hydrogen bond seen only for Trp14 in tuna. Further, the summed fluorescence of the mutant proteins, while spectrally comparable to the wild-type, is 30 ± 5%. The point mutation Trp→Phe may alter packing in the surrounding cluster, causing the other Trp to escape from a proximate quencher. Hence, site-directed mutagenesis can yield misleading data in a protein with coupled tryptophan environments. We will discuss the origins and weight of the species yielding DAS in all of the cases above.

743 - Pos
PROBING HIV-1 REVERSE TRANSCRIPTASE USING FLUORESCENCE SPECTROSCOPY
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Human immunodeficiency virus type-1 reverse transcriptase (HIV-1 RT) is a complex, multifunctional enzyme catalyzing DNA polymerization from a RNA template, DNA polymerization from a DNA primer, RNAase H cleavage, and two separate strand transfer events. A primary goal of our research is to obtain an increased understanding of the interaction between HIV-1 RT and its various nucleic acid substrates at the molecular level. Currently, we are using the fluorescent nucleotide analog 2-amino-4,6-phenylindole (2-AP) to investigate specific binding events in DNA-dependent DNA polymerization. 2-AP does not cause major structural deformation of the nucleic acid strand and its fluorescence is sensitive to n-n stacking interactions. We are also employing fluorescence energy transfer to examine the spatial arrangement of the various substrates throughout the transcription process.

744 - Pos
TIME-RESOLVED FLUORESCENCE OF WT R67 DIHYDROPROPONATE REDUCTASE AND ITS TWO SINGLE-TRYPTOPHAN MUTANTS.
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R67 dihydorolate reductase (DHFR) is a resistance plasmid-encoded version of the enzyme that confers trimethoprim resistance upon bacteria. It has no sequence or structural homology with chromosomal DHFR. R67 DHFR is a 34 kD homotrimer, each subunit of which contains two tryptophan (Trp) residues: W38 at the dimer-dimer interface and W45 at the monomer-monomer interface. We have carried out time-resolved decay associated spectral (DAS) and emission anisotropy measurements on the wt enzyme and its two single-try mutants, W38F and W45F. Experiments were performed under both native and denaturing conditions, and at pH values at which the protein is either an inactive dimer (pH 5) or the active tetramer (pH 8). We find that at either pH value, the native wt decay spectrum is the sum of the spectra of the individual try mutants. The anisotropy decays of the native proteins show a much lower initial anisotropy value (e.g., τ ‖ = 0.07 for the W45F tetramer at pH 8) than typically seen in other proteins. As R67 DHFR possesses Trp symmetry, potential donor and acceptor pairs have identical environments and overlapping spectra. Some very fast (a few picoseconds) energy transfer process is presumably responsible for these low initial anisotropy values.
Fluorescence: Intrinsic & Extrinsic Probes

745 - Pos
PH-INDUCED FLUORESCENCE SHIFTS IN E. COLI THIOREDOXIN MUTANTS. COMPARISON OF EXPERIMENT AND THEORY.

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The distillate redox protein thioredoxin has two tryptophans (Trp) flanking the active distillate group. Trp28 is primarily buried; Asp26 lies interior to it, is very close to the Trp pyrrole ring, and has an unusually high pKa of 7.5. The close proximity of the Asp26 negative charge when pH=9 is predicted by hybrid quantum-molecular dynamics methods to result in a 30-40 nm red shift, because of the much larger dipole of the 1Aa excited state, whose positive end points to the negative charge. Careful measurement of the fluorescence maximum as a function of pH for the oxidized E. coli single Trp mutant W31F, having only Trp 28 reveals no such large shift, but only a 5 nm redshift. The striking absence of such a large shift in the experiment provides a stiff challenge to the theoretical methods. The environment of Trp28 in thioredoxin provides much electrostatic heterogeneity. Interestingly, after 30 ps of dynamics, 13 of the 20 different NMR structures converge to a predicted wavelength maximum of 380 ± 5 nm, but the rms deviation is about 420 nm. The same method predicts the neutral form to have a fluorescence max. at 345 ± 5 nm, more typical of the method and close to experiment.

746 - Pos
EFFECT OF BACKBONE CONFORMATION ON Tryptophan FLUORESCENCE IN RIGID CYCLIC HEXAPEPTIDES

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The structure of [D-PyTTFW] in aqueous solution was studied by H-NMR spectroscopy, and excited-state decay parameters were measured by steady-state and time-resolved fluorescence techniques. The peptide bond is the only functional group in this peptide that quenches indole fluorescence in biomolecular quenching experiments. The fluorescence decay is a triple exponential with lifetimes of 3.7, 1.6, and 0.3 ns and amplitudes of 71, 13, and 15% respectively. These decay amplitudes correlate well with NMR-determined \( \chi_2 \) side-chain rotamer populations of 67, 16, and 17% for Trp in [D-PyTTFW]. The NMR studies show that the peptide backbone is rigid with a major \( \chi_2 \) rotamer of -60° for Trp. There are six low energy conformations of the tryptophan side chain: \( \chi_2 = \pm 60° , 180° \), and \( \chi_2 = \pm 90° \).The lifetimes of the six canonical rotamers of Trp in [D-PyTTFW] were estimated assuming three nonradiative processes: inter-system crossing, solvent quenching, and electron transfer from excited indole to the carbonyl carbon of the peptide bonds. Electron transfer rates were calculated using the Marcus theory. For each \( \chi_2 \) rotamer, the calculated lifetimes of the \( \chi_2 = \pm 90° \) rotamers differ by less than a factor of two. This suggests that the triple exponential decay arises from the three \( \chi_2 \) rotamers. Calculated lifetimes for the -60° and 180° rotamers agree well with the experimental lifetimes of 3.7 and 1.6 ns. These results strongly support the rotamer model of tryptophan photophysics in this cyclic peptide.

747 - Pos
BROADENING OF FLUORESCENCE EMISSION SPECTRA BY EXCITED STATE REEQUILIBRATION.

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Recent efforts by workers in the protein fluorescence and phosphorescence lifetime community have focused on achieving consensus definitions and assessments of the available models for the ubiquitous nonexponential decay of tryptophan fluorescence by single tryptophan proteins. These models include: 1. multiple tryptophan rotomers or other environments that do not interconvert on the nanosecond time scale, 2. solvent relaxation in response to excited state charge distributions, 3. ionization/recombination luminescence or a related heterogeneous dark state process, and 3b. reequilibration of excited state states. In a database of time resolved emission spectra of a large number of single tryptophan proteins these models lead to specific predictions about the statistical prevalence of spectral broadening, red or blue shifting, and negative red edge preexponential factors. In this work the statistical predictions of model 3b, are sharpened by exhaustively examining the model parameter space of an interconverting two excited state system with realistic kinetic parameters and emission band shapes.

748 - Pos
CORRELATION BETWEEN FLUORESCENCE AND STRUCTURAL PARAMETERS OF Tryptophan RESIDUES IN PROTEINS. STATISTICAL CLASSIFICATION OF Tryptophan RESIDUES IN PROTEINS.

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The distribution of spectral components obtained after decomposition of fluorescence spectra into log-normal components of more than 100 proteins confirmed the existence of 5 rather distinct classes of emitting tryptophan residues in proteins. It suggests that it might be a result of various combinations of interactions (specific and universal) of fluorophores of different classes with their microenvironment. The assessment of a set of structural parameters of microenvironment of 137 tryptophan residues based on X-ray structures of 48 proteins was used to reveal structural-defined classes of tryptophan residues by cluster and discriminant functional analyses methods. The results are compared with other classification methods. The figure illustrates the discrimination by the best canonical function of the 137 fluorophores between 5 classes in canonical co-ordinates. The most discriminative parameters are the pKa density (B-factors) and relative medium polarity in the layer of 7 Å from indole ring and its solvent accessibility. The structure-based classification practically coincides with those based on fluorescent parameters. The work was supported by the grant 97-94-94449 from Russia Basic Research Foundation.

749 - Pos
HOW TO MEASURE Tryptophan FLUORESCENCE IN MEMBRANES PROPERLY, AND WHY BOTHER?

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Light scattering associated with vesicular membrane systems presents obstacles to fluorescence studies. Smaller sonicated vesicles (SUV), rather than larger extruded ones (LUV), have been perceived as the most appropriate system because of reduced scattering. However, the high curvatur of SUV is known to produce anomalous peptide partitioning (Seeley & Ganc Blocken, 1991, 30:3935). SUV are more suitable models of biological membranes, but have high scattering. One of the strongest, but often overlooked, effects of scattering is the wavelength-dependent loss of emitted light which hinders measurements of both spectral position and fluorescence intensity. We present experimental schemes that minimize and/or correct for scattering artificats through manipulation of optical path, polarizer orientation, and the use of a reference fluorophore. We demonstrate that the correct spectral distribution of tryptophan can be obtained in the presence of up to 6 nm SUV. The parameters of the spectral position and width are used to evaluate heterogeneity of membrane partitioning. Use of a reference fluorophore allows for the correction of intensity measurements required for determination of energetics of membrane partitioning. Failure to account for scattering effects in the determination of the behavior of SUVs results in a 2-fold error in LUV quantum yield increase and in a 10-fold error in the partitioning coefficient. Supported by GM-46823.

750 - Pos
DNA DYNAMICS MEASURED WITH LONG LIFETIME PROBES

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Fluorescent probes bound to DNA typically display ns decay times and reveal only ns motions. We extended the time range of measurable DNA dynamics using [Ru(ppy)2] which displays a mean lifetime near 200 ns. This probe was used to measure the anisotropy decay of cut, supercoiled and relaxed pTZ plasmid from E. Coli (Figure 1). The supercoiled plasmid displays longer correlation times than the relaxed and cut plasmid, indicating increased segmentated motions in these latter forms.

We also used resonance energy transfer (RET) to investigate the contribution of diffusion motions to the transfer efficiency. We used streptavidin (D) with lifetimes near 5 ns for acridine orange (AO), 20 ns for ethidium bromide (EB) and 180 ns for [Ru(ppy)2]3+, with mule as the acceptor. Comparison of the extent of RET can be used to set limits on the rate of nile blue exchange between binding sites on the DNA.
Ecliptic pH-sensitive green fluorescent protein (EGFP) has two electronic states, protonated and deprotonated, that absorb at 395 and 475 nm, respectively. In this contribution, the flickering dynamics of EGFP have been characterized in solution, as a function of pH, excitation wavelength (\(\lambda_e\)) and excitation intensity (\(I_e\)), using fluorescence correlation spectroscopy. The \(\lambda_e\) and \(I_e\) dependent flicker occurs at rate 5-10 kHz, which increases with intensity below saturation. The flickering fraction (F) depends strongly on \(\lambda_e\) and \(I_e\). At \(\lambda_e\) 5.5 and \(I_e\)=475 nm (deprotonated state excitation), F increases from 0.17 to 0.55 over a two-decade change in intensity. However, while the observed flicker at \(\lambda_e\)=490 nm (protonated state excitation) shows a similar rate, F decreases from 0.70 to 0.42 over the same intensity range. These results suggest that the partition between the ground states of both forms can be shifted in \(\lambda_e\) and \(I_e\), dependent on excitation state. Excited-state time-resolved fluorescence of the deprotonated EGFP builds up on a ~20 picosecond timescale following protonated-state excitation. In contrast, 475-nm excitation accesses only the deprotonated excited state, which decays monoexponentially with a 1.4 ns lifetime. These findings suggest that \(pH\) and \(pK_a\) values measured by fluorescence intensity might depend on \(\lambda_e\) and \(I_e\).


### 753 - Pos

**FLUORESCENCE LIFETIME AND POLARIZATION MEASUREMENTS BY PHASE FLUOROMETRY OF 2'-O- AND 3'-O-CY3-EDA-ATP/ADP ON BINDING TO MYOSIN SUBFRAGMENT-1**


The ribose-modified highly-fluorescent ATP analog, 2'-(3'-O-Cy3)-Cy3-EDA-ATP are good substrates for myosin and actomyosin ATPases with kinetics similar to ATP, and have been used in single molecule kinetic studies (Eccleston et al., Biophys. J. 70, A199, 1996; Owa et al. ibid. 70-76, 1996-9). There are differences in fluorescence intensity changes of separated 2'-O- and 3'-O-isomers on binding to rabbit skeletal muscle myosin subfragment-1 (S1): 2'-O-isomer fluorescence decreases by 12% while that of the 3'-O-isomer increases by 80%, with no absorbance changes or spectral shifts. To elucidate mechanisms for these intensity changes, we measured fluorescence lifetimes of the isomers both free in solution and bound to S1 with 2nm varandate, using multi-frequency phase and modulation fluorometry. Fluorescence lifetimes, excited at 514.5nm, of free 2'-O-Cy3-EDA-ATP and bound 2'-(3'-O-Cy3)-Cy3-EDA-ADP-varadate-S1 complex, were 0.92 and 1.45ns, for free 3'-O-Cy3-EDA-ATP and bound 3'-O-Cy3-EDA-ADP-varadate-S1, 0.99 and 2.16ns, respectively and of free Cy3, 0.2m. Polarization measurements show rotation of the Cy3 moiety of 3'-O-isomer becomes restricted upon formation of the 3'-O-Cy3-EDA-ADP-varadate-S1 complex, with observed polarization increasing from 0.25 for free 3'-O-Cy3-EDA-ATP to 0.45 for the trapped complex. These results suggest that motion of 3'-O-Cy3-EDA-ATP is sterically inhibited when bound to S1 and the increase in fluorescence intensity reflects enhanced quantum yield due to restricted solvent reorientation.

### 754 - Pos

**EFFECTS OF TEMPERATURE ON CALCIUM-SENSITIVE FLUORESCENT PROBES**

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Fluorescent probes are widely used for measuring intracellular cation concentrations. However, problems can arise when such experiments involve variable temperatures. The effects of temperature on the binding equilibria of calcium-sensing dyes has been extensively studied, but there are important temperature-related changes in the photophysics of the dyes that have been largely ignored. We conducted a systematic study of thermal effects on five calcium-sensing dyes under calcium-saturated and calcium-free conditions. Calcium-activated, indo-1, and Fura-2 all show temperature-dependent effects on fluorescence in all or part of the range tested (5-40 °C). Specifically, the intensity of the single-wavelength dyes increased at low temperature. The ratio metric dyes, due to variable effects at the two wavelengths, showed, in general, a decrease in the fluorescence ratio as temperature decreased. The effects of temperature on fluorescence could not be explained by shifts in viscosity or pH of the solution, and were unrelated to oxygen-quenching. The excited state lifetimes of the dyes were studied by the phase-modulation technique. In most cases, low temperature led to increased fluorescence lifetimes. The longer lifetimes at reduced temperature probably contribute significantly to the effects of temperature on the physical properties of the calcium-sensing dyes. Clearly, these temperature effects can affect reported calcium concentration and must therefore be taken into consideration during any investigation involving variable temperatures.


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757 - Pos
UNRAVELING THE MYSTERIES OF THE GREEN FLUORESCENT PROTEIN

We have applied several high resolution fluorescence spectroscopic techniques, including spectral hole-burning, time-correlated single photon counting, two-photon excited fluorescence, and fluorescence correlation spectroscopy, to elucidate the photophysics of the Green Fluorescent Protein and its mutants. Using spectral hole-burning spectroscopy at cryogenic temperatures we have identified the I-form of the wild-type GFP in absorption, established the 0-0 transitions of the A, I and B states, and determined the transitions of interconversion between the three forms (I). Using precise time-resolved spectroscopy we have distinguished the fluorescence lifetimes of the I and B species, and investigated the photochromicity between the A and B forms (2). We have also compared one- and two-photon excited fluorescence of different GFPs; the results indicate that the fluorescence originates from the same distinct excited singlet states, albeit with ground and excited state distributions and decay rates differing for the various GFP species (3). These techniques have been extended to various red-shifted mutants of GFP, including S65T, RSGFP and EYFP (Creemers et al., manuscript submitted).


758 - Pos
QUANTUM EFFICIENCY OF LUMINESCENT LANTHANIDE CHELATES AND FARDY DYES MEASURED BY DIFFUSION-ENHANCED ENERGY TRANSFER
Ming Xiao, Paul R Selvin, University of Illinois at Urbana & Champaign.

Time-discrimination using long-lived (milliseconds) luminescent lanthanide ions, and wavelength discrimination using far-red dyes, are two means to enhance sensitivity of biosassays. Lanthanides are also valuable donors in energy transfer experiments. We have developed a simple method to accurately measure the quantum efficiency of luminescent lanthanide chelates, which is also applicable to conventional organic dyes emitting in the visible to far-red region. This method is based on diffusion-enhanced energy transfer where a lanthanide donor transfers energy to an organic acceptor fluorophore. By knowing the quantum yield of the acceptor, and measuring the energy transfer efficiency by both lifetime and spectrum, the quantum yield of the lanthanide can then be determined. Once the lanthanide quantum yield is known, the experiments can be repeated to determine the quantum yield of an organic acceptor. Using this method, we have determined the quantum yield of a series of luminescent lanthanide complexes based on linear and macrocyclic polynuclearcarboxylate chelates. Terbium quantum yields range from 0.44 to 0.73 in H2O-based buffers and from 0.75 to 0.82 in D2O. Europium quantum yields in H2O range from 0.14 to 0.42, and in D2O from 0.50 to 0.65. Lanthanide photophysical constants, including absolute radiative and nonradiative rates can then be determined. The quantum yield of the far-red dye Cy5 is measured to be 0.4.

MONDAY
SYMPOSIUM 5 - PROTEIN FOLDING: AN URGENT PROBLEM IN THE POSTGENOMIC ERA

760 - Symp
THE ROLES OF LOCAL SEQUENCE AND GLOBAL INTERACTIONS IN THE FOLDING OF A PREDOMINANTLY β-SHEET PROTEIN
Lila M. Giersch, Stephen J. Eyles, Jennifer A. Habink, Kannan Gunasekaran, Melissa Kosinski, Kenneth S. Rotondo, University of Massachusetts, Amherst, MA 01003.

Cellular retinoic acid binding protein 1 (CRABP1) is a member of the intracellular lipid binding protein (ILBP) family, whose structures are comprised of a short helix-turn-helix and two nearly orthogonal five-strand β sheets wrapped around a central cavity. Kinetic analysis by stopped-flow fluorescence and circular dichroism, hydrogen exchange, and probing of ligand binding provides a description of the landscape of refolding of CRABP1. Within 10 msec, the ensemble of conformers is hydrophobically-collapsed and contains significant local secondary structure. Native-like topology, as indicated by ligand-binding, develops in a ca. 100 msec kinetic phase. Strikingly, stable hydrogen bonding in the β sheets forms in a fully cooperative manner in a later (1 sec) phase, during which specific packing interactions also develop. These results have implications for how sequence information encodes β sheets, which is poorly understood at present, and for the relationship of sequence to topology. We are currently exploring the early folding events leading to the hydrophobic collapse, the contributions of specific amino acids to the stability and folding of CRABP1, and the extent to which local sequence specifies native-like structure. We will discuss the model for folding that emerges from our results, in combination with detailed analysis of the extensive sequence and structure information available for the ILBP family.

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PROTEIN FOLDING: FROM LATTICE MODELS TO REAL PROTEINS.
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Protein folding is a field where theoretical physics, chemistry, molecular and evolutionary biology meet. A theoretical study of statistical mechanics of heteropolymers provides fundamental insights into protein folding, evolution and design. We show that there exist deep analogies between protein design/evolution and well-known statistical-mechanical models which make it possible to address, from a fundamental perspective such questions as degeneracy of protein code (i.e. how many sequences can fold to a given structure). Further we present the results of simulation of "Darwinian" evolution towards fast folding sequences which provide valuable hints in a quest for evolutionary messages about protein folding encoded in protein sequences. In particular, using the lessons from model protein evolution we were able to decipher evolutionary "signals" that call for fast folding in the superfamilies of structurally related nonhomologous proteins.