W-Pos1

MYOFIBRILLAR DAMAGE AND THE DISTRIBUTION OF $[Ca^{2+}]_i$ IN SINGLE SKELETAL MUSCLE FIBRES FOLLOWING ECCENTRIC CONTRACTIONS ((C.D. Balnave and D.G. Allen)) Dept. of Physiology, University of Sydney, N.S.W. 2006, Australia.

Stretching intact single fibres during contraction (eccentric contraction) brings about a pronounced decrease in tetanic force production, partly as a consequence of a reduced Ca^{2+} release (C.D. Balnave & D.G. Allen. *J. Physiol.*, 488.1: 25-36, 1995). The maximum Ca^{2+}-activated force is also reduced, probably due to myofibrillar disruption. In the present study single fibres were dissected from the mouse *flexor brevis* muscle and underwent a stretching protocol. The fibres were then examined for ionic and myofibrillar abnormalities using imaging, confocal and electron microscopy. Electron microscopy revealed that there was a considerable variation in the sarcomere lengths of fibres which had undergone the stretching protocol, while in control fibres the muscle structure appeared normal. Imaging fluorescent microscopy demonstrated that the distribution of both tetanic and resting $[Ca^{2+}]_i$ was uniform along the muscle fibres following eccentric contractions. The results show that the single fibre model of eccentric contraction-induced muscle damage is structurally, as well as functionally, akin to the human condition. The results also show that the reduction in tetanic $[Ca^{2+}]_i$ occurs uniformly throughout the fibre and is not isolated to specific damaged regions.

W-Pos2

AN EPITOPE STRUCTURE SHARED BY TITIN AND CALDESMON. ((W.H. Raharjo*, J.J.-C. Lin†, A.S. Maki and J.-P. Jin*)) *Smooth Muscle Research Group and Dept. Medical Biochemistry, Univ. of Calgary, Calgary, Canada T2N 4N1; †Dept. Biological Sciences, Univ. of Iowa, Iowa City, IA 52242; ¶Dept. Biochemistry, Queen's Univ., Kingston, Canada K7L 3N6.

Titin is a giant protein containing two types of repeating motifs that interact with myosin and actin filaments in the striated muscle sarcomere. The I-band portion of titin has been found to contain a long segment of the Class II motifs. Caldesmon is a Ca^{2+}/calmodulin-, actin- and myosin-binding protein found in smooth muscle and nonmuscle cells. A monoclonal antibody (mAb Ti104) generated against a cloned Class II motif of rat cardiac titin (Ti II, Jin, J.-P., J. Biol. Chem. **270**, 6908-6916, 1995) was found to cross-react with smooth muscle caldesmon. To determine whether caldesmon may function as a titin analog to organize smooth muscle and nonmuscle cell contractile elements, we carried out a peptide mapping of the Ti104 epitope. Western blotting and amino acid sequencing of chymotryptic fragments of the Ti II motif revealed that the Ti104 epitope locates in a 37-amino acid peptide. Ti104 and an anti-caldesmon mAb C21 showed very similar reaction patterns to various caldesmons. Using several bacterially expressed deletion mutants of the COOH-terminal fragment (CaD39) of human fibroblast caldesmon, we have identified that the C21/Ti104 epitope is located in the actin-binding domain (Leu_{495}-Leu_{523}, corresponding to Leu_{693}-Leu_{721} of the smooth muscle caldesmon). This is consistent with the previous report that C21 competitively inhibited the binding of caldesmon to F-actin-tropomyosin filaments. The structure/function similarity between Ti II and the actin-binding domain of caldesmon suggests that these two proteins perform analogous roles in organizing the contractile elements and/or in modulating the contraction/motility within muscle and nonmuscle cells. (Supported by the MRC of Canada and the Heart and Stroke Foundation of Alberta)

W-Pos3

RELATIONSHIPS BETWEEN EXPRESSION OF SMOOTH MUSCLE CALPONIN AND CONTRACTION OF THE DEVELOPING RAT AORTA. ((R. Nigam, C.R. Triggle, G.A. McMartin, M.E. Resek and J.-P. Jin)) Smooth Muscle Research Group, Depts. Medical Biochemistry and Pharmacology and Therapuetics, Univ. of Calgary, Calgary CANADA T2N 4N1.

Calponin is a smooth muscle thin filament-associated protein. To elucidate the physiological role of calponin this study examined the relationship between expression of calponin and postnatal development of contractility in isolated blood vessels. Aortic rings denuded of endothelium from 2, 3, 4, 6, 8, and 10 week-old male Wistar rats were cut to approximately 7, 6, 5, 4, 3 and 2 mm in length, respectively, and the exact tissue mass was used to normalize the data from the different age classes. Each ring was carefully prepared for isometric recordings and maintained at the optimal resting tension of 2.0g. Data from each age group indicated that application of preload placed aortic ring preparations on comparable levels of their length-tension curve. Cumulative concentration-response curves for aortic ring contractions were established for NaF, norepinephrine and KCl. Expression of calponin in aortic tissue at the different age groups was assessed by Northern blot analysis using an *h1*-calponin cDNA probe and Western blot analysis using specific antibodies. For all three drugs, no significant contractions were observed in aortic rings from 2 week-old rats. The onset of contraction occured at 3 weeks and maximum contraction was obtained from 4 weeks onward. Western blot analysis revealed that calponin was present in adult aorta but not in 2 week-old or younger aortae. Detailed investigation examining each age group for expression of calponin in rat aortae is undergoing to reveal relationships with the development of contractility. The correlation between the development of sensitivity to contractile agents in aorta and pulmonary artery and the expression of smooth muscle specific proteins is also being assessed. (Supported by MRC of Canada)

W-Pos4

MEASUREMENT OF SARCOPLASMIC RETICULUM CALCIUM UPTAKE & CALCIUM RELEASE IN SKELETAL MUSCLE HOMOGENATES USING FURA-2 ((*Stuart A. Warmington, Mark Hargreaves and David A. Williams.*))
Department of Physiology, The University of Melbourne, Parkville, Australia, 3052.

Muscle homogenates have been used extensively to assess membrane specific functions, in particular those of the sarcoplasmic reticulum (SR). Recent methods using the dual emission fluorescent Ca^{2+} indicator Indo-1 produce large initial artefacts lasting up to 20 seconds, while the use of microelectrodes and Ca^{45} are somewhat limited by their speed of data collection. Our method involves the use of the dual excitation fluorescent Ca^{2+} probe Fura-2 in a microscope based spectrofluorimeter adapted to house a cuvette system (Cairn; UK). The system is heated to 37°C and constantly stirred.

The EDL and soleus muscles from male Sprague Dawley rats were removed intact and stored in Krebs Heinsleit solution bubbled with carbogen until assayed. The muscles were homogenised in 8 volumes of buffer consisting of Sucrose (250mM), Hepes (40mM, pH 7.1), KCl (40mM), N,N,N',N'-tetrakis(2 Pyridyl-Methyl)Ethylenediamine (TPEN) (5μM), Oxalate (5mM) and Sodium Azide (10mM). A 100μl sample was then injected into a cuvette containing the assay buffer to initiate the SR Ca^{2+}-uptake assay. This buffer consisted of Hepes (40mM; pH 7.1); KCl (40mM); MgATP (5mM), $CaCl_2$ (10μM) the level of which was monitored ratiometrically by the fluorescence of the calcium binding dye fura-2 (7.5μM). After a plateau of the fluorescence ratio was reached Ca^{2+}-release was initiated by the addition of silver nitrate ($AgNO_3$) (50μM).

Relative rates of SR Ca^{2+}-uptake conform with the literature with respect to differences apparent between fast- (EDL) and slow- (soleus) twitch muscle fibres. However, we show that addition of $AgNO_3$ to induce Ca^{2+}-release is only effective in fast-twitch muscle fibres of the EDL and thus this method may be unreliable for measuring Ca^{2+}-release in mixed muscle samples such as human biopsies. It is interesting to note that other methods only quote Ca^{2+}-release in muscles such as the white gastrocnemius or a mixture of white and red muscle, suggesting an underestimation to the measurement of Ca^{2+}-release.

These results support the existence of multiple Ca^{2+}-release channel isoforms in fast- and slow-twitch fibres of skeletal muscle which exhibit different sensitivities to chemical inducing factors such as heavy metals, ionic conditions such as free $[Mg^{2+}]$ or adenine nucleotides.

W-Pos5

CALMIDAZOLIUM INCREASES THE CALCIUM SENSITIVITY OF FORCE AND THE RATE OF FORCE DEVELOPMENT IN SKINNED RABBIT SKELETAL MUSCLE FIBERS. ((M. Regnier, P.B. Chase and A.M. Gordon)) Dept. of Physiology & Biophysics and Dept. of Radiology, University of Washington, Seattle, WA 98195.

The 'calcium sensitizer' calmidazolium (Cal) binds to TnC and increases Ca^{2+} affinity at its Ca^{2+} specific sites (El-Saleh & Solaro, 1987, *JBC* 262:17240). The proposed mechanism of Cal action is via a decrease in the Ca^{2+} off rate with little or no effect on the Ca^{2+} on rate (Wahr *et al.*, 1993 *Biophys.J.* 64:A24; Johnson *et al.*, 1994, *JBC* 269:8919). If this mechanism is correct Cal should affect force generation by muscle fibers at low $[Ca^{2+}]$ but not at maximal Ca^{2+} activation. We tested this hypothesis by measuring the steady-state isometric force (P) and the rate of force development (k_{tr}) in Ca^{2+}-activated, skinned fibers from rabbit psoas. At maximal Ca^{2+} activation (pCa 4.0) [Cal] ≤ 30 μM had no discernible effect on either P_o or k_{tr}. At 100 μM Cal both Po and k_{tr} were reduced by 20-30%, an effect that was at least partially reversible. In contrast, P and k_{tr} were both increased at submaximal Ca^{2+} activations in the presence of Cal (relative to control values). At 30 μM Cal the pCa-force curve was shifted to the left by approximately 0.2 pCa units. Additionally, 30 μM Cal increased k_{tr} by 2-3 fold at 50% P_o. These effects at submaximal $[Ca^{2+}]$ were not readily reversible. These results support the hypothesis that the Ca^{2+} binding properties of TnC are important modulators of the rate of force development in skeletal muscle (Chase *et al.*, 1994 *Biophys.J.* 67:1994). Supported by NIH HL52558

W-Pos6

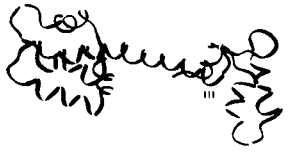
COMPARISON OF THE EFFECTS OF CALPONIN AND 38 kDa CALDESMON-FRAGMENT ON THE FORMATION OF "STRONG BINDING" STATE IN A GHOST MUSCLE FIBER. ((Yu. S. Borovikov, M.I. Khoroshev, K.Y. Horiuchi, and S. Chacko)) Institute of Cytology RAN, St.Petersburg 194064, Russia and Department of Pathobiology, University of Pennsylvania, Philadelphia, PA 19104. (Spon. by E. Holzbaur)

Decoration of thin filaments in "ghost fibers" with NEM-modified myosin subfragment-1 and phosphorylated smooth muscle heavy meromyosin produces structural changes in actin which is typical for the formation of "strong binding" between actin and the myosin head. The effects of acting-binding proteins calponin and 38 kDa fragment of caldesmon on these structural changes was investigated using polarized fluorometry.The F-actin in the myosin-free "ghost fibers" was labeled with fluorescein-maleimide and TRITC-phalloidin. Both actin-binding regulatory proteins inhibited the conformational changes in actin, that are compatible with the formation of "strong binding" between actin and the myosin head. Tropomyosin reduced the effect produced by calponin, but increased the conformational change caused by the 38 kDa caldesmon.The *in vitro* motility assay showed that these regulatory proteins inhibit motility of actin filament over immobilized surface of skeletal muscle myosin heads. It is thus suggested that the inhibition of the formation of "strong-binding" is an important factor in the mechanism for the regulation of smooth muscle contraction. Supp. by grants from RBRF& NIH.

W-Pos7

A MOLECULAR MODEL OF THE STRUCTURE OF BARNACLE TROPONIN C ISOFORM 2. Alan Mandveno*, Bruce Parsons*, Christopher C. Ashley* and James D. Potter*. *University of Miami School of Medicine, Miami, FL 33101 and *University Laboratory of Physiology, Oxford, UK OX1 3PT.

Muscle from the giant barnacle (Balanus nubilus) has been shown to contain two isoforms of Troponin C, $BTnC_1$ and $BTnC_2$ (Ashley *et al.*, 1991). The amino acid sequences of both isoforms have been determined and the Ca^{2+} binding properties of $BTnC_2$ have been characterized (Collins *et al.*, 1991). $BTnC_2$ binds two moles of Ca^{2+}/mole and both sites appear to be Ca^{2+}-specific. Analysis of the amino acid sequence suggests that only sites II and IV bind Ca^{2+}. In order to learn more about the possible structure of this protein, we built a three dimensional model of $BTnC_2$ using a computer program (Homology; Biosym, Inc.) that constructs an unknown protein structure from proteins with homologous sequence and/or conserved structural regions. After constructing our protein we used a unique iterative minimization and dynamics simulation method which prevented loss of structure due to excessive intramolecular stress. Our structure was dumbbell shaped containing N- and C-terminal halves. The N-terminus is shorter than the turkey/chicken crystal structures, however it is also comprised of α-helix. Sites I through IV are all composed of helix-loop-helix structures however site III is altered from a "classic" EF hand motif. Although the D/E linker and region IV have sequences that differ significantly from analogous regions of vertebrates, they appear to be similar in structure. (Supported by NIH grants #AR37701 and AR40727).



W-Pos8

MODELING COOPERATIVITY BETWEEN Ca^{2+} AND MYOSIN BINDING IN SKELETAL MUSCLE. ((D L Morgan* & F J Julian.)) Department of Anesthesia, Brigham & Women's Hospital, 75 Francis St., Boston, MA 02174; *Monash University, Clayton, Vic., 3168, Australia.

Recent experiments have shown 1) that the tension-pCa relation for muscle is even steeper than previously thought, and 2) that movement introduced to a previously isometric fibre during relaxation can cause the myoplasmic $[Ca^{2+}]$ to rise while tension falls. The first of these results implies that activation of muscle must involve a large degree of cooperativity. Modelling results based on Westerblad and Allen (J Physiol 466:611,1993) show that the second result can only be obtained if at least some of that cooperativity is brought about by the binding of myosin affecting the binding of calcium. Effects of bound calcium on calcium binding and bound myosin on myosin binding are not excluded, but cannot of themselves lead to the rise. With this myosin-calcium cooperativity, both the tension and the fraction of troponin with bound calcium for a given myoplasmic $[Ca^{2+}]$ depend on the assumed crossbridge detachment rate constant. As this rate constant represents an average over the crossbridge population, it will be increased in the presence of sarcomere length changes, changing the shape and position of the tension-pCa curve. Variations in the amount of internal movement may acccount for some of the variation in reported shapes of tension-pCa curves.
Supported by NIH HL35032 and a Monash University outside studies program grant.

W-Pos9

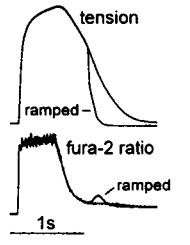
THE TENSION-$[Ca^{2+}]$ RELATION IN INTACT FROG MUSCLE FIBERS. ((D L Morgan*, D R Claflin & F J Julian.)) Department of Anesthesia, Brigham & Women's Hospital, 75 Francis St., Boston, MA, 02174; *Monash University, Clayton, Vic., 3168, Australia.

The steady state relation between intra-cellular $[Ca^{2+}]$ and tension was investigated in intact single frog muscle fibres. A slow decline of tension after a brief tetanus was produced by the sarcoplasmic reticulum Ca pump inhibitor, cyclopiazonic acid (CPA), followed by a series of conditioning contractions. $[Ca^{2+}]$ was estimated using the ratiometric fluorescent dyes fura-2 and furaptra, combined with rapidly alternating excitation wavelengths, to eliminate motion artifact in a single contraction. The tension-pCa relation was extremely steep, with typical Hill coefficients of 20, corresponding to 10% to 90% tension change in less than 0.1 pCa unit. Neither the steepness nor the position of the curve changed consistently over a wide range of tension decay times from a few seconds to over one hundred seconds, indicating that a decline over a few seconds was near steady state. By combining the calcium release inhibitor D600 (methoxyverapamil) with CPA, contractions with a slow rise composed of many small steps, and a slow fall were produced. The tension-$[Ca^{2+}]$ relation on the steps of the rise was very close to that during the fall, indicating that the relation had little or no hysteresis. These extremely steep curves indicate that activation is even more cooperative than previously reported.
Supported by NIH HL35032 and Monash University outside studies program grant.

W-Pos10

SHORTENING APPLIED DURING TENSION RELAXATION AT LONG LENGTH RESTORES THE TRANSIENT Ca^{2+} RISE IN INTACT FROG SKELETAL MUSCLE FIBERS. ((DR Claflin, DL Morgan* & FJ Julian)) Department of Anesthesia, Brigham & Women's Hospital, Boston, MA 02115; *Monash University, Australia.

We reported previously that the transient increase in free myoplasmic $[Ca^{2+}]$ observed during tension relaxation (the "relaxation calcium transient", RCT) in intact skeletal muscle fibers at sarcomere lengths optimal for tension generation is abolished at longer sarcomere lengths (Julian et al., Biophys. J. 61:A294, 1992). We have now performed additional experiments designed to determine whether this reduction in the amplitude of the RCT is due to the reduced number of myosin crossbridges able to interact with the thin filament, or due to a stabilizing effect of the structures that give rise to passive tension at long sarcomere lengths. Experiments were performed on intact fibers isolated from the tibialis anterior muscle of the frog (*R. temporaria*) and maintained at 3°C. $[Ca^{2+}]$ was monitored using the Ca^{2+}-sensitive fluorescent dye fura-2 excited at wavelengths of 344nm and 380nm and reported here as the ratio of the two responses (344/380). At a sarcomere length of 2.8μm, where passive tension is ≈3% of maximum isometric tetanic tension and the RCT is nearly absent, a small ramp shortening movement was applied to one end of the fiber during tension relaxation. The purpose of the ramp was to counter the stabilizing effect of the passive tension, thus simulating the internal movements of fibers at shorter sarcomere lengths. The result of this maneuver, shown in the figure, was the reappearance of a RCT. We conclude that the primary factor responsible for the reduction in the RCT with increasing sarcomere length is the stabilizing effect of passive tension. Supported by: NIH HL35032



W-Pos11

EFFECTS OF GENETICALLY ENGINEERED $BTnC_2$ ON SKINNED MYOFIBRILLAR BUNDLES FROM THE BARNACLE (*B. nubilus*) AT DIFFERENT IONIC STRENGTHS. *C.C. Ashley, *L. D. Allhouse, #T. Miller and #J.D. Potter. *University Laboratory of Physiology of Oxford, UK, OX1 3PT, #Friday Harbor Laboratories, University of Washington, WA 98250, #Molecular & Cellular Pharmacology, University of Miami School of Medicine, Miami, FL 33101

Recombinant $BTnC_2$ ($rBTnC_2$) containing mutations at either Ca^{2+} binding site II or site IV were studied in native TnC depleted muscle fibers. Previous studies have suggested that only site II and site IV bind Ca^{2+} in $BTnC_2$ (Collins *et al., Biochem.* 30:702, (1991); Ashley *et al., J. Mus. Res. Mus. Mot.* 12:532 (1991)). Site II and site IV mutants were prepared where the Ca^{2+} binding site was rendered inactive by insertion of an ALA for an ASP residue in the X coordinating position of each Ca^{2+} binding loop. Myofibrillar bundles (120 μm diam) were prepared from single lateral depressor muscle fibers under mineral oil. Endogenous TnC was extracted with either 10 mM orthovanadate or 2 mM EDTA (Ashley *et al., Biophys. J.* 68:A56 (1995)). Subsequent exposure (2 mg/ml for 30 min) to $rBTnC_2$ lacking site II at normal (0.15M) or reduced (0.075M) ionic strength and with reconstitution in the presence or absence of Ca^{2+}, regulated force. Exposure to $rBTnC_2$ lacking site IV regulated at reduced ionic strength with reconstitution in the presence or absence of calcium, but regulated poorly at normal I values (0.15M), as did $rBTnC_2$ lacking the last 11 amino acids at the C terminus, and hence most of site IV (B TRUNC). Supported by grants from the MRC and NIH (AR37701 & AR40727).

W-Pos12

PHOSPHORYLATION OF THE REGULATORY LIGHT CHAINS OF MYOSIN MODULATES FORCE DEVELOPMENT IN SKELETAL MUSCLE FIBERS. Danuta Szczesna, Jiaju Zhao and James D. Potter, Dept. of Molecular & Cellular Pharmacology, University of Miami School of Medicine, Miami, FL 33136

We investigated the role of phosphorylation of the myosin regulatory light chains (RLCs) in the regulation of skeletal muscle contraction. The RLCs were phosphorylated in skinned skeletal muscle fibers with Ca^{2+}-calmodulin activated myosin light chain kinase (MLCK). This treatment resulted in an ~10-20% increase in steady state force development compared to untreated fibers. The Ca^{2+} sensitivity of force development was also affected by RLCs phosphorylation. Consistent with Metzger *et al.*, J. Gen. Physiol. 93: 855-883 (1989) and Sweeney *et al.*, Am. J. Physiol. 264: C1085-C1095 (1993), the force - pCa relationship was shifted towards lower concentrations of Ca^{2+} compared to non-phosphorylated fibers. The same results were obtained when steady state force measurements were performed on skinned fibers extracted to remove endogenous RLCs (Szczesna *et al.*, Biophys. J. 68: A168, 1995) and reconstituted with either phosphorylated RLCs (+P-RLCs) or non-phosphorylated RLCs that were subsequently phosphorylated with MLCK. In both cases we observed an ~30% increase in maximal force and an increase in the Ca^{2+} sensitivity of force development of ΔpCa_{50}~ +0.13 of the +P-RLCs-reconstituted fibers compared to fibers reconstituted with non-phosphorylated RLCs. These results suggest that although skeletal muscle is primarily regulated by the troponin-tropomyosin complex, phosphorylation of the regulatory light chains of myosin may play an important modulatory role in vertebrate striated muscle contraction. Supported by NIH grant #AR37701.

W-Pos13

CONFORMATIONAL CHANGES INDUCED BY PHOSPHORYLATED AND DEPHOSPHORYLATED HEAVY MEROMYOSIN IN ACTIN IN "GHOST" FIBER ARE MODULATED BY CALCIUM. ((S.V. Avrova, Yu. S. Borovikov, K.Y. Horiuchi and S. Chacko)). Institute of Cytology RAN, St.Petersburg 194064, Russia and Department of Pathobiology, University of Pennsylvania, Philadelphia, PA 19104, USA. (Spon. by W.T. Weber)

The changes in actin conformation induced by the binding of phosphorylated and dephosphorylated heavy meromyosin (HMM) were determined by measuring the polarized fluorescence of rhodamine-phalloidin complex attached to F-actin. Phosphorylated HMM in the presence of Ca^{2+} induced the conformational changes on actin typical for the "on" state of actin monomers in thin filaments, a specific change caused by "strong-binding" of myosin heads. This effect was markedly inhibited in the absence of Ca^{2+} and when HMM light chain was dephosphorylated. Therefore, it is suggested that both Ca^{2+} and phosphorylation of myosin regulatory light chains switch myosin heads from "weak binding" to a "strong-binding" conformation and the binding of myosin heads switches actin monomers from "off" to "on" state. Supported by RBRF and grants from NIH.

W-Pos14

THIN-FILAMENT COOPERATIVITY IN FORCE DEVELOPMENT: STUDIES WITH ASYMMETRIC TnC-EXTRACTION
((A. Babu Akella and Jag Gulati)) The Molecular Physiology Laboratory, Albert Einstein College of Medicine, Bronx, NY 10461

Brandt et al. (JMB, 180, 379-83, 1984) have hypothesized that all 26 troponin-tropomyosin act as a single grand cooperative unit with Ca for concerted force development. Cantino et al. (BJ, 64, 211-22, 1993), tested this by Ca-binding on the thin filament as influenced by rigor cross-bridges. We have further tested the underlying mechanism during force development. The TnC subunits from approximately half length of each thin filament (closest to the Z-line) were selectively extracted (see *Modulation of Cardiac Calcium Sensitivity*, Oxford U Press, 215-241, 1993). The extraction was confirmed with added biotinylated-TnC. The pCa-force relationships were determined. At 2.4μm, where the TnC distribution within the overlap was largely intact, the force was normal (P_o = 80% of the normal). Moreover, neither the pCa_{50} values nor the Hill coefficient (n_H) was altered (control pCa_{50}=5.98, nH=4.25; extracted pCa_{50}=5.96, n_H=4.27). Reconstituting the asymmetrically extracted fiber with an sTnC-1-2 mutant (an sTnC variant with both trigger sites inactivated) did not modify the above contractile response. The findings support Cantino et al. and indicate that the TnC-depletion in the nonoverlap domain had little influence on cooperativity effect of the contiguous regulatory units within the overlap. Thus, the size of Brandt's grand-cooperative unit must vary with sarcomere length and include TnC units confined to the overlap domain. *[Supported by NIAMS]*

W-Pos15

ON A UNIFIED MECHANISM FOR COOPERATIVE ACTIVATION OF THIN FILAMENTS BY CA^{2+} AND RIGOR CROSS-BRIDGES. ((P.W. Brandt and F. Schachat)) Department of Anatomy, Columbia University, NY, NY 10032 and Department of Cell Biology, Duke University, Durham, NC 27710. (Spon. by M. Briggs)

Troponin C (TnC) extraction from thin filaments has similar effects on activation by both Ca^{2+} and rigor crossbridges. In both instances, extraction of as little one TnC per regulatory strand markedly reduces the maximum tension and Hill coefficients of the pCa and pS/tension relationships, n_H and n_S, respectively (Brandt et al., JMB 180:890; JMB 212:473). Substitution of calmodulin (CaM) or a strongly binding chimera of CaM, CaM[3,4TnC], reduces in parallel the maximal Ca^{2+} activation tension and n_H. Here we report that the pS/tension relations of fibers reconstituted with either CaM[3,4TnC] or cardiac TnC (cTnC) also exhibit reduced rigor crossbridge induced force and n_S. Following substitution of CaM[3,4TnC] only half the lost pCa and pS activated tension and half n_H and n_S are recovered. Substitution of cTnC recovers 60% of the pCa and pS tension and about 35% of the lost cooperativity. Thus, all the chimeric substitutions for TnC performed to date have parallel effects on the pCa and pS/tension relationships. This parallel response is unanticipated as Ca^{2+} binding to TnC results in formation of a TnC-TnI complex that weakens TnI's inhibitory influence while rigor crossbridges are thought to force the regulatory strand out of its inhibitory position. The parallel behavior suggests the mechanisms have some steps in common. It has been suggested that in both modes of activation rigor/cycling cross-bridges displace the regulatory strands. However, modeling with the concerted transition formalism shows that, if cycling cross-bridges are activators, relaxation will be incomplete and Ca^{2+} regulation fails. This might work if activation is limited to attachment *per se*.

W-Pos16

PREDICTED EFFECTS OF HIGH CALCIUM ON THE FREE CONCENTRATION OF ATP NEAR THE PLASMA MEMBRANE OF CELLS ((M.E. Kargacin and G.J. Kargacin)) Univ. of Calgary

From our own work with models of Ca^{2+} diffusion and regulation in smooth muscle cells and from the theoretical and experimental work of others, it is becoming apparent that high $[Ca^{2+}]$ can develop near the plasma membrane of cells when Ca^{2+} influx occurs and/or Ca^{2+} is released from nearby intracellular storage sites. This would result in a localized alteration in the free vs bound concentrations of all molecules that bind Ca^{2+} in this concentration range. Although ATP has a higher affinity for Mg^{2+} than for Ca^{2+}, when Ca^{2+} levels are high, the relative concentrations of free ATP, MgATP and CaATP would be expected to be altered. This, in turn, could effect the activity of ATP-dependent processes near a high $[Ca^{2+}]$ site. To test this, we calculated the effects of an increase in $[Ca^{2+}]$ on $[ATP]_{free}$ and $[ATP]_{bound}$ in intracellular buffers. Under equilibrium conditions, a cellular region with a resting $[Mg^{2+}]_{free}$ of 0.1 mM, $[ATP]_{total}$ of 1 mM, and $[Ca^{2+}$ buffer$]_{total}$ of 250 μM (K_d = 1 μM), $[ATP]_{free}$ dropped by approximately 25% when $[Ca^{2+}]_{free}$ was increased from 50 nM to 100 μM. A similar percentage change in $[ATP]_{free}$ was calculated with a starting $[Mg^{2+}]_{free}$ of 0.5 mM. These results indicate that significant changes in the activity of ATP-dependent processes can occur when Ca^{2+} gradients form during intracellular signaling. We are currently modifying our simulations to study dynamic changes in concentration. (supported by MRC (Canada), HSFA, AHFMR)

W-Pos17

TnC-TnI ELECTROSTATIC INTERACTIONS IN THE REGULATION OF CONTRACTION. Bruce Parsons, Alan Mandveno, Michelle Jones, Jiaju Zhao, and James D. Potter. University of Miami School of Medicine, Miami, FL 33101.

It is possible that a Ca^{2+} induced interaction that regulates contraction may occur between the negatively charged residue 52-62 region of mouse cardiac TnC (MCTnC) and the positively charged 96-115 region in TnI. In order to investigate the possible role of electrostatic interactions we have used site directed mutagenesis to change all of the negatively charged helix-C amino acid residues (T53,E55,E56,Q58,E59,D62,E63) in mouse cardiac TnC to neutrally charged alanine residues (SUB5262). Another mutant which deletes residues 52-62 (DEL5262) was also prepared. As shown previously (Parsons, *et al., Biophys. J.* 68:A56, 1995) both SUB5262 and DEL5262 bind to cardiac skinned muscle preparations but were unable to regulate contraction even though they blocked reincorporation of MCTnC. To rule out the possibility that these mutants had altered Ca^{2+} binding at the single Ca^{2+}-specific regulatory site, thereby preventing force activation, we compared the Ca^{2+} binding characteristics of MCTnC and SUB5262, using flow dialysis. Results from these Ca^{2+} binding studies suggest that MCTnC, SUB5262 and DEL5262 have essentially the same Ca^{2+} binding properties (2 high affinity Ca^{2+}-Mg^{2+} and 1 Ca^{2+}-specific site). Thus, the failure of these mutants to activate force may be related to a change in the interaction between TnC and TnI. Molecular modeling (Delphi) of wild type TnC indicates an intense negative electrostatic potential gradient in the 52-62 region surrounded by more neutrally charged areas. It is possible that the reduction in the electrostatic potential in both mutants may account for the alteration in this activity. (Supported by NIH grants HL42325, AR37701 & AR40727).

W-Pos18

SECONDARY AND TERTIARY STRUCTURAL ANALYSIS OF TROPONIN T. ((Melissa R. Snyder, James Potter*, and Franklyn Prendergast)) Mayo Clinic, Department of Pharmacology, Rochester, MN 55905 and *University of Miami, Department of Molecular and Cellular Pharmacology, University of Miami School of Medicine, Miami, FL 33101.

In skeletal and cardiac muscle, contraction and relaxation is regulated by the binding of calcium to troponin C (TnC), the calcium binding subunit of the troponin complex. The conformational change induced in TnC by calcium is transmitted through the remaining two subunits, troponin T (TnT) and troponin I (TnI), eventually reaching actin and myosin, allowing contraction to occur. Recent studies (Potter, et al., J. Biol. Chem. 270: 2557, (1995)) has suggested an important role for TnT in this process. In order to understand these conformational changes, we have chosen to study the structure of TnT. Initial experiments have been performed with TnT isolated from rabbit cardiac and skeletal muscle. Quasi-elastic light scattering experiments indicate that TnT is an extremely elongated molecule, with cardiac TnT being approximately fifty angstroms longer than skeletal TnT. Circular dichroism studies also indicate differences in structure between these two proteins, with TnT from cardiac muscle being approximately 20% more α-helical than skeletal muscle TnT. This work is being continued on recombinant TnT-α, an individual isoform from rabbit skeletal muscle. From this clone, we have also constructed and expressed two deletion mutants, T_1 (residues 1-149) and T_2 (residues 150-250). From the biophysical characterizations of both the intact protein and the deletion mutants, we intend to determine more precisely the secondary and tertiary structure of TnT as a prelude to a full description of the overall structure of the troponin complex.
This work has been supported by NIH Grant AR37701.

W-Pos19

ALTERED KINETICS OF CONTRACTION OF ATRIAL CELLS EXPRESSING VENTRICULAR MYOSIN LIGHT CHAIN2. ((S.H. Buck*, P.J. Konyn*, J. Palermo, J. Robbins, R.L. Moss#)) Depts. of *Pediatrics and #Physiology, University of Wisconsin, Madison, WI 53706, and Division of Molecular Cardiovascular Biology, Children's Hospital Medical Center, Cincinnati, OH 45229.

To investigate the role of myosin light chain2 (regulatory light chain, LC2) as a determinant of the kinetics of cardiac contraction, unloaded shortening velocity was determined by the slack-test method in skinned control murine atrial cells and transgenic murine atrial cells expressing ventricular LC2 (LC2V). Transgenic mice were generated using a 4.5 kb fragment of the murine alpha myosin heavy chain promoter that was able to drive high levels of LC2V expression in the atrium. This resulted in a complete replacement of endogenous atrial LC2 with LC2V. Velocity of unloaded shortening was determined @15°C in maximally-activating Ca^{2+} solution (pCa 4.5) containing in mmol l^{-1} EGTA 7, free Mg^{2+} 1, MgATP 4, creatine phosphate 14.5, imidazole 20; ionic strength 180 mmol l^{-1}, pH 7.0. Plots of slack length versus duration of unloaded shortening were included only if well fit to a straight line. When compared to control murine atrial cells ($n=6$), the unloaded shortening velocity of the LC2V-expressing transgenic murine atrial cells ($n=5$) was significantly greater (5.46 ± 1.08 ML/sec vs. 2.96 ± 1.14 ML/sec, $p < 0.01$). These results provide evidence for a role of myosin light chain2 in regulation of cross-bridge cycling rate. The faster rates of cycling in the presence of ventricular LC2 is consistent with the greater power generating demands of the ventricle as compared to the atrium.

W-Pos20

DECREASED MAXIMAL CA^{2+} ACTIVATED TENSION IN PERMEABILIZED STUNNED MYOCYTES FROM ISOLATED BUFFER PERFUSED RAT HEARTS BUT NOT IN OPEN CHEST PORCINE HEARTS ((W.P. Miller, K.T. Strang, and R.L. Moss)) Section of Cardiology and Department of Physiology, University of Wisconsin, Madison, WI 53706

Decreases in maximal Ca^{2+} activated isometric tension (P_o) and Ca^{2+} sensitivity of isometric tension (pCa_{50}) have both been proposed as mechanisms for the decrease in myocardial function seen in postischemic stunned myocardium. Using an *in-vivo* porcine heart model of regional stunning we have demonstrated in permeabilized myocytes from endocardial biopsies that Ca^{2+} sensitivity was decreased while P_o was unchanged (Circ Res 72,1993; Circ Res 77,1995). To determine the relative importance of P_o as a mechanism of stunning, P_o and pCa_{50} were measured in myocytes obtained from a commonly employed isolated buffer perfused heart model of stunning. Rat hearts contracting isovolumically against an LV balloon were retrogradely perfused at 37°C for 50 min (controls, $n=6$) or for 10 min of control perfusion followed by 20 min of global no-flow ischemia and 20 min of reperfusion (stunned, $n=7$). Developed pressure was zero during ischemia and recovered to only $36 \pm 15\%$ ($p<0.0001$) of control values after reperfusion. Single cell-sized preparations of permeabilized myocardium were obtained by mechanical disruption of a LV biopsy and attached to a force transducer. P_o was markedly reduced (29.4 ± 11.1 kN/m^2 in control vs 43.4 ± 9.9 kN/m^2 in stunned, $p<0.01$), while there was no significant change in pCa_{50} (5.70 ± 0.06 in control vs 5.75 ± 0.19 in stunned). In conclusion, unlike in the more physiological porcine model, P_o decreases and pCa_{50} is highly variable in myocytes obtained from this rat heart model. This suggests that the decrease in P_o may be a model dependent phenomenon.

W-Pos21

DO MUTANTS IMITATING THE MONO- AND BISPHOSPHORYLATED FORM OF HUMAN CARDIAC TROPONIN I MODULATE CALCIUM SENSITIVITY IN RECONSTITUTED SKINNED CARDIAC MUSCLE FIBRES? ((C. Dohet*, H. Kögler*, E. Al-Hillawi#, I.P. Trayer#, J.C. Rüegg*)) *II. Physiologisches Institut, INF 326, 69120 Heidelberg, FRG; #School of Biochemistry, Birmingham B15 2TT, UK

Protein kinase A (PKA) dependent phosphorylation of the two adjacent serine residues within the unique amino-terminal extension of cardiac troponin I (cTnI) is known to reduce Ca^{2+}-sensitivity in skinned cardiac muscle fibres. Mutants imitating the de-, mono-, and bisphosphorylated forms of human cTnI were reconstituted into skinned cardiac muscle fibres and examined with respect to their ability to modulate Ca^{2+}-sensitivity of isometric tension. A mutant Ser23Asp/Ser24Asp induced a rightward shift of the pCa-tension relation of 0.13 pCa unit as compared to fibres reconstituted with wild type cTnI. Similar results were obtained with a mutant Ser23Glu/Ser24Glu. The reduction in Ca^{2+}-sensitivity elicited by these mutants was similar to that obtained by phosphorylation of fibres reconstituted with wild type cTnI. A Ser23Ala/Ser24Ala mutant in contrast did not influence Ca^{2+}-sensitivity. The possible effect of monophosphorylated cTnI was imitated by reconstitution with Ser23/Ser24Ala and Ser23Ala/Ser24 mutants, respectively, and phosphorylation using the catalytic subunit of PKA.

W-Pos22

CALPONIN INHIBITS, AND A CALPONIN PEPTIDE INDUCES Ca^{2+}-INDEPENDENT CONTRACTION OF SINGLE SMOOTH MUSCLE CELLS ((A. Horowitz[1,2], O. Clément-Chomienne[3], M. P. Walsh[3], T. Tao[2], H. Katsuyama[1], and K. G. Morgan[1,2])) [1]Harvard Med. School, Boston, MA 02215; [2]BBRI, Boston, MA 02114; [3]Dept. of Med. Biochemistry, U. of Calgary, Canada, T2N 4N1.

Though actin-binding and actomyosin inhibition *in vitro* by calponin (CaP) are well documented, its function in smooth muscle has not been elucidated. To address this question we exploited the ferret aorta smooth muscle cell, which shows a PKC-dependent contraction in the absence of a change in myosin light chain (MLC) phosphorylation, even at pCa 9.0. The cells were stimulated by phenylephrine (PE) or by ε-protein kinase C (PKCε) at pCa 7.0 or 9.0. Wild type recombinant CaP significantly reduced Ca^{2+}-independent force in both PE and PKCε-induced contractions by 45-60%. When CaP application preceded stimulation, contraction was completely suppressed. On the other hand, CaP phosphorylated at Ser^{175} or mutant CaP with a Ser^{175}—>Ala replacement had no effect on contractile force. We designed a synthetic peptide from a region of the CaP sequence (Leu^{166}-Gly^{194}) thought to be responsible for actin binding, while avoiding the region thought to be essential for ATPase inhibition. Treatment of cells with 50-200 μM of this peptide elicited a dose-dependent contraction, possibly by interfering with the inhibitory function of endogenous CaP. A control peptide of a scrambled sequence of the same residues produced no contractile response. These results indicate that CaP participates in thin filament-mediated regulation of smooth muscle contraction, and that it may be part of a Ca^{2+}-independent pathway downstream of PKCε. (Support: P01-AR41637, HL42293, HL31704, and MRC Canada).

W-Pos23

TRANSIENT CA^{2+} SENSITIZATION OF MYOSIN LIGHT CHAIN (MLC) PHOSPHORYLATION BY AGONIST AND GTPγS IN PERMEABILIZED RABBIT ARTERIAL SMOOTH MUSCLE. ((A. Fujita, Lin Li, T. Murahashi, and T. Kitazawa)) Dept. Physiol. & Biophys., Georgetown Univ., Washington, D.C. 20007

We have reported that either agonists or GTPγS induces MLC phosphorylation and contraction at constant Ca^{2+} with the phosphorylation declining significantly over time to low steady state levels and the force being maintained at high levels (Biophys. J. 68, A217, 1995). In this study, we investigated mechanism(s) for the inactivation of G protein-mediated Ca^{2+} sensitization. DAG and arachidonic acid inhibit MLC phosphatase indirectly via PKC, while the latter also inhibits MLC phosphatase directly. Thus, these two second messengers both increase MLC phosphorylation readily. However, we show that the two, at constant levels, increased phosphorylation *monotonically*, not transiently. Furthermore, microcystin-LR, a direct inhibitor of MLC phosphatase, at a partial concentration also increased the phosphorylation *monotonically*. Together, these two findings suggest that the transient Ca^{2+} sensitization is not due PKC downregulation or to sustained inhibition of phosphatase activity. When MLC kinase was not active, the rate of MLC dephosphorylation was inhibited after a short, 5 min incubation with GTPγS, but not significantly different from the control after a long, 30 min incubation with GTPγS. This result suggests that the inactivation of agonist/G protein-mediated Ca^{2+} sensitization results at least partly from the restoration of inhibited phosphatase activity. Supported by NIH HL51824.

W-Pos24

MYOSIN LIGHT CHAIN PHOSPHORYLATION, HEAT PRODUCTION AND SHORTENING VELOCITY DECLINE DURING ISOMETRIC CONTRACTION OF RAT ANOCOCCYGEUS MUSCLE ((JS Walker, IR Wendt, CL Gibbs & RA Murphy)) Department of Molecular Physiology & Biological Physics, University of Virginia, Charlottesville, VA 22908

The aim of this study was to correlate changes in activation level of the rat anococcygeus muscle with changes in mechanical and energetic parameters. The anococcygeus, a fast, tonic, visceral smooth muscle, was electrically stimulated for periods up to ten minutes. Myosin regulatory light chain phosphorylation (MRLCP) was measured using 2D-PAGE, heat production (h) was measured using a thermopile, unloaded shortening velocity (V) was measured using the slack test. All experiments were conducted at 27 °C. The results for basal conditions and two time points are shown in the table. Both V and MRLCP declined by 50%, while suprabasal h declined by about 30%. Over the same time period force increased by some 50%. The apparent contradiction of a decline in energy expenditure with an increased number of crossbridges can be reconciled if the reduction in cycling rate is larger than the concomitant rise in crossbridge number. The similarity of decline in V and MRLCP is consistent with four-state models that allows for such a reduction in cycling rate. The simultaneous changes described are associated with a three-fold increase in overall tissue economy. *Supported by NIH 5PO1 HL19242*

	basal	10 s	600 s
MRLCP (%)	6.8	89	47
Stress (mN/mm^2)	0	92	143
Heat-rate (mW/g)	2.8	9.3	7.3
Velocity (L_0/s)	0	0.56	0.28

W-Pos25

MEMORY OF ARTERIAL RECEPTOR ACTIVATION INVOLVES REDUCED SENSITIVITY OF CONTRACTIONS TO $[Ca^{2+}]_i$ AND REDUCED Ca^{2+} MOBILIZATION. ((P.H. Ratz, F.A. Lattanzio, Jr., and P.-M. Salomonsky)) Department of Pharmacology, Eastern Virginia Medical School, Norfolk, VA 23501

Rabbit femoral arteries retain a memory of previous maximum receptor activation for up to 3-4 h after complete cessation of the stimulus, as reflected by a reduction in the steady-state contraction produced by a subsequent exposure to KCl (Am J Physiol 269:C417-C423, 1995). The present study examined the hypothesis that this modulatory effect involves alterations in post-receptor signal transduction. To quantify the degree of cellular down-regulation induced by an episode of α_1-adrenoceptor stimulation, tissues were pretreated for 30 min with 10^{-5} M phenylephrine (PE), washed for 10 min to cause complete relaxation and activated with increasing concentrations of KCl. Pretreatment of tissues with PE resulted in a large reduction compared to control tissues in the ability of 20-60 mM KCl to increase steady-state (5 min) stress and MLC phosphorylation. However, only at low (20 and 26 mM), but not high (>26 mM) KCl concentrations did PE pretreatment reduce the ability of KCl to increase steady-state $[Ca^{2+}]_i$. These data support the hypothesis that memory of receptor activation involves reductions in both Ca^{2+}-mobilization and the sensitivity of contractile proteins to $[Ca^{2+}]_i$.
Support: AHA VA Affiliate and Eastern Virginia Medical School.

W-Pos26

REGULATION OF SMOOTH MUSCLE MYOSIN LIGHT CHAIN PHOSPHATASE (SMPP1M) BY ITS 110 kDa (M110) SUBUNIT.
((P Gailly, D Johnson*, CMM Haystead, YH Chen*, X Wu, TAJ Haystead, AP Somlyo, PTW Cohen+, AV Somlyo and P Cohen*)) University of Virginia, Charlottesville and + University of Dundee.

To characterize the interactions between the subunits (110 kDa, M110; 21 kDa, M21 and catalytic, 37 kDa, PP1C) of SMPP1M, we determined the ability of the following fragments of M110 to regulate PP1C-induced relaxation in Triton X-100 permeabilized rabbit portal vein contracted with microcystin: a) M110 purified from pig bladder, b) 72.5 kD N-terminal fragment expressed from rat kidney cDNA, c) 58 kD fragment, the N-terminal degradation product of M110, d) a synthetic peptide consisting of the first 38 aa and e) another (1-309), containing the ankyrin repeat. M110 as well as its 58 or 72.5 kDa fragments decreased approximately 1.6 fold the $t_{1/2}$ of relaxation induced by PP1C. This effect could be reproduced by a combination of the 1-38 and 1-309 peptides but not by these fragments separately, perhaps due to the presence of a GST at the N-terminus of the 1-309 fragment. Arachidonic acid (AA), known to dissociate PP1C from the native holoenzyme, inhibited the regulatory action of the 72.5kDa fragment, but not that of the combined 1-38 and 1-309 peptides. In contrast to the effect of M110 and its fragments, a peptide, corresponding to part of the PP1C binding site of the regulatory G subunit from skeletal muscle, specifically slowed relaxation, induced by flash photolysis of Diazo-2, of Triton X-100 permeabilized femoral artery strips. Consistent with this finding, this peptide can dissociate PP1C from M110 in vitro. We conclude that: 1. the N-terminal sequence (1-309) of M110 enhances the activity of PP1C to myosin, 2. M110 effect can be inhibited by AA acting on a different region (309-668) , and 3. the G subunit can compete with M110 for the same binding site on PP1C.

W-Pos27

IDENTIFICATION OF SMOOTH MUSCLE Ca^{2+}-BINDING PROTEINS.
((B.G. Allen*‡, I. Durussel*, M.P. Walsh* and J.A. Cox*)) *Smooth Muscle Research Group, University of Calgary, Calgary, Alberta, Canada T2N 4N1, ‡Institut de Cardiologie de Montréal, 5000, rue Bélanger, Montréal, Québec, Canada H1T 1C8 and *Department of Biochemistry, University of Geneva, CH-1211 Geneva 4, Switzerland.

Four Ca^{2+}-binding proteins (of 6, 7.5, 16.5 and 21 kDa) which interact with a hydrophobic matrix in a Ca^{2+}-dependent manner were purified from chicken gizzard smooth muscle and identified as calcyclin, calgizzarin, calmodulin and neurocalcin, respectively. Calcyclin and calgizzarin, members of the S100 family of Ca^{2+}-binding proteins, were characterized. Two isoforms of calcyclin (A and B), which differ with respect to the presence or absence of a *C*-terminal lysine, were identified. Calcyclin exists as non-covalently associated homo- (AA and BB) and heterodimers (AB) under non-denaturing conditions. Calcyclin and calgizzarin bind Ca^{2+} as shown by ^{45}Ca gel overlay, gel filtration ± Ca^{2+}, urea/glycine gel electrophoresis ± Ca^{2+}, UV difference spectroscopy and flow dialysis. Ca^{2+}- binding induces exposure of a hydrophobic patch presumed to interact with a target protein. At physiological ionic strength and pH, both calcyclin and calgizzarin bind Ca^{2+} with low affinity (K_d ≈ 0.3 mM) and slight positive cooperativity (n_H = 1.3-1.4). Mg^{2+} has no effect on the Ca^{2+}binding properties. Calcyclin is expressed in several chicken tissues, being most abundant in smooth muscle tissues. Calgizzarin is also widely distributed, but is most abundant in lung.

W-Pos28

CGMP-INDUCED CA^{2+} DESENSITIZATION OF CONTRACTION AND MYOSIN LIGHT CHAIN PHOSPHORYLATION IN PERMEABILIZED SMOOTH MUSCLE.
((T. Kitazawa, M. Lee, L. Zhang, M. Masuo and L. Li)) Dept. Physiol. & Biophys., Georgetown Univ., Washington, D.C. 20007

Using permeabilized arterial smooth muscle, we investigated mechanism(s) for the cGMP-induced Ca^{2+}-desensitization of the regulatory/contractile apparatus. As expected, this cyclic nucleotide and its non-hydrolyzable analog 8Br-cGMP dose-dependently reduced the contractile sensitivity to Ca^{2+}. When applied to permeabilized strips at pCa 6, 10 μM 8Br-cGMP reduced the relative MLC phosphorylation from 69 ± 3.3 to 41 ± 2.5% and reduced the contractile force by 57 ± 4.3% with a half time of 3.7 ± 0.3 min. The PKG inhibitor Rp-8-PCPT-cGMPS significantly decreased this cGMP inhibitory effect on the contractile force at pCa 6.3. When MLC kinase activity was blocked, the rates of both MLC dephosphorylation and relaxation were significantly accelerated by 8Br-cGMP. When MLC phosphatase activity was blocked, the rate of contraction at pCa 6.7 was not significantly affected. These results suggest that cGMP activates the MLC phosphatase via PKG. This work was supported by NIH HL51824.

W-Pos29

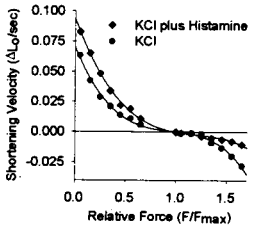
THE DISTRIBUTION OF MYOCYTE PHENOTYPES IN SMOOTH MUSCLES IS A DETERMINANT OF TISSUE CONTRACTILITY. ((A.J. Halayko, E. Rector* and N.L. Stephens)) Dept Physiology, Flow Cytometry Lab*, U Manitoba, Winnipeg, MB, R3E 0W3 and Inspiraplex, Montreal, PQ, H2X 2P4.

Broad differences in the contractile properties of smooth muscles form various sources exist. Smooth muscle is not comprised of phenotypically homogeneous populations of myocytes; differences in expression of isoforms of proteins which comprise or regulate the cytocontractile apparatus exist between sub-populations of smooth muscle cells (SMC) (Frid et al, Circ Res 75:669,1994). We compared the distribution of SMC phenotypes in adult canine pulmonary smooth muscles possessing different contractile properties. Myocytes were dispersed from airways and arteries and subsequently fixed with ethanol for FACS analysis or total protein homogenates were prepared for immunoblot analysis. Tetraploid SMCs comprised 7-15% of the total population in all tissues examined; these cells did not stain positively using anti-cyclin B antibodies indicating that the myocytes were not arrested in G_2 phase of the cell cycle. Greater than 95% of all diploid and tetraploid airway's SMC stained positively for smooth muscle myosin heavy chain, sm-α-actin, calponin and *h*-caldesmon. However, the mean cellular contents of these markers were 20% higher in tracheal SMCs than in bronchial SMCs ($p<0.05$) and 35% higher than measured in main pulmonary arterial SMCs. Coincidentally, shortening velocity and shortening capacity of tracheal smooth muscle is 20-30% greater than for bronchial and pulmonary arterial muscles. These data suggest that smooth muscle contractility is correlated with the distribution of phenotypically distinct myocyte populations (Supported by the Medical Research Council of Canada).

W-Pos30

CROSS-BRIDGE PHOSPHORYLATION DETERMINES LENGTHENING AS WELL AS SHORTENING VELOCITIES IN SWINE CAROTID MEDIAL RINGS. ((J.D. Strauss, N.T.S. Flecker, R.A. Murphy)) Molecular Physiology and Biological Physics, University of Virginia, HSC 449, Charlottesville, VA 22908

A Cambridge Technology Servo motor was used to allow isotonic shortening and lengthening at a series of loads between 5% and 165% of F_{max} (initial isometric tension at L_o). Velocities were derived from exponential fits of length data during a 1 sec force clamp. Tissues "yielded" at approx. 1.7 F_{max} after which the rings did not recover F_{max}, under isometric conditions at L_o, suggesting mechanical damage. The graph depicts a distinct divergence of relative "lengthening" as well as shortening velocity for a single ring stimulated with 109 mM KCl (KCl) versus stimulation with KCl + 30 μM histamine (HIS). The initial lengthening region of this relation for 7 tissues was approximately linear and the slope for HIS was approx. half that of KCl (-0.026 versus -0.054). Myosin phosphorylation values for rings in parallel isometric experiments were 23.2 ± 2.5% (±SEM, n=8) for KCl and 34.25 ± 2.6% (n=8) for HIS. Assuming forced detachment of crossbridges occurs during lengthening, these results are consistent with the hypothesis that phosphorylation is necessary for cross-bridge reattachment. Supported by NIH 5P01 HL19242 (RAM) and AHA VA-93-F-13 (JDS).



W-Pos31

NMR STUDIES OF TROPONIN I(96-115): FREE IN SOLUTION AND BOUND TO TROPONIN C. ((G. Hernández, S. E. Rokop, P. A. Springer, C. J. Unkefer, and J. Trewhella)) Los Alamos National Laboratory, Los Alamos, NM 87545

We proposed a low resolution model of $4Ca^{2+}$•TnC•TnI based on small-angle neutron scattering studies (Olah & Trewhella, *Biochemistry 33*, 12800, 1995). We wish to use this model now to guide high resolution structural studies using NMR and isotope labeling to gain deeper insight into the molecular basis for TnC's Ca^{2+}-dependent regulatory function. A complete NMR analysis of the TnC•TnI complex is very difficult due to its large molecular weight, highly helical structure, and the presence of two structurally homologous domains. We are therefore focusing on complexes of TnC with key segments of TnI. Initially, we have used the inhibitory peptide sequence corresponding to residues 96-115 in TnI. TnI(104-115) has already been studied by Sykes and co-workers (*J. Mol. Biol. 227*, 405, 1991) who used transferred NOE data to propose a helical structure for this shorter peptide sequence bound to TnC. TnI(96-115) binds with an order of magnitude higher affinity than the shorter inhibitory sequence, and we have prepared complexes of $TnC_{(deuterated)}$ with TnI (96-115) to study the structure of the longer peptide in the complex. NMR resonances of TnI(96-115) have been assigned in the free and bound peptide. NOESY spectra suggest a nascent helical structure for the free peptide. Comparison of the chemical shifts following the empirical rule of Wishart D.S. et al (*Biochemistry 31*, 1647, 1992) also agree with some helix formation. Aggregation of the complexe was eliminated using a concentration of only 0.7mM and 1% TFE. At this low concentration, 750 MHz NMR spectra were required to resolve all the peptide resonances. NOESY spectra shows that the turns present on the free peptide are conserved in the complex.

W-Pos32

THE ROLE OF METAL BINDING TO THE Ca^{2+} BINDING SITES OF TnC ON ITS STRUCTURE AND INTERACTION WITH TnI ((James D. Potter[1], Walter F. Stafford[2], Todd Miller[3], Herman Ellemberger[4], Georgianna Guzman[3] and Danuta Szczesna[3])) [1]Dept. of Mol. & Cell. Pharm., Univ. of Miami Sch. of Med., Miami FL 33136; [2]Boston Biomed. Res. Inst., Boston MA 02114; [4]Colgate Univ Hamilton, NY 13346

We investigated the role of the Ca^{2+} specific sites (I and II) and the high affinity Ca^{2+}-Mg^{2+} sites (III and IV) of TnC on the interaction of TnC with TnI. Wild type (WTnC) and two mutants (TnC1,2-, TnC3,4-) of chicken skeletal TnC (Potter *et al.*, Bioph. J. 68, A70, 1995) were examined for metal-dependent interactions with TnI. The TnC's and the TnC-TnI complexes were run on polyacrylamide gels in non-dissociating conditions (no urea, no SDS). We found that migration of WTnC and TnC1,2- (inactivated Ca^{2+} specific sites I and II) was significantly faster in Ca^{2+} than in Mg^{2+}. The COOH-terminal mutant of TnC having inactivated Ca^{2+} binding sites III and IV (TnC3,4-) showed only a small change in mobility in the presence of Ca^{2+}. All TnC's bound to TnI in the presence of Ca^{2+} and less so in Mg^{2+}. In competition experiment between pairs of these TnC's, TnC3,4- was least able to compete for complex formation with TnI independent of Ca^{2+} or Mg^{2+}. In another approach we have employed sedimentation velocity analysis, carried out using the time derivative method (Stafford, Anal. Bioch.203, 295-301, 1992) with an analytical ultracentrifuge (Beckman Instruments Model XL-A) equipped with Rayleigh optics (Stafford and Liu, SPIE Proceedings 2386, 130-135, 1995). The sedimentation coefficients (in svedbergs) for the TnC's in the Ca^{2+}-, Mg^{2+}- and apo (no metal)- states and complexed with TnI are listed in the table.

	Ca^{2+}	Mg^{2+}	apo	+TnI in Ca^{2+}
WTnC	2.18	1.91	1.55	2.71
TnC 1,2-	1.97	1.85	1.55	2.81
TnC 3,4-	1.83	1.70	1.66	2.81

The binding of both Mg^{2+} and Ca^{2+} to TnC results in a more compact structure of the protein. Inactivation of either sites I and II or III and IV reduces these effects.

W-Pos33

Flexibility and Rigidity of the Troponin Complex Conformation Effected by Calcium ((X. Zhao, T.Kobayashi, H.Malak, I.Gryczynski, J.Lakowicz, R.Wade and J.H.Collins)) Dept. of Biol. Chem., Univ. MD, School of Medicine and Med. Biotech. Center, Univ. MD. Biotech. Inst., Baltimore, MD 21201.

Ca^{2+} regulation of vertebrate striated muscle contraction is initiated by conformational changes in the Ca^{2+}-binding protein troponin C (TnC) and subsequent changes in the interaction of TnC with the inhibitory protein TnI and the tropomyosin-binding protein TnT. We used the frequency domain method of fluorescence resonance energy transfer to measure TnT-TnC, TnT-TnI and TnI-TnC distances and distributions, in the presence of Ca^{2+}, Mg^{2+}, or EGTA, in TnC-TnI-TnT and TnC-TnI complexes. We formed ternary complexes using the following recombinant subunits whose sequences were based on those of rabbit skeletal muscle: wild-type TnC; TnT_{25}, a mutant C-terminal 25-kDa fragment of TnT containing a single Trp-212 which was used as the energy donor; Trp-less TnI mutants which contained either no Cys or a single Cys at position 9, 96, or 117. A binary complex was formed with wild-type TnC and mutant TnI which contained no Cys and a single Trp 106. Energy acceptor groups were introduced into TnC or TnI by chemical modification. Our results indicate that the troponin complex is relatively rigid in relaxed muscle, but becomes much more flexible when Ca^{2+} binds to regulatory sites in TnC. This increased flexibility may be propagated to the whole thin filament, releasing the inhibition of actomyosin ATPase activity and allowing the muscle to contract.

E-C COUPLING: SMOOTH MUSCLE

W-Pos34

STAUROSPORINE-SENSITIVE PROTEIN KINASE C ACTIVITY REGULATES MYOFILAMENT Ca^{2+} SENSITIVITY IN ARTERIAL SMOOTH MUSCLE ((Michael R. Samardzija and Robert S. Moreland)) Bockus Research Institute, Graduate Hospital, Philadelphia, PA 19146. (Spon. by S. Moreland)

It is widely accepted that an increase in $[Ca^{2+}]$ initiates vascular smooth muscle contraction. In addition, it is known that myofilament Ca^{2+} sensitivity can be increased by a receptor and G-protein dependent mechanism. The goal of this study was to test the hypothesis that the increase in myofilament Ca^{2+} sensitivity is due, in part, to activation of protein kinase C (PKC). To test this hypothesis, we measured force, myosin light chain (MLC) phosphorylation, and PKC activity in rabbit mesenteric arteries permeabilized with alpha toxin. PKC activity was assayed by measuring ^{32}P incorporation into myelin basic $protein_{4-14}$ that was introduced into the cytosolic space of the permeabilized artery. Force increased monotonically in response to 0.7 μM Ca^{2+} alone or plus 10 μM NE plus 10 μM GTP. In contrast, MLC phosphorylation levels increased transiently in response to either mode of stimulation; steady state levels were greater during exposure to NE plus GTP. Staurosporine completely abolished the enhanced force in response to NE plus GTP but only blocked the transient increase of MLC phosphorylation levels; steady state levels were unaffected. Levels of PKC activity increased during stimulation by Ca^{2+} alone and were further increased by the addition of NE plus GTP. We suggest that staurosporine-sensitive PKC activity is involved in the receptor-mediated enhancement of myofilament Ca^{2+} sensitivity. Moreover, our results suggest that the initial peak increase in MLC phosphorylation levels but not steady state levels are important for regulation of myofilament Ca^{2+} sensitivity. Support: HL 37956; HL 46704.

W-Pos35

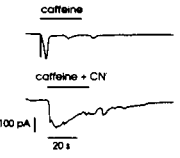
MEMBRANE POTENTIAL, POTASSIUM AND CALCIUM CHANNELS OF THE RAT INTERLOBAR ARTERY ARE ALTERED IN THE DOCA MODEL OF HYPERTENSION. ((Jeffrey R. Martens and Craig H. Gelband)) Department of Physiology, University of Florida College of Medicine, Gainesville, FL 32610.

Little data is available on ion channel function in resistance blood vessels of the kidney. The patch clamp technique and contractile assays were used to examine the differences in interlobar arteries from Sprague Dawley (SD) and deoxycorticosterone acetate (DOCA, 100 mg/kg) treated rats. The mean systolic blood pressures of the SD and DOCA rats were 115 ± 7 and 165 ± 10 mm Hg, respectively (n = 15). KCl (80 mM), angiotensin II (10 nM), phenylephrine (1 μM) and arginine vasopressin (10 nM) caused contraction of isolated arteriolar rings. When contraction was normalized to muscle stress, the DOCA segments produced significantly more force than the SD (n = 7). In current clamp experiments (n = 6), the resting membrane potential of the DOCA cells was 24 ± 3.4 mV more positive than the SD cells. In voltage clamp experiments, DOCA cells exhibited an increased peak Ca^{2+} current which activated at more negative potentials (n = 5). With 4-AP (10 mM) and niflumic acid (100 μM) present to inhibit $K_{(v)}$ and $Cl_{(Ca)}$, DOCA $K_{(Ca)}$ was significantly larger and activated at more negative potentials than the SD (n = 5). Conversely, with ChTX (100 nM) and niflumic acid (100 μM) present to inhibit $K_{(Ca)}$ and $Cl_{(Ca)}$, DOCA $K_{(v)}$ was significantly smaller than the SD (n = 5). These results suggest that membrane potential, K^+ and Ca^{2+} currents are altered in DOCA hypertensive rat renal interlobar arteries. (Supported by an Initial Investigatorship from the AHA, Florida Affiliate).

W-Pos36

PROLONGATION OF Ca^{2+}-ACTIVATED Cl^- CURRENTS IN RAT PULMONARY ARTERY CELLS BY METABOLIC INHIBITION. ((Qiang Wang and Michael I. Kotlikoff)). Dept. of Animal Biology, Laboratory of Pharmacology, Univ. of Pennsylvania School of Vet. Medicine, Philadelphia, PA 19104.

Ca^{2+}-activated Cl^- currents were recorded from isolated rat pulmonary artery cells using the nystatin-perforated and standard whole-cell patch clamp techniques. At a holding potential of -50 mV, which was close to the resting membrane potential, norepinephrine (NE) and caffeine evoked typical currents which had multiple phases in K^+-containing pipette and bath solutions. The evoked inward currents were usually followed by an outward current and another inward current. Removal of K^+ from the system diminished the evoked outward currents, indicating that K^+ was the major charge carrier for the outward current. In K-free conditions, however, only inward currents to NE and caffeine were recorded. The reversal potential of agonist-induced inward currents followed the shift of the anion but not the cation gradient across the membrane. In addition, the inward current was never observed when the pipette solution contained 10 mM EGTA, suggesting this inward current is a Ca-activated Cl current. Bath application of the metabolic inhibitor CN^- (2-10mM) also induced inward currents ranging from 15pA to 263pA (n=7). Moreover, the caffeine-induced Cl currents were substantially prolonged in the presence of CN^- (see the inserted figure, data obtained from the same cell). The present study suggests that metabolic inhibition may interfere with the energy-dependent Ca extrusion or re-uptake process, which results in an elevated intracellular calcium concentration and subsequent activation and prolongation of the Ca-activated Cl channels.



W-Pos37

NON-SELECTIVE CATION CHANNELS IN CANINE COLON SMOOTH MUSCLE CELLS. ((B.W. Frey, A. Carl and K.M. Sanders)) University of Nevada Reno, School of Medicine, Physiology Dept., Reno, NV 89557

Using the Amphotericin method of perforated patch whole cell recording, we observed large holding currents at -60 mV and inward tail currents following step depolarizations of the cell membrane potential to +50 mV. These residual currents had not been seen in earlier studies using dialyzed whole cell patch clamp technique. 10 mM TEA, 5 mM 4-AP and 2 mM $MnCl_2$ were included in the bath solution to block I_K and I_{Ca}. In order to determine the nature of these currents, we replaced 140 mM NaCl in the bath solution with 140 mM N-Methyl-D-Glucamine. This cation substitution reduced the holding current 90.7±3.4% (n=6) and abolished the inward tails. We also replaced 140 mM NaCl in the bath solution with 140 mM Na-Isethionate, shifting the calculated Cl^- equilibrium potential to +42 mV. This anion substitution was without effect on holding current or inward tails. The Cl^- channel blocker niflumic acid did not reduce tail currents at 10 uM (98% of control current) but slightly reduced current at 100 uM (75% of control current). This current was also insensitive to Gd^{3+} up to 100 uM. In addition to these currents, a few cells also displayed "creep" currents, slowly increasing in amplitude over 1 to 4 sec step depolarization to > +40 mV. Creep currents were associated with cell swelling, were followed by large inward tails on repolarization and were completely abolished by 10 uM Gd^{3+}.

These data suggest the existence of at least two types of non-selctive channels in colonic smooth muscle cells. (i) a Gd^{3+} insensitive current I_{X1} active at resting membrane potentials and (ii) a Gd^{3+} sensitive current I_{X2} observed only at very positive membrane potentials and associated with cell swelling. The relationship of these currents with ACh and SP induced non-selective currents described previously by H.K. Lee and K.M. Sanders in colonic muscle (1993, 1995) remains to be established.
Supported by NIH DK-41315.

W-Pos38

ACTIVATION OF MUSCARINIC M_2 RECEPTORS BY METHACHOLINE OPENS NON-SELECTIVE CATION CHANNELS IN AIRWAY SMOOTH MUSCLE CELLS THROUGH PTX-SENSITIVE G PROTEINS. ((Y.-X. Wang, B.K. Fleischmann, and M.I. Kotlikoff)) Department of Animal Biology, School of Vet. Med., Univ. of Penn., Philadelphia, PA 19104-6046. (Sponsored by M.I. Kotlikoff)

Activation of muscarinic receptors induces a non-selective cation current in smooth muscle cells. We examined the signalling pathway associated with the activation of this current using combined measurements of current and calcium in voltage-clamped, equine tracheal myocytes. Application of methacholine (mACH, 50 μM) to cells clamped at -60 mV produced a large, rapidly inactivating chloride current (I_{Cl})and a sustaining non-selective cation current (I_{Cat}), as well as a large transient increase in $[Ca^{++}]_i$ followed by a sustained elevation of about 30% of the peak. The M_2 receptor antagonist, methoctramine (0.2 mM), almost fully inhibited both I_{Cat} and the sustained increase in $[Ca^{++}]_i$ concentration, whereas the I_{Cl} and the transient calcium elevation were unaffected. In the presence of the M_2 receptor antagonist, hexahydro-sila-difenidol (0.4 mM), mACH failed to induce either the I_{Cl} or I_{Cat}, or a rise in $[Ca^{++}]_i$, whereas simultaneous application of caffeine (8 mM) and mACH evoked I_{Cl} and I_{Cat} as well as the transient and sustained elevation of calcium. Caffeine application alone evoked only the I_{Cl} and transient elevation of $[Ca^{++}]_i$. Similarly, application of mACH to cells pretreated with pertussis toxin (0.5 mg/ml for 4-6 hours) evoked I_{Cl} and the transient elevation of $[Ca^{++}]_i$, but not I_{Cat} or the tonic elevation of $[Ca^{++}]_i$. I_{Cat} was isolated by voltage clamping cells at the chloride equilibrium potential. Under these conditions, mACH induced I_{Cat} without I_{Cl}, and inclusion of $G_{\alpha i1,2}$ or $G_{\alpha i3}$ subunit antibody in the patch pipette blocked I_{Cat} and the tonic calcium elevation. These results provide direct evidence that activation of muscarinic M_2 receptors leads to the opening of non-selective cation channels through pertussis toxin-sensitive G_{α} proteins in smooth muscle cells. A rise in $[Ca^{++}]_i$ is necessary but not sufficient for activation of the cation current.

W-Pos39

CALCIUM SPARKS RELAX ARTERIAL SMOOTH MUSCLE ((A.D. Bonev[1], V.A. Porter[1], M. Rubart[2], H. Cheng[3], L.F. Santana[3], H.J. Knot[1], W.J. Lederer[3],and M.T. Nelson[1])) [1]Dept. Pharmacol., Univ. of Vermont, Colchester, VT 05446, [2]Krannert Institute of Cardiology, University of Indiana, 1111 West 10th St., Indianapolis, IN 46202, [3]Dept. of Physiol. and The Medical Biotechology Center, 519 Howard Hall, University of Maryland, 660 W. Redwood St., Baltimore, MD 21201

The functional role of ryanodine-sensitive SR Ca^{2+} release channel in arterial smooth muscle is unknown. Using a laser scanning confocal microscope and the fluorescent calcium indicator fluo-3, we detected localized increases in the intracellular Ca^{2+} ("Ca^{2+} sparks") in smooth muscle cells isolated from small cerebral arteries (100-150 µm). Ryanodine (Ry) (10 µM) and thapsigargin (100 nM) inhibited Ca^{2+} sparks, suggesting that they derived from SR. The mean rise-time and half-time of decay of Ca^{2+} sparks were 20.2 ms ± 2.3 ms and 48.0 ± 2.6 ms (n = 11), respectively. The mean peak $[Ca]_i$ during the spark was 303 ± 27 nM. The mean spread of the spark at the peak was 2.38 ± 0.14 microns. The majority of the sparks (59%) appeared close to the sarcolemmal surfaces (within 1 µm). Ca^{2+} activated K^+ (K_{Ca}) channels that exist in smooth muscle cell membrane should be activated by the local increase in $[Ca]_i$ produced by Ca^{2+} spark. Using perforated patch clamp technique, we recorded spontaneous transient outward currents (STOCs) which had similar time-course to that of Ca^{2+} sparks. STOCs were through K_{Ca} channels because they were blocked by iberiotoxin (100 nM) and reduced to 50% by TEA (0.2 mM). Ry (10 µM) and thapsigargin (100 nM) inhibited STOCs suggesting that Ca^{2+} sparks activate K_{Ca} channels. Furthermore, we recorded simultaneously sparks and STOCs. K_{Ca} channels activated by Ca^{2+} sparks appeared to hyperpolarize and dilate pressurized arteries because Ry and thapsigargin depolarized and constricted these arteries to similar extent as did the blockers of K_{Ca} channels. In the presence of iberiotoxin ryanodine and thapsigargin were without effect. We conclude that Ry sensitive Ca^{2+} release channels in smooth muscle SR have a central role in limiting muscle contraction by activating K_{Ca} channels.
Supported by the NIH and American Heart Association, VT Affiliate

W-Pos40

NITROGLYCERIN RELAXES RAT TAIL ARTERY PRIMARILY BY DECREASING $[Ca^{2+}]_i$ SENSITIVITY AND PARTIALLY BY REPOLARIZATION AND INHIBITING Ca^{2+} RELEASE. ((Xiao-Liang Chen & Christopher M. Rembold)) Cardiovascular Div., U. of Virginia, Charlottesville, VA 22908

There are at least five mechanisms hypothesized to account for cGMP induced smooth muscle relaxation: 1) repolarization, 2) inhibition of Ca^{2+} release, 3) inactivation of L type Ca^{2+} channels, 4) enhancement of Ca^{2+} efflux/sequestration, and 5) decreasing the $[Ca^{2+}]_i$ sensitivity of force. We studied the physiologic relevance of these five mechanisms in the intact rat tail artery stimulated with phenylephrine or high $[K^+]_o$ and then relaxed with nitroglycerin. We measured E_m, $[Ca^{2+}]_i$ with Fura 2, and isometric force. Decreases in the $[Ca^{2+}]_i$ sensitivity of force accounted for most of the nitroglycerin induced relaxation of tissues prestimulated with maximal (1 μM) phenylephrine or 30 mM $[K^+]_o$. In submaximally (0.1 - 0.3 μM) phenylephrine prestimulated tissues, the nitroglycerin induced relaxation was caused primarily by a decrease in the $[Ca^{2+}]_i$ sensitivity of force and partially by repolarization and the resultant decrease in $[Ca^{2+}]_i$. Nitroglycerin also partially attenuated transient increases in $[Ca^{2+}]_i$ and force induced by 100 μM phenylephrine in the absence of extracellular Ca^{2+}. Nitroglycerin induced relaxation was not associated with inactivation of Ca^{2+} channels or enhancement of Ca^{2+} efflux/sequestration. These data suggest that nitroglycerin primarily relaxes precontracted rat tail artery primarily by decreasing the $[Ca^{2+}]_i$ sensitivity of force.

W-Pos41

CYCLIC NUCLEOTIDES RELAX CONTRACTIONS OF ALPHA-TOXIN PERMEABILIZED ARTERIES WITHOUT A PROPORTIONAL CHANGE IN MYOSIN LIGHT CHAIN PHOSPHORYLATION. ((Xiaoling Su, Surender S. Katoch, and Robert S. Moreland)) Bockus Research Institute, Graduate Hospital, Philadelphia, PA 19146.

The two cyclic nucleotides, cAMP and cGMP, have long been known to produce relaxation of contracted vascular smooth muscle. Most investigations have suggested that cyclic nucleotides induce relaxation by decreasing intracellular $[Ca^{2+}]$. However, recent studies have shown that cyclic nucleotides may also induce relaxation by a pathway that is independent of Ca^{2+}. The goal of this study was to determine if either cAMP or cGMP relax vascular smooth muscle at constant $[Ca^{2+}]$ and if so, are the relaxations associated with a change in the levels of myosin light chain (MLC) phosphorylation. We used the alpha-toxin permeabilized mesenteric artery of the rabbit. This preparation has the distinct advantage of allowing precise control of intracellular $[Ca^{2+}]$ while maintaining intact receptor and G-protein mediated signal transduction pathways. Both cAMP and cGMP relaxed Ca^{2+}-induced contractions of the permeabilized artery in a concentration-dependent fashion. The addition of norepinephrine (NE) plus GTP has been shown to significantly enhance myofilament Ca^{2+} sensitivity in this permeabilized arterial preparation. Both cAMP and cGMP also relaxed these Ca^{2+} plus NE plus GTP-induced contractions. Relaxations of the Ca^{2+} alone and Ca^{2+} plus NE plus GTP-induced contractions were not associated with proportional decreases in MLC phosphorylation levels. These results demonstrate that cyclic nucleotides can relax contraction of vascular smooth muscle by a pathway that is independent of both Ca^{2+} and MLC phosphorylation. This study was supported in part by funds from NIH HL 37956 and HL 46704.

W-Pos42

THAPSIGARGIN CAUSES ENDOTHELIUM-DEPENDENT HYPERPOLARIZATION IN RAT MESENTERIC SMALL ARTERIES. ((H. Nilsson and M.J. Mulvany)) Department of Pharmacology and the Danish Biomembrane Research Centre, University of Aarhus, Denmark

Intact intracellular calcium stores have been shown to be essential for the activation of portal vein smooth muscle by agonists. Conversely, the emptying of intracellular calcium stores has been shown to elevate intracellular calcium levels ($[Ca]_i$) in several other cell types. To determine the role of intracellular stores for activation of vascular smooth muscle, the effects of store depletion by thapsigargin (TG; 1 μM for 10 min) on the smooth muscle response was studied in intact and de-endothelialised preparations of rat small mesenteric arteries mounted in a myograph. Membrane potentials were measured by conventional intracellular techniques. $[Ca]_i$ was determined using Fura-2. In the intact vessel, mechanical and $[Ca]_i$ responses to noradrenaline (NA; 2.5 mM external Ca) vanished 10-20 min after TG treatment, while responses to ATP or high-K solutions remained. The smooth muscle gradually hyperpolarized, by 16±2 mV (n=4) after 30 min; this hyperpolarization lasted for more than 1 hour. After endothelial denudation, mechanical and $[Ca]_i$ responses to NA in 2.5 mM Ca were not affected by TG treatment, and membrane potential responses were normal. However, the response to NA in Ca-free solution disappeared regardless of the presence of endothelium. The results show strong, long-lasting activation of endothelial hyperpolarization by TG, with no evidence of sustained enhanced Ca influx in the smooth muscle.

W-Pos43

CYCLIC ADP-RIBOSE RELEASES Ca^{2+} FROM SARCOPLASMIC RETICULUM OF PORCINE CORONARY ARTERY SMOOTH MUSCLE CELLS ((M.S. Kannan, A.M. Fenton, Y.S. Prakash and G.C. Sieck)) Dept. of Veterinary PathoBiology, University of Minnesota, St. Paul, MN 55108 and Depts. of Internal Medicine, Anesthesiology and Physiology & Biophysics, Mayo Foundation, Rochester, MN 55902. (Spon. J. Mickelson).

Ca^{2+} release from sarcoplasmic reticulum (SR) results from receptor-gated, G-protein-coupled activation of inositol trisphosphate (IP_3) and Ca^{2+}-induced Ca^{2+} release through ryanodine receptors (RYR). Cyclic ADP-ribose (cADPR), a metabolite of NAD^+, is known to regulate RYR. In freshly isolated single, ß-escin permeabilized coronary artery smooth muscle (CSM) cells, we examined SR Ca^{2+} release in response to IP_3 and cADPR using a video fluorescence imaging technique. The ratio of fura-2 emissions when excited at 340 and 380 nm was used as an index of $[Ca^{2+}]_i$. Permeabilized cells were exposed to a pCa 6.3 solution to load the SR and then returned to a pCa 9.0 solution. Exposure to IP_3 (18-72 μM) or cADPR (0.3-2.0 μM) resulted in elevation of $[Ca^{2+}]_i$. Prior exposure to 0.5 mg/ml heparin prevented the IP_3 response but not the cADPR response. Addition of 100 μM ryanodine did not block either the IP_3 or cADPR responses. Exposure to caffeine (50 mM) also did not affect the cADPR response. Exposure to both NAD^+ (2 μM), the precursor, and ADPR (2 μM), the metabolite, had no effect on $[Ca^{2+}]_i$. These results provide evidence for cADPR-induced SR Ca^{2+} release in CSM cells through a mechanism that is independent of IP_3 and RYR activation. Supported by NIH grants (HL51736& HL07111), Mayo Fdn. and Univ. of Minnesota. MSK was a recipient of an Abbott Fellowship.

W-Pos44

HISTAMINE-INDUCED DESENSITIZATION OF PORCINE CAROTID ARTERY SMOOTH MUSCLE TO DEPOLARIZING STIMULI. ((R.L. Wardle, J.D. Strauss, C.M. Rembold and R.A. Murphy)) Molecular Physiology and Biological Physics, University of Virginia, Charlottesville, VA 22908

Phenylephrine-induced desensitization of rabbit femoral artery smooth muscle to depolarizing stimuli has been reported (Ratz, P.H., Am. J. Physiol. 269:C417-C423, 1995). Our aims were to see if this phenomena occurred in another arterial smooth muscle and to elucidate the underlying mechanism(s). We found that porcine carotid artery smooth muscle exhibits histamine-induced desensitization to depolarizing stimuli. We measured isometric force generated by medial rings in response to raising extracellular KCl to 40 mM both before and after exposure to histamine. The rate of force development in response to KCl was significantly attenuated following histamine and was dependent upon [histamine] (10^{-6} to 10^{-3} M), duration of histamine exposure (10-80 min), and duration of the interval between histamine and KCl (20-120 min). Medial strips were loaded with the Ca^{2+} indicator aequorin and we measured simultaneously isometric force and aequorin luminescence during 40 mM KCl-induced contractions both before and after histamine (10^{-3} M, 30 min). The attenuated rate of force development in desensitized muscle strips can be attributed predominantly to an attenuated rate of rise of myoplasmic $[Ca^{2+}]$. The mechanisms underlying this attenuated response remain to be elucidated. Supported by NIH 5P01 HL19242, NIH HL 38918, NIH T32 HL07284 traineeship to RLW, and AHA VA-93-F-13 to JDS.

W-Pos45

A CAGED PEPTIDE INHIBITOR OF CALMODULIN PREVENTS Ca^{2+}-DEPENDENT ENHANCEMENT OF Ca^{2+} CURRENT IN SMOOTH MUSCLE CELLS. ((R.M. Drummond, R. Sreekumar*, J.W. Walker*, R.E. Carraway, M. Ikebe#, and F.S. Fay)). Dept. of Physiology, Univ. Massachusetts. Med. Center, Worcester, MA 01605, *Dept. of Physiology, Univ. of Wisconsin, Madison, WI 53706, and #Dept. of Physiology and Biophysics, Case western Reserve Univ., Cleveland, OH 44106.

To further investigate the role of calmodulin in the persistent enhancement of inward current, which we have previously described (Nature 357, 74), we have developed a caged peptide inhibitor of calmodulin. The peptide which is inactive before photolysis, is based on the calmodulin inhibitory peptide RS-20, with the exception that tryptophan at position 5 is replaced with a caged tyrosine. This substitution reduces the inhibitory potency of RS-20 by at least two orders of magnitude. However, following photolytic removal of the protecting group, the tyrosine variant of RS-20 retains a high inhibitory potency toward calmodulin. The effect of the caged peptide (cRS-20) on Ca^{2+} current was studied in freshly isolated smooth muscle cells from the stomach of *Bufo marinus*, under conditions where membrane potential was controlled by tight-seal, whole cell recording. The experimental protocol commenced with a reference 3 sec depolarization (-110 to +10 mV). After approximately 15 secs, an 8 sec conditioning train of pulses (-110 to +10 mV; 0.5Hz) was delivered and this was followed 12 sec later by a 3 sec test pulse (-110 to +10 mV). In smooth muscle cells which had been pressure injected with cRS-20 via the patch pipette, the peak current increased from 490 ± 76 pA to 744 ± 127 pA (n=7, P<0.05) following the train . After 15 sec exposure to UV, the enhancement of the inward current normally seen after the train was absent, being 562 ± 86 pA before and 505 ± 97 pA (n.s.) after the train respectively. Thus, the facilitation of the inward current normally seen following the conditioning train, was blocked after photolysis of the caged peptide inhibitor of calmodulin. The caged peptide offers the advantage of being able to use the same cell as both control and test of the calmodulin dependence of specific cellular processes. In addition it should prove to be a useful tool for investigating the spatial and temporal aspects of calmodulin involvement in various functions of smooth muscle and other cell types. Supported by an AHA postdoctoral fellowship (RMD) and NIH HL 14523 and HL 47530 (FSF).

W-Pos46

Ca^{2+}-INDUCED Ca^{2+} RELEASE DOES NOT PLAY A SIGNIFICANT ROLE IN RAT PORTAL VEIN SMOOTH MUSCLE CELLS DURING DEPOLARISATION. ((T. Kamishima and J.G. McCarron)) IBLS, University of Glasgow, G12 8QQ, Scotland

We investigated Ca^{2+}-induced Ca^{2+} release's role in vascular smooth muscle Ca^{2+} homeostasis. Single smooth muscle cells were dissociated from rat portal vein. Whole-cell Ca^{2+} current (I_{Ca}) and cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) were simultaneously measured. A depolarising pulse from -70 mV to 0 mV evoked inward current and increased $[Ca^{2+}]_i$. The magnitude of both inward current and increase in $[Ca^{2+}]_i$ were extracellular Ca^{2+}-concentration dependent, enhanced by Ca^{2+} channel agonist, Bay K 8644, and blocked by Ca^{2+} channel antagonists, nimodipine and cadmium. Thus, the Ca^{2+} transient occurs as a consequence of I_{Ca} through voltage-dependent Ca channels. The relationship between the expected $[Ca^{2+}]_i$ increase (calculated from the time-integrated I_{Ca}) and actual increase in $[Ca^{2+}]_i$ was determined in the absence and presence of 30 μM ryanodine (which eliminates Ca^{2+}-induced Ca^{2+} release). During the first 200 ms of the depolarising pulse, $[Ca^{2+}]_i$ increased to the range of 180 nM - 260 nM in both control cells and ryanodine-treated cells. Over this Ca^{2+} range, the ratio of expected increase in $[Ca^{2+}]_i$ / measured increase in $[Ca^{2+}]_i$ was about 140 for the control cells, and not significantly different from the ratio for ryanodine-treated cells (about 170). Therefore, it was concluded that Ca^{2+}-induced Ca^{2+} release does not play an important role in rat portal vein smooth muscle cells during depolarisation. The ratio also provides some estimation of cytosolic Ca^{2+} buffering, i.e., about 1/140th of Ca^{2+} entering is appearing as free Ca^{2+}. We estimated that the contribution of Fura-2 (35 μM) to this Ca^{2+} buffering capacity was, at most, about 33 % of the total. The hypothesis that Ca^{2+} transient is tightly coupled with I_{Ca} was further supported by the observation that the voltage-dependency of increase in $[Ca^{2+}]_i$ was identical to that of I_{Ca}. When measured during brief pulses, increase in $[Ca^{2+}]_i$ peaked at a pulse to 0 mV, while at steady-state holding potential, $[Ca^{2+}]_i$ was highest at -30 mV. *Supported by the Wellcome Trust.*

W-Pos46a

STIMULUS--CONTRACTION COUPLING AMONG GIANT CILIATES (*Spirostomum ambiguum*): PULSE-COUPLED OSCILLATORS. **((S.R. Taylor and D.S. Lake)) Department of Pharmacology, Mayo Foundation, Rochester, MN, 55905.**

Several classes of bacterivorous ciliates are capable of exceptionally rapid contraction (< 5 ms) by a mechanism fundamentally different from muscle contraction. Contraction occurs spontaneously, or after electrical, mechanical, or chemical stimulation, and has no known function. Ciliates thrive in water that is viscous for their relative size (i.e., at a low Reynold's number). This has raised the possibility that the purpose of contractility is to refresh the organism's external environment by moving so rapidly that turbulence develops in spite of small cell size. We studied *Spirostomum ambiguum* using a MOS image sensor (HR Deltaron Camera; Fuji Photo Co.) operating at a frame rate of 1.7 kHz (128 x 128 pixels). The ciliates were grown in pasteurized spring water containing lissamine rhodamine B200, which filled cells with dye particles of assorted size and aided the measurement of segment length changes. Groups of mechanically coupled (physically entwined) cells began to contract with the same rhythm (~0.2 Hz at 23°C) when the density of bacteria and ciliates were both high, or after adding Zn^{2+} (10^{-6}M). The cell-to-cell coupling velocity, measured from fields of cells with no member out of phase by more than 0.6 ms, was greater than 2 m/s. A purpose for this rhythmic communal synchrony may be to produce more turbulence in the environment than created by contraction of an isolated cell. **[We thank the NSF (IBM 92-13160) for support and Fuji Medical Systems (Stamford, CT) for the advance of the HR Deltaron 1700.]**

W-Pos47

LUMINAL Ca^{2+} REGULATES GATING OF CANINE CARDIAC SARCOPLASMIC RETICULUM CALCIUM RELEASE CHANNEL *((R Singh, I. Györke and S. Györke))* Department of Physiology, Texas Tech University HSC, Lubbock, 79430 TX (Spon. by A. Neely)

Gating of the sarcoplasmic reticulum (SR) Ca^{2+} release channel (CRC) is modulated by several cytosolic ligands including Ca^{2+} and ATP. In addition, an intraluminal Ca^{2+} regulatory site may account for spontaneous SR Ca^{2+} release during Ca^{2+} overload in various myocardial preparations. However, studies involving the action of luminal Ca^{2+} on the CRC have been contradictory. Here, we examined the role of luminal Ca^{2+} on the open probability (P_o) of the CRC using cytoplasmic Ca^{2+} and ATP to activate the channel. Cardiac CRC were incorporated into lipid bilayers. Conductance and P_o were measured in symmetrical $CsMeSO_3$. In the presence of ATP (3 mM total) and Ca^{2+} (50 nM free) on the cytoplasmic side, elevating luminal free [Ca^{2+}] from 30 μM to 0.5-10 mM increased P_o from 2 to 10 times ($K_{1/2} \approx 2$ mM). This increase in P_o was due to an increase of mean open time and frequency of events. In channels activated solely by 0.5-50 μM Ca^{2+} (no ATP), the same increments of luminal Ca^{2+} failed to increase P_o significantly. Our results suggest that the effect of intraluminal Ca^{2+} on CRC involves allosteric interactions between ATP- and Ca^{2+}- binding sites on both sides of the channel. Since ATP is an endogenous constituent of the cytoplasm, the observed ATP-mediated increase in P_o by elevating luminal Ca^{2+} could account for or contribute to the spontaneous Ca^{2+} release which occurs during Ca^{2+} overload in heart. Supported by AHA and NIH (HL52620).

W-Pos48

RAPID SOLUTION CHANGE ACTIVATES RYANODINE RECEPTORS WITHIN 20 ms. ((Derek Laver and Brian Curtis)) Division of Neurosciences, John Curtin School of Medical Research, Australian National University, Canberra, ACT 2601 Australia.

We describe a puffing method for activating ryanodine receptor (RyR) channels in lipid bilayers which allows simultaneous measurement of channel activity and solution change. Rapid ionic changes induced a shift in bilayer potential which generated a transient current under voltage clamp conditions. This current depended on the ion species and bilayer composition and was accurately described by Stern-Gouy-Chapman theory. Sarcoplasmic reticulum vesicles and two 0.5 mm perfusion tubes were added to the grounded *cis* chamber containing 250 mM CsCl. The *trans* chamber contained 50 mM CsCl and was held at -40 mV (membrane PD = +40mV). Flowing solutions containing 100 μM Ca^{2+} under 15 cm H_2O pressure onto skeletal RyRs increased pCa from ~6 (RyR activation threshold) to ~5 (maximum P_o) in 20 ms. Following activation (P_o = 0.45), P_o declined in 74% of trials with time constants between 2 and 15 ms When 2 mM Mg^{2+} was present before and during the Ca^{2+} step, (184 trials of 7 channels) the initial P_o was 0.21 followed by a similar declining phase. This inhibition was reversed in 2 mM Ni^{2+}. The Po of cardiac and skeletal RyRs following activation by 1-10μM Ca^{2+} in 0-1 mM Mg^{2+} did not decline. During rapid (~15 ms) plunges in pCa from ~1 to ~7, RyRs did not turn on when switched from one inhibited state to the other.

Supported by an Australian Research Council Senior Research Fellowship and grants from the National Heart Foundation and the Illinois Affiliate, American Heart Association.

W-Pos49

SLOW CALCIUM-INDUCED CALCIUM RELEASE (CICR) IN CHINESE HAMSTER OVARY (CHO) CELLS EXPRESSING SKELETAL RYANODINE RECEPTOR (RyR) AND CHIMAERIC DIHYDROPYRIDINE RECEPTOR (DHPR). ((N. Suda[1], M. Bödding[1], A. Fleig[1], D. Franzius[1], M. Hoth[1], S. Nishimura[2], K. Imoto[3], H. Takeshima[4] and R. Penner[1])) [1]Max-Planck-Institut für biophys. chem. Göttingen, FRG. [2]Nippon Boehringer Ingelheim Co. LTD. Kawanishi, Japan. [3]National Institute for physilogical Sciences, Japan.[4]Dept. Pharmacol, Saitama Medical School, Saitama, Japan.

Combined patch-clamp and fura-2 measurements were performed on CHO cells expressing both DHPR-Ca^{2+} channels and skeletal muscle RyR-Ca^{2+} release channels. To ensure expression of a functional L-type Ca^{2+} channel, we expressed a chimaeric DHPR in which the putative cytoplasmic regions between repeats II and III were of skeletal origin and the rest of the protein was of cardiac origin. There was no clear indication of a direct mechanical interaction between these two channels [e.g., Depolarization failed to induce a Ca^{2+} transient (CaT) in the absence of $[Ca^{2+}]_O$]. However, in the presence of $[Ca^{2+}]_O$, depolarization-induced CaTs (DICTs) exhibited a bell-shaped voltage-dependence, indicating a functional coupling via a CICR mechanism. In 3-5 mM $[Ca^{2+}]_O$, DICTs (<30 % of total cells tested) showed two kinetic components: a transient increase in $[Ca^{2+}]_i$ followed by a sustained increase. The slower component was abolished upon addition of 100 μM tetracaine, suggesting that this component reflects release of Ca^{2+} from internal stores. Although the activation rate of the Ca^{2+} current was similar to that of native cardiac muscle, the time course of CICR was slower than that of cardiac muscle. These results suggest that the skeletal muscle RyR isoform supports CICR, but that the distance between the DHPRs and the RyRs is much larger in the transformed CHO cells than in cardiac or skeletal muscle.

W-Pos50

ENDOGENOUS PEPTIDE CARNOSINE IS CARDIOINOTROPIC, RELEASES SR Ca^{++} AND MODULATES THE RYR2 CALCIUM RELEASE CHANNEL. ((G. Zaloga, P. Roberts, G. Zapata-Sudo, R.T. Sudo, M. Lin and T.E. Nelson)) Dept. Anesthesia, Bowman Gray School of Medicine, Winston-Salem, NC 27157.

Carnosine (B-alanyl-l-histidine) is a dipeptide found in muscle tissue at mM concentration and its exact function is unknown. A carnosine-sensitization of contractile proteins to Ca^{++} has been reported.[1] The following findings in our laboratory suggest that carnosine may be an important regulator of cardiac contractility and that RYR2 is affected: In Langendorff-prepared, spontaneously beating rat hearts, 1-20 mM carnosine perfusion increases dP/dt_{MAX} for contraction and relaxation in a concentration dependent manner. In saponin-skinned cardiac muscle cells, carnosine releases Ca^{++}, produces tension and this effect is blocked by procaine; suggesting an effect on the calcium release channel. At mM concentration carnosine is as effective as AMP-PCP for increasing the affinity for [^{3}H]-ryanodine binding to cardiac SR membrane vesicles. When applied to single RYR2 channels in a lipid bilayer, carnosine (1-5 mM) increased Po by 4-fold. The most remarkable effect of carnosine on RYR2 channel behavior was the alteration of RYR2 response to [Ca^{++}]. In the absence of carnosine and pCa = 2, the channel is submaximally active. In the presence of carnosine, the channel is inactivated as pCa is changed from 4.5 to 2.0. Conversely at pCa=7 RYR2 is inactive in the absence of, and active in the presence of carnosine. Carnosine may be sensitizing RYR2 to [Ca^{++}] in a manner that favors calcium release at high pCa and that induces channel closure at low pCa. These responses may reflect carnosine's inotropic effects. These in vitro effects of carnosine and its high concentration in muscle tissue in vivo suggest that it may play an important role in regulation of contractility. (1). C Lamont, DJ Miller J Physiol 1992;454:421.

W-Pos51

EFFECTS OF SARCOPLASMIC RETICULUM (SR) CALCIUM RELEASE MODULATORS ON THE L-CURRENT IN CULTURED MOUSE MYOTUBES ((E.M. Balog and E.M. Gallant)) Depts. of Vet. PathoBiol. and Physiol. Univ. of Minn. St. Paul, MN 55108

We studied whether drugs which modulate SR Ca^{2+} release might affect the sarcolemmal L-current. Myotubes were cultured from mouse limb muscles and patch clamped in the whole cell configuration. Ruthenium red and high concentrations of ryanodine were used to block SR Ca^{2+} release. The inclusion of 1 mM ryanodine in the patch pipette led, over time, to a significant enhancement of the L-current, at test potentials more negative than that eliciting the peak current. From 2 to 5 min after the establishment of the seal the current in response to a test pulse to +10 mV increased 43% (from -6.89±0.85 to -9.82± 0.90 pA/pF; n=18). The inclusion of 200 μM ruthenium red in the pipette had a similar effect although it required a longer time. The current in response to a test pulse to +10 mV increased 127% from 2 to 20 min after the seal was established (-2.99± 0.72 to -6.76 ±1.51 pA/pF; n=5). In the absence of drug, currents did not change significantly over a similar time period. When the concentration of EGTA in the pipette was reduced (from 8.4 to 0.1 mM) myotubes contracted in response to periodic stimulation for 20 minutes. The inclusion of either ryanodine or ruthenium red, over time, eliminated contraction. Caffeine (1 mM) was added to the bath to enhance SR Ca^{2+} release. Addition of caffeine significantly decreased the current in response to test pulses in the range of +10 to +40 mV. At +10 mV the current was reduced 34% (control = -11.76 ±2.32, caffeine=-7.71 ±1.36 pA/pF; n=9). Changing the solution without including caffeine did not alter the current. In a low EGTA internal solution, caffeine lowered the contractile threshold (-3.3±1.1; -9.2±0.8; -4.2±1.5 mV for control, caffeine and wash respectively). This data suggests that SR Ca^{2+} release can modulate the L-current in cultured mouse myotubes.

W-Pos52

EXPRESSION AND ASSOCIATION OF CALCIUM CHANNEL SUBUNITS IN THE NORMAL AND HYPERTROPHIED HUMAN HEART. ((H. Haase, A. Hohaus, H.D. Schulte, M. Maier, P.E. Lange and I. Morano)) Max Delbrück Center for Molecular Medicine, 13122 Berlin, Germany.

Interaction between the α1- and ß-subunits influences basic properties of L-type Ca^{2+} channels. We studied the expression and association of α1-, ß2- and ß3-subunits on the protein level in cardiac preparations from normal human heart ventricles (NH) and from the hypertrophied septum of patients with hypertrophic obstructive cardiomyopathy (HOCM). 1,4-Dihydropyridine (DHP) binding and immunorecognition by antibodies raised against the specific C-terminal amino acid sequences of the ß2- and ß3-subunits were used for detection and quantification of α1-, ß2, and ß3-subunits, respectively. Bmax of high-affinity DHP binding was 35 ± 2 and 20 ± 2 fmol/mg of protein in HOCM and normal human hearts ($p < 0.05$), respectively. The ß2-subunit directed antibody immunoprecipitated 80 % and 25 % of DHP-labeled Ca^{2+} channels from rabbit heart and both normal and HOCM ventricles, respectively. A fractional immunoprecipitation (35%) was also observed in pig hearts. Immunoblot analysis showed that the ß2-subunit was copurified with the cardiac DHP-receptor/Ca^{2+} channel from rabbit, pig and human heart. In neither of the purified cardiac Ca^{2+} channels the ß3-subunit isoform was detected. Our results demonstrate that in human myocardium the ß2-subunit is expressed and is complexed with the α1c-subunit similar to other models of mammalian heart.

W-Pos53

THE STRUCTURAL DETERMINANTS FOR RYANODINE BINDING ARE DIFFERENT THAN THE MOLECULAR FEATURES WHICH CORRELATE TO CHANNEL ACTIVATION

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Ryanodine is a modulator of the ryanodine receptor (RyR). At sub-micromolar concentrations, ryanodine increases channel activity by stabilizing an open subconductance state. In contrast, the channel persists in a closed state in the presence of high concentrations of ryanodine. The interactions of ryanodine with the skeletal and cardiac RyR isoforms have been modeled by using comparative molecular field analysis (CoMFA), a three-dimensional QSAR technique. The CoMFA technique measures the steric and electrostatic fields around a basis set of molecules. These fields are correlated to a biological activity by partial least squares analysis. Previously, a CoMFA model was developed which described the molecular determinants of tight binding to the skeletal RyR. A CoMFA study of the modulating effects of ryanodine analogs on the RyR calcium channel is presented here. The functional state of the channel was assessed by measurement of the calcium uptake rates of SR microsomes. We present evidence that the determinants of ryanodine binding to RyR are different from the molecular features that correlate to channel activity. For example, substitutions at the 10-position of ryanodine minimally perturb binding to the RyR. In contrast, these same modifications show large differences in the ability of ryanoids to open or close the RyR.

W-Pos54

FAST AND SLOW GATING CHANNEL ACTIVITIES OBSERVED FROM THE SR MEMBRANE FRACTIONS OF CANINE LATISSIMUS DORSI MUSCLE

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Ryanodine receptor/channel (RyR) mediates the release of calcium from the sarcoplasmic reticulum (SR) in both skeletal and cardiac muscle cells. There are three isoforms of the RyR: RyR1, RyR2, and RyR3. RyR1 is specifically expressed in skeletal muscles and RyR2 in cardiac muscles. Single channel recordings of RyR1 and RyR2 reconstituted in artificial lipid bilayer show that the characteristics of two isoforms are very distinct.

In this study, we isolated the heavy SR membranes from canine latissimus dorsi muscles and investigated the single channel activities from the heavy SR membranes fraction using Cs^+ as a charge carrier. Two different types of the activities were observed. Fast-gating activity (FGA) with the mean open time of 0.9ms was more frequently recorded (n=12) than slow-gating activity (SGA) with the mean open time of 269.2ms. From the I-V relation, the slope conductance of the FGA was calculated to 514.7pS and the SGA, 625.6pS. The activity of the fast gating type increased by raising the concentration of Ca^{2+} in the cis-solution up to 100μM.

The appearance of the SGA in the canine heavy SR membrane fraction suggests a possibility that two types of RyR isoform, RyR1 and RyR2, are co-expressed in mammalian skeletal muscle as well as in avian, amphibian, and piscine fast twitch muscles.

W-Pos55

FUNCTIONAL INTERACTION BETWEEN TRIADIN AND RYANODINE RECEPTOR IN SARCOPLASMIC RETICULUM MEMBRANE OF SKELETAL MUSCLE.

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We examined the effect of antibodies directed against triadin on calcium release from triads vesicles. Antibodies were raised against synthetic peptides corresponding to the N-terminal (residues 2-17) and C-terminal (residues 691-706) ends of rabbit skeletal muscle triadin. We previously shown that the N-terminal region of triadin is exposed on the cytoplasmic side of the vesicles while the C-terminal end is in the lumen of the vesicles (*Marty et al. Biochem. J. 1995, 307, 769-774*). Triads vesicles were incubated 2h at 37°C in presence or absence of anti triadin antibodies directed against either the N-terminal peptide (A-Nter antibodies) or the C-terminal peptide (A-Cter antibodies) and then, passively loaded with ^{45}Ca. Ca release was measured using the rapid filtration technique. Ca release was induced at pH 7 by pCa 5 (Ca induced Ca release), in presence or absence of 5mM ATP. After incubation of the vesicles with A-Nter antibodies we observed a significant (up to 66%) inhibition of the Ca release induced in presence of ATP. This inhibition was reversed when A-Nter antibodies were pre-incubated with N-terminal peptide. In contrast, Ca-induced Ca release, measured in absence of ATP, was unaffected by the A-Nter antibodies. Incubation of the vesicles with the A-Cter antibodies did not significantly affect Ca release induced either in presence of ATP or in presence of Ca alone. These results demonstrate a functional interaction between triadin, putatively *via* its cytoplasmic domain, and ryanodine receptor, in the sarcoplasmic membrane. The effect of these antibodies on the hypothesized interaction between triadin and calsequestrin is currently studied. (Supported by Association Française contre les Myopathies)

W-Pos56

STRUCTURE OF SKELETAL MUSCLE FIBERS IN TRANSGENIC MICE HOMOZYGOTS FOR A TARGETED NULL MUTATION (*CCBL1^{tm1Uw}*) OF THE DIHYDROPYRIDINE RECEPTOR (DHPR) β_1 SUBUNIT

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Lack of the β_1 subunit of DHPRs results in reduced L-type currents and lack of e-c coupling (Biophys. J. 68, A372, 1995). We compared the ultrastructure of diaphragm and leg muscles in normal and mutant (*ccbl1^{tm1Uw}/ccbl1^{tm1Uw}*) mice. In normal mice, the diaphragm at days 15-17 of gestation (E15-17) has numerous peripheral couplings between the SR and the surface membrane. These disappear at later ages, while T tubules and triads develop. The leg muscles have a similar but slower time scale. In freeze-fractures of normal diaphragms at E16-17 and leg muscles at E18, 35 out of 54 and 68 out of 88 observed fiber segments have several groups of tetrads (presumably DHPRs). These are located in domed domains at sites of peripheral couplings. In the homozygous (*ccbl1^{tm1Uw}/ccbl1^{tm1Uw}*), the overall fiber structure resembles that of dysgenic (DHPR α_1 deficient) and dyspedic (RyR deficient) mice. The myofibrils are not well developed and often split; triads are disordered and scarce; peripheral couplings are present. In freeze-fractures, domed domains are found in 8 out of 39 fiber segments in E16-17 diaphragm, and in 28 out of 53 in E 18 leg muscle. On the domains there are clustered large particles of the same size as the presumed DHPRs in normal junctions. However, the domains are rare, particles in each domain are few and disordered, and tetrads are rarely seen. We conclude that association of DHPRs with junctional membrane domains may occur, but it is rare and not well organized, perhaps due either to scarcity of DHPRs, or to weak binding.

W-Pos57

INFLUENCE OF INSULIN LIKE GROWTH FACTOR-1 (IGF-1) ON DIHYDROPYRIDINE RECEPTOR (DHPR) FUNCTION AND SR CALCIUM RELEASE ON RAT SKELETAL MUSCLE FIBERS AT DIFFERENT AGES.

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We have demonstrated that skeletal muscle comprised of only type-II fibers undergoes a progressive excitation-SR calcium release uncoupling with age (Delbono *et al.*, J. Membrane Biol. *In press*). This process substantially contributes to the decline in skeletal muscle contractility with age. We proposed that the expression of the DHPR is regulated by trophic factors such as IGF-1 and thatdecreased responsiveness in old muscle fibers to IGF-1 reduces the number of functionally active DHPRs probably through a decrease in *c-fos* and *c-jun* mRNAs. This results in a decreased intracellular Ca^{2+} mobilization. In this work, we measured charge movement (I_Q) and I_{Ca} (double Vaseline gap voltage-clamp technique) in young (7), middle age (18) and old (28 months) F344XBN rats. I_Q was not affected upon cell exposure to 20 ng/ml IGF-1, however, I_{Ca} was potentiated 215 ± 32% (n = 15) in young and middle age fibers and non significant changes were recorded in older muscles (97 ± 7%, n = 15). SR Ca^{2+} release was measured with photodiode and ratio imaging techniques. A significant potentiation of the rate of Ca^{2+} release was recorded in the first two groups upon stimulation (189 ± 23%; n = 12) while no significant differences were recorded in older fibers. [^{125}I]IGF-1 binding studies were performed to define whether the decrease in response to IGF-1 was due to alterations in number of IGF-1R and/or affinity for its specific ligand. These studies demonstrated a conserved number of IGF-1R and similar K_D with age. These data suggest a functional alteration of the signal transduction cascade triggered by activation of the IGF-1R in aged animals. These data also support the hypothesis that a decline in muscle fiber response to IGF-1 is associated with DHPR activation-SR Ca^{2+} uncoupling with age. Supported by grants from NIH, AHA and MDA.

W-Pos58

REGENERATING RAT SKELETAL MUSCLE EXPRESSES mRNA ENCODING THE CARDIAC ISOFORM OF THE DIHYDROPYRIDINE RECEPTOR - CALCIUM CHANNEL.

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One of the the major properties of mature skeletal muscle is its ability to regenerate after injury, and some of the structural and functional characteristics of regenerating muscle are similar to those of normal myogenesis during embryological and post-natal development. The purpose of the present study was to determine whether the expression of the dihydropyrine receptor - calcium channel (DHPR) gene is upregulated by regeneration as it is during normal development. Extensor digitorum longus (edl) muscle of adult rats was surgically injected with 0.75 % bupivacaine to induce the degeneration-regeneration process. mRNA was isolated from the injected and the contralateral control edl muscles 3, 7, 15 and 30 days after injection using guanidine-isothiocyanate. Northern Blot analyses were carried out with a rat DHPR cDNA specific for skeletal muscle, and RNase protection assays were performed with a 152-bp cDNA corresponding to part of the cytoplasmic loop linking repeats II and III of the cardiac isoform of the DHPR α1-subunit, subcloned from a mouse cardiac specific calcium channel cDNA kindly provided by Dr. N. Chaudhari. We observed during the first week a marked increase of DHPR skeletal muscle isoform. Moreover, we showed that regenerating skeletal muscle expresses mRNA for the DHPR cardiac isoform, mainly at the beginning of regeneration (3 days after bupivacaine injection). Similar findings were previously reported from experiments conducted during early developmental stages in skeletal muscle [Chaudhari & Beam, *Dev Biol* 155:507-515, 1993]. These results raise the question of the functional role of this cardiac isoform in skeletal muscle and whether it could explain certain cardiac-like aspects of excitation-contraction coupling of developing skeletal muscle [Cognard *et al*, *Pflugers Archiv* 422:207-9,1992] and whether similar behavior should be expected in regenerating skeletal muscle. Supported by grants from the Ministère des Affaires Etrangères (Programme Lavoisier, Paris, France), the Philippe Foundation, and AFCPH (Nantes, France)

W-Pos59

CHARACTERISATION OF TWO Mg^{2+}-INHIBITION MECHANISMS IN RYANODINE RECEPTOR Ca^{2+} CHANNELS ((Derek R. Laver, Timothy M. Baynes)) Division of Neuroscience, John Curtin School of Medical Research. The Australian National University, Canberra, ACT 2601, Australia (Spon. by Peter H. Barry).

Ryanodine receptor (RyR) gating depends on myoplasmic $[Ca^{2+}]$ and $[Mg^{2+}]$. Both skeletal (rabbit) and cardiac (sheep) isoforms are inhibited by Mg^{2+}, activated byμ M $[Ca^{2+}]$ and inhibited by mM $[Ca^{2+}]$. Cardiac and skeletal RyRs were incorporated into lipid bilayers. Analysis of RyR open probability and open and closed frequency distributions revealed two Mg^{2+}-dependent gating mechanisms which mediate RyR inhibition: 1) Mg^{2+} competes with Ca^{2+} for the high affinity site and prevents activation of RyRs by Ca^{2+}. 2) Mg^{2+} and Ca^{2+} share a common inhibitory mechanism by binding at the low affinity Ca^{2+} site which does not discriminate between Mg^{2+} and Ca^{2+}. Both mechanisms gate independently and appear to be associated with different parts of the protein structure. Whilst they both contribute to Mg^{2+}-inhibition their relative importance depends on myoplasmic $[Ca^{2+}]$. When $[Ca^{2+}]$ is low (<100 nM in skeletal and <10 μM in cardiac muscle) Mg^{2+}-inhibition is determined mainly by ion competition at the high affinity site whereas at higher concentrations it is mainly determined Mg^{2+} binding at the low affinity divalent ion site. It appears that both mechanism are significant in the Mg^{2+}-inhibition of skeletal RyRs under physiological conditions.

W-Pos60

IMMUNOSUPPRESSANT/MACROLIDE ACTIVATION OF RYANODINE RECEPTOR INDEPENDENT OF FK506-BINDING PROTEIN (FKBP12) ASSOCIATION ((Gerard P. Ahern, Pauline R. Junankar & Angela F. Dulhunty)) Division of Neuroscience, John Curtin School of Medical Research, The Australian National University, Canberra, ACT 2601, Australia.

Four FK506 binding protein (FKBP12) monomers tightly associate with the ryanodine receptor (RyR) and regulate its activity. We have investigated the effects of FKBP12-RyR association and macrolide drugs on single channel RyR activity in lipid bilayers. Terminal cisternae vesicles were stripped of FKBP12 by preincubation with rapamycin at 37°C for 15 mins. FKBP12 remaining was estimated by Western Blotting and vesicles were incorporated into lipid bilayers. Channel open probability and substate activity increased in proportion to FKBP12 dissociation. Substates were clustered at levels ~25, 50 and 75 % of the maximum conductance. Stripped channels could still be blocked by ruthenium red and activated by ATP. Ryanodine reduced the conductance of fully stripped channels but did not abolish substate activity and rapamycin (1-10mM) reversibly increased channel activity suggesting an additional action independent of FKBP12. Consistent with this hypothesis, related macrolide drugs, ivermectin and midecamycin, reversibly activated unstripped RyRs but did not induce FKBP12 dissociation. The results provide evidence for a macrolide binding sites(s) on the RyR and suggest that FK506/rapamycin may activate RyRs directly, in addition to their effect of dissociating FKBP12.

W-Pos61

CHARACTERIZATION OF RYR3 RYANODINE RECEPTOR ISOFORM IN MAMMALIAN BRAIN. ((T. Murayama and Y. Ogawa)) Dept. Pharmacol., Juntendo Univ. Sch. Med., Tokyo 113, Japan. (Spon. by M. Konishi)

While the RNA for the third isoform of ryanodine receptor (Ryr3) was detected in specific regions of mammalian brain, the protein remains to be elucidated. We investigated properties of Ryr3 in rabbit brain, using an antibody against the synthetic peptide corresponding to amino acid sequence 4375–4387 of rabbit Ryr3. The antibody reacted with bullfrog β-RyR, a homologue of Ryr3, but not with rabbit Ryr1, Ryr2 or bullfrog α-RyR. The antibody specifically precipitated a single polypeptide of a molecular mass similar to that of β-RyR from solubilized rabbit brain microsomes, confirming the existence of Ryr3. Sedimentation pattern of Ryr3 through sucrose gradients and negative reaction with anti-Ryr2 antibody revealed formation of homo-tetramer. Consistent with its RNA distribution, Ryr3 was abundantly expressed in hippocampus, corpus striatum, and diencephalon. The amount of Ryr3 was estimated to be only 1.6% of total RyR in rabbit brain. Ryr3 demonstrated Ca^{2+}-dependent [^{3}H]ryanodine binding. Caffeine markedly sensitized Ryr3 to Ca^{2+}. The Ca^{2+}-sensitivity was also enhanced by 1 M NaCl, as observed with β-RyR. These results confirm the functional expression of Ryr3 in mammalian brain, which has properties similar to nonmammalian β-RyR.

W-Pos62

GENERATION AND ANALYSIS OF RYR-1 NULL MICE AND SKELETAL MUSCLE ((P.D. Allen, *†, I. N. Pessah‡, E. D. Buck‡, P.C. Dolber*, J.R. Sommer* and H.T. Nguyen*)) *Department of Anesthesia, Brigham and Women's Hospital, †Department of Cardiology, Children's Hospital, Boston MA 02115, *Department of Medicine Duke University, Durham NC 27705 and ‡Department of Molecular Biosciences, School of Veterinary Medicine, Univ. of California, CA 95616

Ryanodine receptors (RyRs) are intracellular calcium release channels that are involved in regulating cytosolic calcium levels. There are three RyR isoforms of which one, RyR-1, is the dominant form in skeletal muscle. To define more precisely its functional role in skeletal muscle, we have disrupted the RyR-1 gene in ES cells using a replacement type targeting construct. J1-ES clones, which were heterozygous for the targeted allele were injected into C57BL/6 mouse blastocysts and germline transmission of the mutated allele was obtained. Homozygous RyR-1 null mice die perinatally because they lack skeletal E-C coupling. Skeletal muscle from these mice and their wild type littermates was examined for: 1. expression of RyR-1, RyR-2, and myosin using immunohistochemistry and western blot analysis 2. Ryanodine binding and 3. Histological and ultrastructural changes. Muscle from control mice was shown to express RyR-1 and myosin but not RyR-2, while homozygous mutant mice express myosin but NOT RyR-1 or RyR-2. In control muscle, equilibrium binding experiments performed with 1 nM [^{3}H]ryanodine shows specific binding (B_{max} = 0.34 pmol/mg, Kd = 18 nM), but there is NO specific binding in RyR-1 null muscle. Histologically, when compared to muscle from normal littermates, RyR-1 null muscles have smaller and more disorganized muscle fibers with centrally located nuclei.. EM studies of mutant muscles demonstrate an absence of the normal periodic junctional processes in the triad and a narrowed junctional gap. These data demonstrate that RyR-1 is necessary for normal E-C coupling, and skeletal muscle from these mice will provide an excellent model to study structure function relationships of RyR-1 (MDA, HL12486; VA Res.Serv)

W-Pos63

COMPARATIVE STUDIES OF THE C-TERMINAL TRACT OF A RYANODINE RECEPTOR FROM THE INSECT PEST *HELIOTHIS VIRESCENS*. ((E. Puente, J.D. Windass)) Zeneca Agrochemicals, Jealott's Hill Research Station, Bracknell, Berkshire, RG12 6EY, UK.

New knowledge about the ryanodine receptor (RyR) family has been obtained by cloning cDNAs encoding the mammalian skeletal, cardiac, and brain isoforms. The comparative analysis of other vertebrate RyR cDNAs and also the corresponding *D.melanogaster* genomic DNA sequence reveal substantial conservation of RyR protein and mRNA features between different species.
We have cloned cDNAs encoding the C-terminal 1172 amino acids of a RyR from the lepidopteran pest, *Heliothis virescens* (tobacco budworm), and present a comparison with the corresponding regions of the *D.melanogaster* and mammalian counterparts. This shows 77.6% amino acid identity with *D.melanogaster* and 50.2-51.5% with the rabbit isoforms. However, this homology is not uniformly distributed. Interestingly, there is a relatively poorly conserved region whose position is also maintained between the other receptors isoforms. Hydropathicity profile analysis of the available *H.virescens* sequence and corresponding tracts of *D.melanogaster* and mammalian isoforms are also consistent with a high level of structural similarity. Several functionally important regions of RyRs, have been provisionally located within this region of RyR1. The very high conservation of this part of the RyRs from *D.melanogaster* and *H.virescens* would be consistent with this interpretation, since it would be expected to be a region subjected to very strong evolutionary pressures. These and other features concerning heterologous expression of the available *H.virescens* RyR cDNA will be discussed.

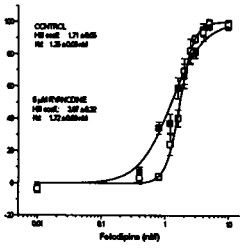
W-Pos64

METHIOSULFONATE (MTS) COMPOUNDS PROBE THE PORE OF THE RYANODINE RECEPTOR AND INDUCE SUBSTATES. **((K. E. Quinn and B. E. Ehrlich)) Univ. of CT, Farmington, CT.**

Methiosulfonate (MTS) compounds were added to ryanodine receptors (RyR) incorporated into planar lipid bilayers. The MTS compounds altered channel conductance without affecting regulation by ATP, caffeine or calcium. These hydrophilic compounds were only effective when the channel was open suggesting that the compounds bind in the water-filled channel pore. Following the addition of the MTS compounds, channel conductance was reduced in multiple steps. The alteration was irreversible and the decrease in current was cumulative over time, eventually leading to the complete block of channel current. If the compound was washed out before a complete block was achieved, there was no further decrease in current, suggesting that the change in conductance was due to the accumulated modification of residues by the MTS compounds. The MTS-modified steps are similar to the four subconductance states observed in the native channel. These results suggest that the MTS compounds are causing a serial modification of pore residues over the course of exposure and that each subunit contributes a portion of the channel's unitary conductance. These studies will further allow us to examine how the four subunits form the conduction pathway in the RyR.
This work was supported by NIH and The Grass Foundation.

W-Pos65

FLUORESCENT-PROBING OF THE DHP RECEPTOR - ITS CONFORMATIONAL INTERACTION WITH THE RYANODINE RECEPTOR IN SKELETAL MUSCLE. I. Minarovic and L.G. Mészáros; Department of Physiology and Endocrinology, Medical College of Georgia, Augusta, GA.



The binding of felodipine, a DHP antagonist to the receptor was associated with a marked increase in felodipine fluorescence. With isolated cardiac sarcolemma and skeletal heavy sarcoplasmic reticulum (SR) preparations, the latter containing SR T-tubule junctions, the fluorescence increase revealed the presence of high-affinity felodipine binding sites with apparent K_d's of 0.35 and 1.25 nM, respectively (and low dissociation rates; k_{off} < 1/min). Characteristics of felodipine fluorescence were altered by other DHP ligands (diltiazem, isradipine) in sarcolemma and by ligands of the Ca^{2+} release channel, ryanodine (fig. 1) and poly-lysine, in triadic skeletal muscle SR preparations. The results indicate that i) the detection of changes in felodipine fluorescence is useful for probing DHP receptor conformational changes and ii) protein conformational changes can be transmitted between the DHP and the ryanodine receptors, as proposed earlier to be the basis of skeletal-type excitation-contraction coupling.

W-Pos66

ALTERNATIVELY SPLICED VARIANTS OF THE BRAIN CA^{2+}-RELEASE CHANNEL (RYANODINE RECEPTOR) EXPRESSED IN SMOOTH MUSCLE TISSUES. ((S. R. Wayne Chen)) The Department of Medical Biochemistry, the University of Calgary, Calgary, Alberta, Canada, T2N 4N1

The primary structure of the brain Ca^{2+} -release channel (ryanodine receptor) (RYR3) expressed in various smooth muscle tissues was investigated using the reverse transcriptase mediated polymerase chain reaction (RT-PCR). Total RNA isolated from rabbit uterus was reversibly transcribed to generate first strand cDNA, which was used as a template for the amplification of the entire coding region of RYR3 cDNA by PCR using eight pairs of primers. Overlapping PCR fragments were subcloned into the Bluescript plasmid and analyzed by restriction enzyme digestions and DNA sequencing. When compared with the reported cDNA sequence of RYR3 isolated from rabbit brain, the sequences of PCR fragments of RYR3 isolated from uterus display several deletions. Some deletions appear to be tissue-specific, since they could be readily detected in smooth muscle tissues including uterus, aorta and vas deferens, but not in heart. Further studies on the tissue specificity, developmental regulation and functional consequence of these alterations should shed new insights into the structure, function and regulation of RYR3. (Supported by the Medical Research Council of Canada and the Alberta Heritage Foundation for Medical Research)

W-Pos67

EMBRYONIC E-C COUPLING IN CULTURED SKELETAL MUSCLE CELLS ((Ruth Cordoba-Rodriguez, Donald R. Matteson, Hugo Gonzalez-Serratos)) Department of Physiology, University of Maryland School of Medicine, Baltimore, Maryland 21201

During e-c coupling, adult skeletal muscle cells do not require $[Ca^{2+}]_o$ to contract (Nature 298, 292-294, 1982). Yet, the role of $[Ca]_o$ in e-c coupling at early stages of skeletal muscle development is not clear. The purpose of this work is to study the relationship between $[Ca]_o$, I_{Ca}, and skeletal e-c coupling in cultured cells from *Xenopus Laevis* embryos. These myocytes showed slow I_{Ca} that increase in amplitude from about 50pA to 2000pA with time in culture. In order to assess the importance of $[Ca]_o$ in e-c coupling in embryonic cells we performed two types of experiments. First, we investigated the existence of contraction at E_{Ca} in the presence of 1mM $[Ca]_o$. One day after culture, cell contraction was small and maintained during the pulse. After one week in culture, contractions were strong and finished before the depolarizing pulse ended. In the second set of experiments we stimulated the cells in the presence of low $[Ca]_o$ (10^{-3}M) and normal $[Ca]_o$ (1mM). Early in culture, $[Ca]_i$ was not increased when cells were stimulated in the presence of low $[Ca]_o$. Later in culture, $[Ca]_i$ increased during external stimulation whether $[Ca]_o$ was low or normal. We explored $[Ca]_i$ further by studying calcium transients during 700ms depolarizations. In this experiment, cells showed a maintained increase in $[Ca]_i$ during depolarization early in culture. After one week in culture $[Ca]_i$ also increased during depolarization but biphasically. Caffeine was used to investigate the functional development of the SR in these myocytes. Two protocols were used for this. In the first experiment caffeine contracture thresholds were higher in one day old cells (20mM) than in one week old cells (1mM). In the second experiment $[Ca]_i$ was imaged. We found that younger cells needed higher caffeine concentrations to show similar $[Ca]_i$ levels as older cells. We conclude that there is a clear difference in e-c coupling between embryonic and adult cells. The above indicates that the T-system and SR junction may not be well formed in the early stages of embryonic development. Therefore, calcium channels may function as a pathway for external calcium to trigger the release of calcium from immature stores within the cell. Supported by NIH grant NS17048.

W-Pos68

DOWNREGULATION AND RECOVERY OF KINASE C (PKC) ISOFORMS DISSOCIATE PHORBOL ESTER-INDUCED FROM GTPγS-INDUCED Ca^{2+}-SENSITIZATION. ((P.E. Jensen, M.C. Gong, A.V. Somlyo and A.P. Somlyo.)) Dept. Mol. Phys. & Biol. Phys., Univ. of Virginia, Box 449, Charlottesville, VA 22908.

Conventional (α, β) and novel (θ, ε) PKC isoforms were detected in Western blots of rabbit femoral artery, portal vein (PV) and ileum. PKC ζ was probably also present, although the specificity of this antibody was poor. In PV PKC η was also detected. Chronic treatment with phorbol esters caused significant down-regulation of each isoform, except ζ. Down-regulation of the proteins was associated with abolition of the PDBu-induced increase in contraction at constant $[Ca^{2+}]$ in α-toxin-permeabilized muscles, without significantly affecting agonist or GTPγS-induced Ca^{2+}-sensitization. Following termination of down-regulation with PDBu, the recovery of conventional PKCs, observed in Western blots of PV, was associated with 70% recovery of PDBu-induced Ca^{2+}-sensitization. The Ca^{2+}-sensitizing effect of PDBu (1 μM) in controls was associated with increased phosphorylation (~25%) of the regulatory myosin light chain, and down-regulation abolished only the PDBu-induced, but not the endothelin- or GTPγS-induced, increases. In α-toxin-permeabilized smooth muscle, PDBu caused significant translocation of each isoform, except PKC ζ, to the membrane fraction. Our results are consistent with PKC having only limited or no role in G-protein-coupled Ca^{2+}-sensitization. Experiments designed to identify the PKC isoforms involved in inhibition of muscle myosin light chain phosphatase leading to Ca^{2+}-sensitization are in progress. Supported by NIH HL48807.

REGULATION OF CARDIAC EXCITABILITY

W-Pos69

CALCIUM RELEASE FROM THE SARCOPLASMIC RETICULUM OF PACEMAKER CELLS ISOLATED FROM THE RABBIT SINOATRIAL NODE. ((J. Li and R. D. Nathan)) Department of Physiology, Texas Tech University Health Sciences Ctr., Lubbock, TX 79430.

Ca^{2+} release from the sarcoplasmic reticulum (SR) and its effect on pacemaker activity were studied in single, cultured pacemaker cells. Indo-1/AM was used to indicate changes in intracellular Ca^{2+} concentration, (Ca_i^{2+}), and the perforated-patch recording technique (nystatin) was used to monitor spontaneous electrical activity. During simultaneous recordings of electrical activity and the fluorescence ratio (F405/F485) in 10 cells, ryanodine (10 μM, 10 min) had no effect on the action potential (AP), but slowed spontaneous firing by 38 ± 4% (mean ± SEM, $P < 0.01$), increased the time constant for decay of the Ca_i^{2+} transient (τ_d) by 76 ± 9% ($P < 0.01$), and decreased the amplitude of the Ca_i^{2+} transients by 23 ± 2% ($P < 0.01$) while increasing the diastolic level. Thapsigargin (5 μM) also slowed the firing rate and increased τ_d in 11 cells, by 31 ± 6% ($P < 0.01$) and 67 ± 19% ($P < 0.01$), respectively; however, it failed to affect the amplitude of the Ca_i^{2+} transient significantly (change = 2 ± 3%). In the presence of 2 mM Ni^{2+} to block the influx of Ca^{2+} through voltage-gated channels, 10-20 mM caffeine elicited a Ca_i^{2+} transient that was only 35 ± 4% (n = 16) of the AP-induced transients and could be blocked by pretreatment with ryanodine (10 μM) or thapsigargin (5 μM). In perforated-patch voltage clamp recordings, 10 μM ryanodine failed to affect either hyperpolarization-activated or time-independent inward current. However, it significantly reduced total Ca^{2+} current at -30, -20 and -10 mV (holding potential = -80 mV) but did not affect L-type Ca^{2+} current (holding potential = -50 mV). Our results confirm the existence of modest internal stores of Ca^{2+}, the contents of which can be released by caffeine and depleted by ryanodine or thapsigargin. In contrast to other types of cardiac myocytes, SA node pacemaker cells seem to exhibit Ca_i^{2+} transients produced mainly by Ca^{2+} entry through voltage-gated channels rather than by Ca^{2+} release from the SR. Disruption of such release by ryanodine slows pacemaker activity, in part by reduction of T-type Ca^{2+} current.

W-Pos70

EFFECTS OF BLOCKERS OF DIASTOLIC TIME-DEPENDENT CURRENTS ON THE PACEMAKER POTENTIAL OF SINO-ATRIAL NODE. ((H. Zhang and M. Vassalle)) SUNY, HSC at Brooklyn, NY 11203. (Spon. by P. Gootman)

The effects of blockers of different diastolic time-dependent currents on diastolic depolarization (DD) were studied in the sino-atrial node (SAN) of guinea pig and rabbit. The SAN was isolated from surrounding atrial tissue and membrane potentials recorded by means of a microelectrode technique. In Tyrode solution, Cs^+ (10 mM, a blocker of I_{Kdd} and I_f) abolished DD and caused depolarization in SAN subsidiary pacemakers, but did not stop the SAN. Adding Ba^{2+} (0.5-5 mM, a blocker of I_{Kdd} and I_K) to the Cs^+ solution led to a progressive decrease in maximum diastolic potential and even to stoppage of discharge. High $[K^+]_o$ (10-12 mM, a blocker of I_{Kdd} but not of I_f) abolished DD in subsidiary pacemakers, which then assumed the characteristics of dominant pacemakers. Adding 10 mM Cs^+ to high $[K^+]_o$ did not stop the SAN. Adding E4031 (1 μM, a blocker of I_{Kr}) to the high $[K^+]_o$-Cs^+ solution led to depolarization and quiescence in the SAN of the rabbit, but not in that of the guinea pig. Quiescence was brought about by a gradual decrease in maximum diastolic potential, a decrease in action potential amplitude and slower repolarization. Later, only sinusoidal deflections were present with a period similar to that of the control action potentials. Eventually, the membrane potential was steady. During recovery, the oscillations reappeared and the upstroke was superimposed on their depolarizing phase. These findings appear consistent with the following conclusions: I_{Kdd} may have a role in DD of SAN subsidiary pacemakers, but not in DD of dominant pacemakers; I_f does not play a substantial role in DD either in SAN subsidiary or dominant cells; I_K has a primary role in SAN pacemaking, I_{Kr} being important in rabbit SAN and possibly I_{Ks} in guinea pig SAN.

W-Pos71

ACH EFFECTS ON NA/K PUMP CURRENT IN GUINEA PIG VENTRICULAR MYOCYTES. ((J. Gao, R.T. Mathias, I.S. Cohen & G.J.Baldo)) Dept. of Physiology, SUNY at Stony Brook, NY 11794-8661.

Typically, the sympathetic and parasympathetic autonomic systems have antagonistic actions on heart function. We have previously shown the activation of ventricular muscle β-receptors by the sympathomimetic agent isoproterenol (Iso) affects Na/K pump current (I_p) in a Ca^{2+}-dependent manner. At low $[Ca^{2+}]_i$ (15 nM), Iso reduces I_p by about 25% at all physiologically-relevant membrane potentials (V_m). At high $[Ca^{2+}]_i$ (1.4 μM), Iso shifts the I_p-V_m relationship about 25 mV negative at V_m < 0 mV, with no change in I_p at $V_m \geq 0$ mV. A combination of these effects is observed at intermediate $[Ca^{2+}]_i$. At -60 mV for example, the net effect of Iso is to increase I_p when $[Ca^{2+}]_i$ = 1.4 μM, and reduce I_p when $[Ca^{2+}]_i$ = 15 nM.

In the present study we used the whole cell patch clamp technique to investigate the effects of the parasympathetic transmitter ACh on I_p. At -60 mV, ACh in the absence of Iso has no effect on I_p at either high or low $[Ca^{2+}]_i$. In the presence of Iso, however, ACh reverses both the stimulation of I_p seen at high $[Ca^{2+}]_i$ and the inhibition seen at low $[Ca^{2+}]_i$. These ACh effects are inhibited by the muscarinic blocker atropine. Hyperpolarizing voltage ramps (from V_m = 0) and step changes in V_m showed ACh has no effect on the I_p-V_m relationship in the absence of Iso, regardless of $[Ca^{2+}]_i$, whereas ACh does reverse the reported Iso-induced changes in I_p at all voltages studied. Supported by NIH grants HL54031 and HL20558

W-Pos72

α_1-ADRENERGIC ACTIVATION INHIBITS β-ADRENERGIC STIMULATED UNITARY CALCIUM CHANNEL CURRENTS IN CARDIAC VENTRICULAR MYOCYTES. ((L. Chen, N. El Sherif, M. Boutjdir)) V.A. Med. Ctr. & SUNY/Hlth. Sci. Ctr., Brooklyn, N.Y. 11209. (Spon. by M. Boutjdir).

We previously showed that whole cell L-type Ca current which was initially stimulated by β-adrenergic receptors, was negatively modulated by α_1-adrenergic activation. Here, we investigated the kinetic basis of this modulation at the single channel level in adult rat ventricular myocytes using Ba as the charge carrier. Unitary current sweeps were evoked by 300 ms depolarizing pulses to 0mV, from holding potential of -50mV at 0.5Hz. During control, 33±4% of total sweeps showed channel activity. Unitary current amplitudes varied with applied potential, the I-V relations being linear with a channel conductance of 27.7±0.7pS (n=5). Extrapolated reversal potential was 56.5 mV. Macroscopic current obtained by the ensemble average of single channel recordings showed an amplitude of 0.18±0.01 pA (n=7). To achieve β-adrenergic stimulation (β-effect), cells were superfused with norepinephrine (NE, 10 μM) in the presence of prazosin (Pz, 10 μM), an α_1-adrenergic blocker. β-adrenergic stimulation resulted in an overall increase in channel activity to 65±7% leading into an enhanced ensemble average current (from 0.18±0.01 to 0.75±0.04 pA, p<0.05, n=7). β-adrenergic stimulation increased the open time and decreased the close time constants. To activate α_1-receptors while maintaining the β-adrenergic stimulation, cells were superfused with NE alone (α_1+β effects). α_1-adrenergic activation reduced overall channel activity to 54±8% resulting in reduction in ensemble average current from 0.75±0.04 to 0.41±0.03 pA (p<0.05, n=7). The open time constants were decreased and close time constants were increased by α_1-adrenergic activation. The conductance was not affected by β-or α_1-adrenergic activation. We conclude that α_1-adrenergic activation exerts an inhibitory effect on β-adrenergic stimulated unitary Ba current at the single channel level. The shortening of the open time and the lengthening of the closed time constants and the increase in blank sweeps may, in part, explain the inhibition of the Ca channel activity and the reduction in whole-cell Ca current previously reported. This regulatory process may serve as negative feedback control during physiological and pathological settings.

W-Pos73

C2-REGION-DERIVED PEPTIDES OF β-PROTEIN KINASE C REGULATES CARDIAC CALCIUM CHANNELS. ((Z.H. Zhang, N. El-Sherif, D. Ron*, D. Mochly-Rosen*, and M. Boutjdir)) V.A. Med. Ctr. & SUNY/Hlth. Sci. Ctr., Brooklyn, N.Y. 11209 and *Stanford Univ. Med. Ctr., Stanford, CA. 94305. (Spon. by K. Chinn).

We have previously established that α_1-adrenergic activation inhibited β-adrenergic stimulated L-type Ca current, I_{Ca}. This inhibition could, in part, be mediated through diacylglycerol activation of protein kinase C (PKC). To verify this hypothesis, we recorded whole-cell $I_{Ca/Ba}$ in rat ventricular myocytes in the presence of intracellularly applied C2-region-derived peptides of βPKC (βC2-2, βC 2-4, 0.1μM). These peptide specifically inhibit the translocation and function of C2-containing PKC isozyme, termed cPKC (D. Ron & D. Mochly-Rosen. J.Biol Chem. In Press. Oct 1995). As a negative control, we used scrambled pseudo-substrate peptide (sPS01, 0.1μM) and as a positive control, pseudo-substrate peptide (PS01, 0.1μM) that inhibits the activity of all the PKC isozymes. The effects of PMA (a PKC activator, 0.1μM) on basal or isoproterenol (ISO, 0.1 μM) stimulated $I_{Ca/Ba}$ were evaluated in the absence and presence of the peptide in the pipette solution. With normal internal solution or sPS01, PMA resulted in about 48% inhibition of basal and ISO-$I_{Ca/Ba}$. With βC 2-2 or 2-4, PMA inhibition of basal and ISO-$I_{Ca/Ba}$ was only about 24% and 29% respectively (p<0.05). With PS01, PMA inhibition of ISO-$I_{Ca/Ba}$ was reduced to 16% (p<0.05). The results are summarized in the table. Therefore, blockade of a cPKC translocation by C2-region derived peptides antagonized PMA effect on Ca channels. We conclude that a cPKC, probably βPKC, negatively regulates cAMP-activated Ca channels and could participate in the reported α_1-adrenergic inhibition of β-adrenergic stimulated I_{Ca}.

Ext.Solt	Basal-$I_{Ca/Ba}$ (%)	ISO-$I_{Ca/Ba}$ (%)	Pipt.Solt
PMA	-36.9±7.8 n=3	-48.9±7.8 n=6	Normal
PMA	-47.9±12.9 n=3	-48.0±19.1 n=6	sPS 01
PMA	-23.6±2.2 n=2	-29.3±7.8 n=7	βC 2-4
PMA	-25.0±6.1 n=3	-29.9±6.6 n=6	βC 2-2
PMA	-------	-16.7±7.4 n=5	PS 01

W-Pos74

DOES Ca^{2+} MODULATE β-ADRENERGIC STIMULATION OF Na/K PUMP CURRENT? ((J.R. Stimers and M. Dobretsov)) Dept. Pharmacology & Toxicology, Univ. of Arkansas for Medical Sciences, Little Rock, AR 72205.

Previously we have found that isoproterenol (ISO) stimulates Na/K pump current (I_p) with intracellular free Ca (Ca_f) of 20 nM in adult rat cardiac myocytes; however, it has been reported that in guinea pig myocytes ISO inhibits I_p when Ca_f is low and only stimulates I_p when Ca_f is above 150 nM (Gao et al., J. Physiol. 1992, 449:689) We have investigated the effect of Ca_f on β-adrenergic receptor (β-AR) mediated stimulation of Na/K pump current. Myocytes were whole cell patch clamped at -40 mV with 50 mM Na, 5 mM ATP, 0.5 mM GTP in the patch pipette and 5.4 mM K, 0 Ca, 2 mM Ba, and 0.2 Cd in the bathing solution. In the pipette solution Ca_f was bufferred with EGTA to either 20 nM, 100 nM or 1 μM. I_p was measured as the change in holding current elicited by exposure to 1 mM ouabain. At all concentrations of Ca_f tested I_p was stimulated by 50 μM ISO. At 20 nM Ca_f I_p was increased 1.44 ± 0.05 fold by ISO. Increasing Ca_f to 100 nM did not significantly change the response to ISO; I_p was increased 1.34 ± 0.06 fold. At 1 μM Ca_f ISO consistently stimulated I_p; however, due to the high incidence of spontaneous contractions of the myocytes it was not possible to quantify this effect. Two obvious differences between our results of those of Gao are 1) ISO was never found to inhibit I_p and 2) ISO did not activate an inward current. Further study is needed to clarify these differences. [Supported in part by NIH grant HL44660]

W-Pos75

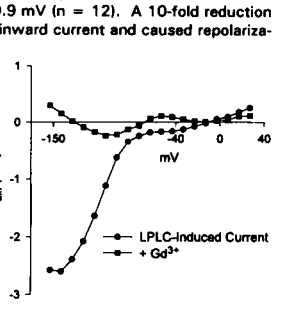
ADRENERGIC STIMULATION OF Na/K PUMP CURRENT IN CARDIAC MYOCYTES IN TISSUE CULTURE. ((M. Dobretsov and J.R. Stimers)) Dept. Pharmacology & Toxicology, Univ. Arkansas Med. Sci., Little Rock, AR 72205.

Some properties of cardiac myocytes change with time in culture. To determine the stability of Na/K pump current (I_p) and its stimulation mediated by adrenergic receptors (AR), cardiac myocytes were isolated from adult rats and placed in culture in 60% M199, 36% Ca-free Earls solution and 4% fetal bovine serum at 36 °C with 5% CO_2/95% air. Myocytes, freshly isolated (day 0) or cultured for 1-3 days, were whole-cell patch clamped at -40 mV with 50 mM Na, 5 mM ATP, 0.5 mM GTP in the patch pipette and 5.4 mM K, 0 Ca, 2 mM Ba, and 0.2 Cd in the bathing solution. On day 0 myocytes have a "broken brick-like" appearance, while on day 3 all cells are "rod-shaped" with rounded edges and most have lamellipodia at the ends. Total membrane capacitance (C_m) was 193 ± 16 pF on day 0 (n=10) and showed a significant decline with time in culture with a slope of -13 pF/day. Despite this decrease in C_m there was no change in I_p density with time in culture averaging 1.53 ± 0.05 pA/pF (n=35) for all days. Similarly, the stimulation of I_p by 50 μM norepinephrine (NE) was not affected by time in culture with an average increase in I_p of 48 ± 3% (n=28). The change in C_m with time may be due to poor survival of larger cells or myocyte atrophy in culture. Nevertheless, I_p density and AR stimulation of I_p is constant for at least 3 days in culture supporting the use of cultured cells to study adrenergic stimulation of the Na/K pump current. [Supported in part by NIH grant HL44660]

W-Pos76

LYSOPLASMENYLCHOLINE (LPLC) INDUCES AUTOMATICITY AND A $[Na^+]_o$-DEPENDENT, Gd^{3+}-SENSITIVE CURRENT IN VENTRICULAR MYOCYTES. ((R.A. Caldwell and C.M. Baumgarten)) Medical Coll. of Virginia, Richmond, VA.

PLA_2 activated during ischemia is plasmalogen-selective and produces 1-alkenyl-LPLC in preference to 1-acyl-lysophosphatidylcholine (LPC). We found that exogenous LPLC induced automaticity in rabbit ventricular myocytes. Automaticity developed with a median time of 8.2 (n=62), 16.1 (n=41), 27.3 (n=36) and >60 min (n=25) on exposure to 10, 5, 2.5 and 1 μM LPLC. At 2.5 and 5 but not 10 μM, LPLC caused automaticity significantly faster than equal concentrations of LPC (>60 min, n=29; 38 min, n=48; 7.3 min, n=12). Whole-cell voltage clamp revealed that LPLC induced and inward current which reversed at -18.5 ± 0.9 mV (n = 12). A 10-fold reduction of $[Na^+]_o$ (N-methyl-d-glucamine) decreased the inward current and caused repolarization. However, 10 μM tetrodotoxin (n=4) and 1 μM saxitoxin (n=3) failed to prevent or reverse the LPLC-induced current. Pretreatment with 100 μM Cd^{2+} also failed to prevent or delay automaticity in 10 μM LPLC (n=12). In contrast, Gd^{3+}, a stretch-activated channel blocker, decreased the LPLC-induced current by 68.5 ± 9.5% (n=4) at -83 mV. Thus, LPLC-induced depolarization depends on a guanidinium toxin-insensitive Na^+ flux that may involve stretch-activated channels. Other actions of Gd^{3+} cannot be excluded at present, however. Sarcolemmal accumulation of LPLC may contribute to dysrhythmias during myocardial ischemia. (NIH HL-46764)



W-Pos77

PKC-INDEPENDENT INHIBITION OF THE β-ADRENERGICALLY ACTIVATED CARDIAC CFTR Cl^- CURRENT BY α-ADRENERGIC STIMULATION
((L.M. Oleksa, L.C. Hool, and R.D. Harvey)) Department of Physiology and Biophysics, Case Western Reserve University, Cleveland, OH 44106-4970

The cardiac isoform of the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel is activated by β-adrenergically-stimulated protein kinase A-dependent phosphorylation . Although cardiac myocytes are exposed to neurotransmitters acting at both α- and β-adrenergic receptors *in vivo*, the effect of α-adrenergic stimulation on this channel is not known. Therefore, we used the whole cell patch clamp technique to investigate the role of α-adrenergic receptor stimulation on the cardiac CFTR Cl^- current in guinea-pig ventricular myocytes. Our studies show that although methoxamine alone activates no Cl^- current, this α_1-adrenergic receptor agonist effectively inhibits the Cl^- current activated by 30 nM isoproterenol, with a K_i of 6.7 μM. This inhibition is blocked by prazosin, confirming that the response to methoxamine is mediated specifically through α_1-adrenergic receptors. Experiments using pertussis toxin (PTX) show that this inhibitory response is mediated by a PTX-sensitive G-protein. This PTX-sensitive response is not linked to protein kinase C (PKC) activation, as (1) direct stimulation of PKC with phorbol ester does not mimic the effect of methoxamine, and (2) inhibition of PKC by bisindolylmaleimide does not prevent the methoxamine-induced response. Additional studies suggest that α-adrenergic inhibition is specific for β-adrenergic responses, as the inhibition is not seen with either histamine- or forskolin-stimulated current. These results demonstrate a unique mechanism for α_1-adrenergic receptor regulation of cardiac Cl^- channels. (Supported by NIH grant HL45141 and the AHA)

W-Pos78

NOREPINEPHRINE REGULATES THE CARDIAC CFTR CHLORIDE CURRENT THROUGH α AND ß ADRENOCEPTORS. ((L.C. Hool and R.D. Harvey)) Department of Physiology and Biophysics, Case Western Reserve University, Cleveland, OH 44106-4970. (Spon. by G. Dubyak)

The sympathetic nervous system modulates the activity of ion channels in the heart through α- and ß-adrenoceptors. However, the interaction of these receptors on ion channel function has not been well characterized. We investigated the effects of the physiological neurotransmitter norepinephrine (Nor) on the cardiac CFTR Cl^- current in guinea pig ventricular myocytes. Using the whole-cell patch-clamp technique we recorded current induced by Nor over a concentration range of 0.001 to 10 μM. The Cl^- current was activated in a concentration dependent manner with a $K_{0.5}$ of 59 nM. To examine if α_1 receptor stimulation contributed to the response, we incubated myocytes with prazosin (1 μM) and measured the Cl^- current elicited by increasing concentrations of the agonist. Blockade of α_1 receptors shifted the $K_{0.5}$ to 20 nM. A similar increase in sensitivity to Nor was measured after incubation of myocytes in pertussis toxin (2 μg/ml) for 3-8 hr ($K_{0.5}$ 8.7 nM). These results indicate that the α_1 receptor modulates the activity of the β-adrenoceptor regulated Cl^- current in cardiac myocytes, and this response appears to be mediated via a pertussis toxin-sensitive G protein. This demonstrates that stimulation of both α and ß-receptors contributes significantly to the Nor-dependent regulation of cardiac ion channels. (Supported by NIH grant HL45141 and the AHA)

W-Pos79

ACETYLCHOLINE-INDUCED REBOUND OF Cl^- CURRENT IN GUINEA-PIG VENTRICULAR MYOCYTES ((S.I. Zakharov and R.D. Harvey)) Department of Physiology and Biophysics, Case Western Reserve University, Cleveland, OH 44106-4970. (Spon. by T. Gerken)

Acetylcholine (ACh) induced rebound of the cAMP-regulated Ca^{2+} current has been reported in cardiac atrial myocytes, even in the absence of adenylate cyclase stimulation (Wang and Lipsius, *Circ. Res.* **76**:634, 1995). We studied the properties of ACh-induced rebound of the cAMP-regulated Cl^- current in ventricular myocytes. The conventional whole-cell patch-clamp technique was used together with a method for rapid exchange of extracellular solutions. Unlike the response in atrial cells, in the absence of adenylate cyclase stimulation, exposure and subsequent removal of ACh had no effect on membrane current. However, when myocytes were first exposed to a subthreshold concentration of isoproterenol or histamine to increase basal cAMP production, a transient or rebound activation of the Cl^- current was consistently observed following removal of ACh. More than 10 nM ACh was required for rebound to occur, and saturating effects were obtained with micromolar concentrations of ACh. Rebound was also induced when atropine was applied in the continued presence of ACh, indicating that the response is associated with muscarinic receptor activation. Rebound stimulation of the Cl^- current was not observed in pertussis toxin-treated myocytes. Furthermore, rebound was not blocked by the nitric oxide synthase inhibitors L-NMMA and L-NAME. These results suggest that ACh-induced rebound of the cAMP-regulated Cl^- current in ventricular myocytes is mediated by muscarinic receptors acting through a PTX-sensitive G-protein, but the response does not appear to involve the production of nitric oxide. (Supported by NIH grant HI45141 and the AHA)

W-Pos80

EFFECTS OF NITRIC OXIDE ON PROTEIN PHOSPHORYLATION IN RAT CARDIOMYOCYTES. ((M.O. Stojanovic, B.M. Wolska, G.M. Wahler and R.J. Solaro)) Department of Physiology & Biophysics, University of Illinois, Chicago, IL 60612-7342.

Beta-adrenergic stimulation enhances contractility and speeds up relaxation. Nitric oxide (NO) has been shown to inhibit there effects. We studied the effect and mechanism of action of NO on rat ventricular myocytes responding to β-adrenergic stimulation. SIN-1 (3-morpholinosydnonimine), an active metabolite of Molsidomine, was used as a source of NO. SIN-1C, a breakdown product of SIN-1 that does not liberate NO, served as a control. Isoproterenol (ISO) was used as β-adrenergic agonist. Isolated myocytes were metabolically labeled with ^{32}P and incubated with ISO alone, ISO + SIN-1, and ISO+SIN-1C. ISO (10^{-9}-$5x10^{-7}$M) alone produced a dose dependent increase in phosphorylation of cardiac troponin I (cTnI) and phospholamban (PLB). Decrease in the levels of ^{32}P incorporation into cTNI and PLB were observed in the myocytes exposed to ISO+SIN-1 (10^{-7}M or $5x10^{-7}$M ISO and 200μM SIN-1) compared to the myocytes incubated with same concentrations of ISO alone or ISO+SIN-1C. Phosphorylation of myosin P-light chain was unchanged. Our results indicate that the anti-adrenergic effects of NO involve reduction of phosphorylation of cTnI and PLB.

W-Pos81

β_2-ADRENERGIC RECEPTOR MODULATION OF THE ACTION POTENTIAL IN RAT VENTRICULAR MYOCYTES ((Michael A. Laflamme and Peter L. Becker)) Dept. of Physiology, Emory University School of Medicine, Atlanta, GA

β_2-adrenergic receptor (AR) activation results in a pronounced lengthening of action potential duration in rat cardiomyocytes under conditions of near-physiological calcium buffering. The purpose of this investigation was to elucidate the mechanism(s) of this action. Fura-2 loaded cells were studied under current-clamp conditions using the perforated-patch technique. The selective β_2-AR agonist zinterol (Zint; 10 μM) increased $[Ca^{2+}]_i$ transient amplitude (↑49±18%) and action potential duration (↑APD_{90} from 80±16 msec to 144±24 msec). β_2-AR activation modestly enhances the magnitude of the L-type Ca^{2+} current (↑I_{Ca} 40 ± 22%) and, thus, some AP prolongation would be expected. However, Zint slowed the rate of repolarization at all potentials, even those where one would expect calcium channels to be closed, indicating that some other current was affected. Because no evidence of modulation of potassium currents could be detected, we tested the hypothesis that an increased inward Na^+/Ca^{2+} exchanger current, resulting from the ↑$[Ca^{2+}]_i$, was responsible for Zint's effect on the late phase of repolarization. Elimination of I_{Ca} (0 $[Ca^{2+}]_o$, 10 μM nifedipine, 300 nM Cd^{2+}) prevented the AP prolongation by Zint. Conversely, the Ca-channel agonist BAY K 8644 influenced late repolarization in a manner qualitatively akin to Zint. Finally, rapidly lowering $[Ca^{2+}]_i$ in Zint-stimulated myocytes by photolysis of the caged Ca^{2+}-chelator diazo-2 accelerated late repolarization, an observation consistent with the predicted action of a modified Na^+/Ca^{2+} exchanger current. Therefore, it appears that β_2-AR prolongation of APD is achieved exclusively by enhancement of the I_{Ca} and of the Na^+/Ca^{2+} exchanger current.

W-Pos82

SIGNAL TRANSDUCTION MECHANISMS UNDERLYING MUSCARINIC RECEPTOR-MEDIATED STIMULATION OF THE HEARTBEAT IN SINGLE CULTURED NEONATAL RAT VENTRICULAR MYOCYTES. ((H. M. Colecraft and S-S. Sheu)) Department of Pharmacology, University of Rochester Medical Center, Rochester, NY 14642.

In single spontaneously beating cultured neonatal rat ventricular myocytes (CNRVMs) pretreated with pertussis toxin, carbachol (CCh) elicits a muscarinic receptor (mAChR) mediated positive chronotropic response (EC_{50}=50μM) as measured by the frequency of fura-2-reported Ca^{2+} transients. We have examined the mAChR subtype, the G-protein type and second messenger system involved in generating this response. The receptor subtypes expressed in CNRVMs were assessed by reverse transcripion of mRNA followed by the polymerase chain reaction (RT-PCR). Fibroblasts contaminating the culture were found to express functional mAChRs, therefore RT-PCR was carried out in single CNRVMs. The results indicated the expression of m_1, m_2 and m_4 mAChRs in these cells. The mAChR subtype mediating the CCh-induced stimulatory response was determined with the use of antisense oligonucleotides targeted against the expressed receptor subtypes. The delivery of fluorescein-tagged oligonucleotides to their sites of action in the cytoplasm and nucleus was achieved with the aid of cationic liposomes and assessed by fluorescence microscopy. Antisense oligonucleotides directed against mRNA for the m_1, but not the m_2 or m_4, mAChRs resulted in a significant inhibition (70%) of the CCh-induced positive chronotropic response. The response was further inhibited by antisense oligonucleotides directed against Gqα and agents, such as neomycin and U73122, that prevented phospholipase C (PLC)-induced phosphoinositide (PI) hydrolysis. These results indicate that the CCh-induced positive chronotropic response in CNRVMs is mediated through an m_1 mAChR coupled through a Gq protein to PLC-induced PI turnover.

W-Pos83

EFFECTS OF ISOPRENALINE, CARBACHOL AND Cs$^+$ ON INTRACELLULAR Na$^+$ ACTIVITY AND PACEMAKER POTENTIAL IN SINO-ATRIAL CELLS ISOLATED FROM THE RABBIT HEART ((H.S. Choi and C.O. Lee)) Dept. of Life Science, POSTECH, Pohang, Korea

Effects of isoprenaline, carbachol and Cs$^+$ on intracellular Na$^+$ activity (a^i_{Na}) and spontaneous action potentials were studied in multicellular preparations and single cells isolated from the rabbit S-A nodes. a^i_{Na} were measured with double barrelled Na$^+$-selective microelectrodes and fluorescent Na$^+$ indicator, SBFI. In spontaneously beating cells, a^i_{Na} measured with Na$^+$-selective microelectrodes and SBFI were 4.5±1.2 (mean±S.D., n=21) and 3.9±1.1 mM (n=18) respectively. The measurements with Na$^+$-selective microelectrodes showed the following results. Isoprenaline (10^{-7}-10^{-6}M) increased a^i_{Na} from 4.7 ±1.2 to 5.5±1.6 mM (mean±S.D., n=20, p<0.005) and shortened the action potential cycle length (ACL) from 344±45 to 240±63 msec (n=20, p<0.005). Carbachol ($5x10^{-6}$M) decreased a^i_{Na} from 4.6±1.4 to 3.9±1.2 mM (n=26, p<0.001) and prolonged the ACL from 320±44 to 687±123 msec (n=26, p<0.001). Cs$^+$ (6mM) decreased a^i_{Na} from 4.9±1.3 to 3.9±1.3 mM (n=23, p<0.005) and prolonged the ACL from 343±30 to 464±100 msec (n=23, p<0.005). In the measurements with SBFI, isoprenaline, carbachol and Cs$^+$ produced a^i_{Na} changes that were similar to those measured with Na$^+$-selective microelectrodes. Our results suggest that (1) isoprenaline increases a^i_{Na} possibly by enhancing the inward Na$^+$ movement (presumably by *i$_f$*), causing an increase in the slope of the spontaneous depolarization; (2) carbachol and Cs$^+$ decrease a^i_{Na} possibly by inhibiting the inward Na$^+$ movements (presumably by *i$_f$*), causing a decrease in the slope of the spontaneous depolarization.

W-Pos84

THE ROLE OF PHOSPHORYLATION IN THE REGULATION OF I_F IN RABBIT SA NODE MYOCYTES: ((Y.M. Liu, H. Yu and I.S. Cohen)) Physiology and Biophysics, HSC SUNY at Stony Brook 11794-8661.

Recent experiments in Purkinje fibers and isolated Purkinje and ventricular myocytes suggest that i_f is regulated in these preparations by phosphorylation (Chang *et al.* J. Physiol **440**: 367-384, 1991, Yu *et al.* Pflugers Arch. **422**: 614-616, 1993, Yu *et al.* J. Physiol. **485**: 469-483, 1995). In sinus node, little is known about the role of phosphorylation in regulating i_f, although direct gating by cAMP has been reported (DiFrancesco & Tortora, Nature **351**: 145-147, 1991). We therefore undertook our study on rabbit SA node (SAN) myocytes to examine what role, if any, phosphorylation plays in i_f regulation. Calyculin A, a non-specific phosphatase inhibitor (0.5-1.0 μM) reversibly increased i_f in 5 SAN myocytes, while the non-specific kinase inhibitor H-7 (25 -100 μM) reversibly reduced the magnitude of i_f in 5 SAN myocytes. Thus as in other cardiac preparations phosphorylation state regulates i_f in SAN myocytes.

We next examined the actions of isoproterenol (ISO, 1.0-1.5 μM) on i_f in SAN myocytes. Neither H-7, nor H-8 could eliminate the actions of ISO on i_f, thus suggesting an important role for direct cAMP gating. Nevertheless, preliminary results in 3 SAN myocytes comparing the actions of the Rp and Sp isomers of cAMP (Rp does not activate the A kinase, while Sp does) suggest that Sp has a larger effect. This result suggests that the A-kinase may also play an important role in the actions of beta agonists. In conclusion, i_f is regulated by phosphorylation state in SA node myocytes. The actions of beta agonists appear to be mediated by both phosphorylation and direct cAMP gating. Supported by HL20558 and HL28958 from the NHLBI.

W-Pos85

EFFECTS OF BRADYKININ ON DIASTOLIC MEMBRANE CURRENTS IN CANINE AND RABBIT CARDIAC MYOCYTES. ((J. Wu, F. Chang, and I.S. Cohen)) Physiology and Biophysics HSC SUNY at Stony Brook, NY 11794.

Bradykinin (BK) has been shown to reduce the incidence of ventricular tachycardia (Vegh *et al.* Br. J. Pharmacol. **113**: 1167-72, 1994). We therefore studied the actions of BK on the diastolic membrane currents, iK_1 and i_f, in canine and rabbit cardiac myocytes as well as canine Purkinje fibers in an attempt to see whether alterations in these membrane currents might underlie this cardioprotective effect.

BK at a low concentration (2.5μM) reversibly decreased iK_1 in both canine Purkinje and ventricular myocytes recorded with the whole cell patch clamp technique at -100 mV by 18%±4%, n=8(Mean±SEM), and 15%±2%, n=10, respectively. The same low concentration of BK reversibly reduced i_f recorded in rabbit SA node myocytes with the permeabilized patch clamp technique at -75 mV by 36%±9%, n=8. Similar results were obtained on iK_1 and i_f in canine Purkinje fibers with the two microelectrode voltage clamp technique. These results are mediated through a B_2 receptor since HOE 140 (10μM), a B_2 receptor antagonist, reversed the decrease in iK_1 and i_f induced by BK in the isolated ventricular, Purkinje, and SA node myocytes.

BK at a higher concentration (20μM) reversibly increased iK_1 recorded at -100mV in canine ventricular myocytes (16%±4%, n=4), and also reversibly increased i_f recorded at -75mV in rabbit SA node myocytes (19%±5%, n=4). Both of these higher concentration effects were inhibited by HOE 140 (80μM). Thus BK has a concentration dependent effect on diastolic membrane currents. The intracellular mediators of these effects as well as the role of these changes in the cardioprotective effect of BK remains to be determined. Supported by HL20558 and HL28958 from NHLBI.

TRANSMEMBRANE SIGNALING - MECHANISM AND THEORY

W-Pos86

THE INTERACTION OF MN^{2+} WITH CALCINEURIN STUDIED USING NMR PROTON RELAXATION ENHANCEMENT. ((A. Haddy, R. R. Sharp and F. Rusnak)) Mayo School of Graduate Studies, Rochester, MN 55905 and University of Michigan, Ann Arbor, MI 48109.

Calcineurin (protein phosphatase 2B) contains an Fe-Zn dimetal cluster at the active site and is stimulated by added Mn^{2+}. In this study, the interaction of Mn^{2+} with calcineurin was characterized in three calcineurin preparations, two native bovine brain and one recombinant. Each preparation contained substoichiometric quantities of Fe and Zn. The binding of Mn^{2+} to calcineurin was examined at room temperature by measuring the electron paramagnetic resonance signal due to unbound hexaquo-Mn^{2+} and by direct detection of bound Mn^{2+} using NMR proton relaxation enhancement, which provided a more accurate and sensitive characterization of the high affinity sites. Two types of sites were revealed, 0.2 high affinity sites with K_d of 1-5 μM and a large number (≥7) of low affinity sites with K_d in the mM range. Treatment of one native preparation with $FeCl_3$ decreased the number of high affinity sites to 0.1. The high affinity sites showed enhancements ε_b of 4.5 to 5.3 depending on the preparation, consistent with the presence of exchangeable water within the inner coordination sphere of bound Mn^{2+}. The low affinity Mn^{2+} sites probably represent nonspecific binding. The high affinity Mn^{2+} sites are consistent with binding at a catalytic site that is not fully occupied by Fe and/or Zn, suggesting that Mn^{2+} directly coordinates phosphate as its mode of stimulation.

W-Pos87

LYSOPHOSPHATIDYLCHOLINE AFFECTS THE SARCOLEMMAL PHOSPHOINOSITIDE PATHWAY. ((S.-Y. Liu, C.-H. Yu and V. Panagia)) Division of Cardiovascular Sciences, St. Boniface G.H. Research Centre, University of Manitoba, Winnipeg, Canada R2H 2A6.

Although lysophosphatidylcholine (lyso-PtdCho) accumulates in sarcolemma (SL) during myocardial ischemia compromising SL function, its effects on SL signalling processes are scarcely known. We have examined the in vitro action of lyso-PtdCho on the enzymes of the phosphoinositide pathway in SL membranes purified from rat heart. 100 μM lyso-PtdCho significantly decreased both phosphatidylinositol (PtdIns) kinase and phosphatidylinositol 4-phosphate (PtdIns4P) kinase activities. 1-Oleoyl lyso-PtdCho was a more potent inhibitor than 1-palmitoyl and 1-stearoyl lyso compounds. Oleate and L-α-glycerophosphate did not affect the enzymes. Although the pattern of the concentration (10-200 μM)-dependent effect of oleoyl lyso-PtdCho was similar for the two kinases, PtdIns4P kinase was significantly more depressed than PtdIns kinase. None of the tested lyso-PtdCho species did alter PtdIns(4,5)-bisphosphate hydrolysis by SL phospholipase C (PLC). The results suggest that phospholipase A_2-dependent intraSL accumulation of lyso-PtdCho during myocardial ischemia may negatively effect the availability of phosphoinositide substrate for the production of second messengers by receptor-linked PLC. (Supported by H.S.F. of Manitoba)

W-Pos88

PHOSPHOLIPASE C ISOFORMS IN VASCULAR SMOOTH MUSCLE ((E.Polyak and E.F.LaBelle)) Bockus Research Inst., Graduate Hospital, Philadelphia,PA 19146

Receptor-mediated inositol 1,4,5-trisphosphate (IP_3) formation in most tissues is dependent on a variety of phospholipase C (PLC) isoforms. To determine which PLC isoforms were present in vascular smooth muscle compared to brain, liver and spleen, we extracted proteins from these tissues, separated and identified the PLC isoforms by immunoblotting. Aliquots of rat tail artery were examined by this procedure together with aliquots of rat liver, spleen, cerebral cortex, hippocampus, cerebellum, aorta, and mesenteric artery. PLC γ1 was shown to be present in all of these tissues, while PLC β1 was shown to be limited to fractions from brain. PLC δ1 was detected in rat tail artery, mesenteric artery, aorta and brain. PLC β2 was found in rat tail artery, mesenteric artery, aorta, liver, and brain. This is the first report of PLC β2 in tissues other than HL60 cells. Since G proteins activate IP_3 production via stimulation of PLCβ isoforms in many tissues, and agonist-stimulated IP_3 production in smooth muscle requires G protein activation, PLCβ2 is most likely required for agonist-stimulated force production in vascular smooth muscle. We find $G\alpha_{q/11}$ in rat tail artery, which has been shown by other investigators to activate PLC β in different tissues. When rat tail artery proteins were separated by two-dimensional gel electrophoresis and probed with antibodies to phospholipase C isoforms, phospholipases Cγ1, δ1, and β2 were all identified and were all shown to exist as multiple spots, indicative of phosphorylation. (Supported by Grant HL 37413 from NIH)

W-Pos89

PHOSPHOLIPASE D IS ACTIVATED BY G PROTEIN AND NOT BY CALCIUM IONS IN VASCULAR SMOOTH MUSCLE ((E.F.LaBelle, R.M.Fulbright, R.J.Barsotti,and E.Polyak)) Bockus Research Inst., Graduate Hospital, Philadelphia, PA 19146. Spon. by E.Murer

We assessed the sensitivity of phospholipase D activity in vascular smooth muscle to cytosolic calcium by increasing cytosolic calcium levels independently of agonist stimulation. When rat tail artery was preloaded with the calcium indicator Fluo-3AM, the addition of high extracellular K+, caffeine, or norepinephrine rapidly enhanced cytosolic calcium levels. Neither increased extracellular K+ nor caffeine addition increased phosphatidylethanol production, indicating that cytosolic calcium elevation alone did not stimulate phospholipase D. In contrast, norepinephrine stimulated phosphatidylethanol production in this tissue. In strips of tail artery permeabilized with α toxin and incubated in solutions containing free calcium concentrations observed during physiological stimulation (pCa=6.4), phospholipase D was not stimulated, while incubation with GTPγS at pCa 7.0 activated this enzyme. Aluminum fluoride stimulated phospholipase D and this activity was insensitive to pertussis toxin after stimulation by either norepinephrine or aluminum fluoride. These results indicate that phospholipase D in vascular smooth muscle is activated by norepinephrine via stimulation of a pertussis toxin-insensitive G protein and not via an increase in intracellular calcium levels. (Supported by Grant HL 37413 from NIH)

W-Pos90

TUBULIN - G PROTEIN ASSOCIATION AND NUCLEOTIDE TRANSFER: A FLUORESCENCE STUDY. ((M. E. Knight and M. M. Rasenick)) University of Illinois at Chicago, Chicago, IL, 60612.

The demonstration of specific binding between tubulin and specific G proteins, $G\alpha_{t1}$, $G\alpha_s$, and $G\alpha_q$, the transfer of guanine nucleotide between tubulin and $G\alpha_{t1}$ or $G\alpha_s$, as well as the stimulation of a GTPase upon binding, implicates a significant role for tubulin in the regulation of G-protein mediated signal transduction. Based on these observations, a model for tubulin-$G\alpha_{t1}$ interaction is proposed for binding, nucleotide transfer, and GTPase stimulation. Fluorescently labeled proteins and guanine nucleotides were used to elucidate each step in the model. Fluorescence resonance energy transfer (FRET) was used to characterize the interaction between purified brain tubulin labeled with coumarin maleimide (CM) or fluorescein-5-maleimide (FM) and purified recombinant $G\alpha_{t1}$ labeled with rhodamine isothiocyanate (RITC). When both proteins were in the GDP-bound form, the $K_{D,app}$ was determined to be 500 nM, while when both proteins were in the GTPγS-bound form, the $K_{D,app}$ was shifted to the μM range. This decrease in affinity when in the GTPγS forms suggests that GTP/GDP binding regulates this interaction. It also reflects the binding observed between $G\alpha_{t1}$ subunits, in which association between subunits is not observed in the GTPγS form (S. Roychowdhury and M.M. Rasenick, unpublished observations). Fluorescently labeled guanine nucleotides were used to compare nucleotide transfer from tubulin to $G\alpha_{t1}$ with the binding of free nucleotide. The binding of mant-GTPγS to $G\alpha_{t1}$ was found to have k_{on} of 3.7×10^6 $M^{-1}min^{-1}$ and k_{off} of 0.17 min^{-1}. However, when mantGTPγS was transferred from tubulin to $G\alpha_{t1}$, the k_{on} was doubled to 6.6×10^6 $M^{-1}min^{-1}$, while the off rate was unchanged. This is consistent with the hypothesis that tubulin activates $G\alpha_{t1}$ in a manner similar to that seen for an activated receptor.

W-Pos91

KINETICS AND THERMODYNAMICS OF G PROTEIN ACTIVATION

((Bogumil Zelent*, Heidi Hamm+ and Paul A. Liebman*)) *Dept. of Biochemistry and Biophysics, Univ. of Pa. Medical Center, Phila.,PA and +Dept. of Physiology and Biophysics, Univ. of Ill.@ Chicago

G proteins are held quiescent in a GDP-bound form when their heptahelical receptor is not activated. Nevertheless, a very slow rate of spontaneous dissociation of GDP allows GTP binding to generate a background level of spontaneous activity of these important signal coupling proteins. In this study, we have measured the thermal dependence of this process for recombinant $G_{i\alpha1}$ and compared it with similar measurements on $G_{t\alpha}$ using both radionucleotide and fluorescence enhancement methods. Arrhenius plots of data in the temperature range 20-40°C. give activation enthalpies of +28 kcal/mole and +38 kcal/mole with activation entropies of +17 e.u. and +40 e.u. and Q_{10}'s of 4.2 and 5.5 respectively for G_i and G_t. The high activation enthalpy for G_t rivals that of its receptor, rhodopsin, and accounts for its needed stability. Differential scanning calorimetry shows the heat capacity of both proteins to rise steeply with an extremely low melting temperature (Tm=49.5 °C.) The complex non-2 state, steeply rising heat capacity curve at moderate to near physiologic temperatures may belie floppy structural states related to the large positive activation entropy. Easy formation of these states may drive GDP dissociation against the formidable enthalpic barrier. GTPγS stabilizes (T_m>60°C.) and simplifies the calorimetric domain character as might be expected from the more ordered Xray structure of $G_{t\alpha}$•GTPγS.

Supported by NIH grants EY00012/EY01583 (PL) and EY06062/EY10291(HH).

W-Pos92

FLUORESCENCE QUANTUM YIELDS OF TRANSDUCIN ($G_{t\alpha}$) IN SEVERAL DIFFERENT FUNCTIONAL STATES. ((Bogumil Zelent, Robert Sharp and Paul A. Liebman)) Dept.of Biochemistry and Biophysics, Univ. of Pa. Medical Center, Phila., Pa.19104. (sponsored by John W. Weisel)

Previous intrinsic fluorescence studies of G proteins have failed to establish conditions of protein homogeneity, high functional activity and appropriate excitation wavelength for evaluation of quantum yield. We have purified $G_{t\alpha}$ and tested functional efficacy to assure 100% activity for more systematic excitation and emission fluorescence study. After solving problems of long term cuvette stability, we find the net Trp quantum yields of Trp127 and Trp207 for $G_{t\alpha}$•GDP, $G_{t\alpha}$•G_γS and $G_{t\alpha}$•GDP•AlF_4 to be 0.21, 0.40 and 0.37 respectively. Only Trp207 is thought to reposition within the molecule upon activation by G_γS or AlF_4. Trp207's quantum yield must therefore exceed 0.4 in the activated state of $G_{t\alpha}$. Tyrosine emission is largely internally quenched in $G_{t\alpha}$ but tyr-trp intramolecular energy transfer is reduced from 0.144 to 0.072 on activation. Analysis of dipole orientation for all 13 tyr and 2 trp of the crystal structure suggests that Tyr254/Trp207 is the likely pair whose efficiency is changed.

Supported by NIH grants EY00012 and EY01583.

W-Pos93

A GENERAL METHOD FOR MAPPING TERTIARY CONTACTS IN MEMBRANE PROTEINS. ((Hongbo Yu, Masahiro Kono, Timothy D. McKee, Daniel D. Oprian)) Graduate Department of Biochemistry and Volen Center for Complex Systems, Brandeis University, Waltham, MA 02254. (Spon. by I. Levitan)

We report a general method for mapping the tertiary contacts between helices of membrane proteins using rhodopsin as a model. The idea is to use Cys-scanning mutagenesis in combination with split-rhodopsin mutants in which the protein is assembled from two separately expressed gene fragments. If Cys residues from separate fragments are close to each other in the three dimensional structure of the protein then a disulfide bond will form and covalently link the two fragments. The disulfide cross-linked fragments can be readily identified by an electrophoretic mobility shift on Western blots of SDS gels.

To test the disulfide cross-linking assay, we used a rhodopsin mutant split in the loop between helices 5 and 6, and substituted Cys residues for Val204, located in helix 5 of the amino-terminal fragment, and Phe276, located in helix 6 of the complementary carboxy-terminal fragment. The selection of Val204 and Phe276 was based on the report that a metal-ion binding site could be engineered between these two positions in the tachykinin NK-1 receptor [Elling et al. (1995) *Nature* 374, 74-77]. Treatment of the Cys-subsituted split rhodopsin mutant with copper phenathroline oxidant resulted in a disulfide cross-link formed specifically between Cys204 and Cys276 which was easily identified by a mobility shift on Western blots of SDS gels using an antibody to the carboxy terminus of rhodopsin.

W-Pos94

INTERACTIONS BETWEEN HELIX 5 AND HELIX 6 OF RHODOPSIN USING CYSTEINE-SCANNING MUTAGENESIS OF SPLIT RECEPTOR MUTANTS. ((Masahiro Kono, Hongbo Yu, and Daniel D. Oprian)) Graduate Department of Biochemistry and Volen Center for Complex Systems, Brandeis University, Waltham, MA 02254

We have recently developed a procedure for mapping tertiary interactions between the transmembrane segments of membrane-embedded proteins. The proteins used in this approach are assembled from two separately expressed gene fragments to form mutant heterodimers that nonetheless display wild-type structure and function. Cys residues are systematically substituted for the amino acids in the transmembrane segments of each fragment and the isolated proteins monitored for disulfide bond formation between juxtaposed Cys by their electrophoretic mobility on Western blots of SDS gels.

Using this approach, we have initiated a systematic examination of the contacts between transmembrane segments 5 and 6 in rhodopsin. To date, we have identified contacts between Phe276 of segment 6 and Asn200 and Val204 of segment 5. A disulfide bond forms spontaneously between Cys200 and Cys276, whereas cross-linking of Cys204 and Cys276 requires treatment of the protein with a copper phenanthroline oxidant. Preliminary data for other amino acids in this area of the protein suggest the presence of helical secondary structure.

W-Pos95

RHODOPSIN PHOTOACTIVATION IS MODULATED BY WATER ACTIVITY. ((S.C. Myers-Payne, D.C. Mitchell, and B.J. Litman)) NIAAA, NIH, Bethesda, MD 20892.

Rhodopsin (rho) is an integral membrane protein whose photoactivation is modulated by compositionally-determined bilayer lipid properties, such as acyl chain packing free volume. Herein we demonstrate that rho's osmotic environment also effects its photoactivation. In the presence of increasing concentrations of three different non-ionic solutes, glycerol, sucrose, and stachyose, the equilibrium of metarhodopsin I (MI) to metarhodopsin II (MII) was shifted in favor of MII. This shift was directly proportional to osmolality, suggesting modulation by water activity. Non-ionic solutes can dehydrate both lipid bilayers and proteins, thereby altering acyl chain packing free volume and protein conformation. To test if the equilibrium shift was due to changes in the lipid bilayer, PDPC vesicles were examined in the absence and presence of non-ionic solutes. Each of the non-ionic solutes tested decreased acyl chain packing free volume, as measured by DPH anisotropy decay. Previous observations show that increases in acyl chain packing free volume result in a shift in the equilibrium of photoactivated MI toward MII. If the rho equilibrium shift were due solely to bilayer dehydration, the solute-induced decrease in acyl chain packing free volume would favor MI. However, the observed equilibrium shift favoring MII is in the opposite direction and therefore not attributable to bilayer dehydration. Therefore, the effect of dehydration on the protein appears to overcome the induced decrease in acyl chain packing free volume for a net equilibrium shift toward MII.

W-Pos96

PHOSPHORYLATION OF RHODOPSIN SHIFTS THE MI/MII EQUILIBRIUM TOWARDS MII. ((Scott K. Gibson, John A. Parkes, and Paul A. Liebman)) Department of Biochemistry and Biophysics, University of Pennsylvania Medical Center, Philadelphia, PA 19104

Upon light activation, rhodopsin decays into a proton-driven equilibrium between two spectral intermediates, Metarhodopsin I (MI) and Metarhodopsin II (MII). MII is the intermediate that activates the G-protein, transducin. After activation, rhodopsin is phosphorylated at multiple sites by rhodopsin kinase to control the amount of G-protein activation. A previous report suggests that phosphorylation of rhodopsin does not change the MI/MII equilibrium in reconstituted phosphotidylcholine (PC) vesicles. Using regenerated phosphorylated rhodopsin in native rod disk membranes (RDM), we have determined that the equilibrium between MI and MII is shifted towards MII in proportion to the phosphorylation level of rhodopsin. The difference from previous results may be due to charge contribution from phosphatidylserine (PS) in RDM as well as to single-sided orientation of rhodopsin in RDM. A Gouy-Chapman model of the surface potential of RDM shows that phosphorylation of the C-terminal tail of rhodopsin can result in an appreciable change in membrane surface potential. The change in surface potential may in turn lower local pH which would increase the MI/MII equilibrium constant.

Supported by NIH grants EY00012/EY01583 to PL and predoctoral grant GM08275 to SKG

W-Pos97

G PROTEIN TRYTOPHAN FLUORESCENCE QUENCHING IN THREE DIFFERENT FUNCTIONAL STATES. ((Bogumil Zelent, Robert Sharp and Paul A. Liebman)) Dept.of Biochemistry and Biophysics, Univ. of Pa. Medical Center, Phila., Pa.19104. (sponsored by David F. Wilson)

G proteins can exist in three functional states: G•GDP, G•GTP and G•GDP•AlF_4^-. The external quenchers, I^-, acrylamide and Cs^+, were used to probe accessibility of the two tryptophans (Trp) of transducin alpha subunit ($G_{t\alpha}$) to the aqueous environment in these functional states. A method was developed to allow very stable step titration of these sometimes unstable proteins with quencher. Each quencher titration was compared to its companion effect on N-acetyl-L-tryptophanamide, (NATA), a freely accessible form of Trp. Trp's of all species of $G_{t\alpha}$ were found to be shielded from quencher access (K_Q) in the increasing order I^-, acrylamide, Cs^+, respectively ca. 1/3, 1/7 and 1/10 as accessible as NATA. Quencher order is reversed for I^- and acrylamide relative to that for NATA suggesting that I^- may be electrostatically enhanced by excess positive surface charge near both Trp127 and Trp207. Cs^+ may be repelled for the same reasaon. "Grasp"© views of surface electrostatics confirm this expectation. Since Trp127 is known to remain stationary in the helical domain upon $G_{t\alpha}$ activation while Trp207 appears to fold into a hydrophobic cleft of the GTP'ase domain in the GTPγS or AlF_4^- form, it may be possible to distinguish Trp127 from Trp207 with current quenchers. Both static and dynamic quenching mechanisms appear in our quenching curves.

Supported by NIH grants EY00012 and EY01583.

W-Pos98

Two-Photon 3-D Single Particle Tracking: A Scanning Microscopy Approach. ((Peter T. C. So, Tim Ragan, Weiming Yu and Enrico Gratton)) Laboratory For Fluorescence Dynamics, Department of Physics, University of Illinois at Urbana-Champaign, Urbana, IL 61801

Single particle tracking is a powerful approach to study membrane protein diffusion, membrane compartmentalization and protein-cytoskeleton interaction. Tracking the dynamics of a single particle can resolve diffusive, directed or restricted motions. This type of information cannot be obtained with conventional techniques where the statistically average behavior of particles is monitored. These studies typically use wide field illumination and monitor the motion of either colliodial gold or fluorescent particles using area detectors. This type of technique suffers from two major limitations. First, only two dimensional motions can be followed. Many interesting biological processes, such as endocytosis kinetics, intracellular transports and diffusion in cytoplasm, can not be studied. The development of 3-D particle tracking using cylindrical optics by Verkman's group (Biophys. J. **67**, 1994) has somewhat alleviated this problem. However, the axial tracking of this method is still confined to a range of 5 to 10 μm. Second, the time-resolution of the fastest available instrument is limited to the video rate. In this study, we propose to develop a 3-D particle tracking instrument utilizing the inherent localization of two-photon excitation. Two-photon point spread function using a 1.25 NA objective has a FWHM of 0.3 μm in the radial direction and 0.9 μm in the axial direction. Using a galvanomotor x-y scanner and a piezo-controlled z-stage, the dynamic response in positioning of this sub-femtoliter excitation volume can be on the order of 100-1000 Hz. Using a real-time feedback system, we can track the position of the diffusing fluorescent particle by maximizing detected intensity. This system has been used to study the processes of antigen endocytosis of macrophages and 3-D diffusion in cytoplasm of fibroblasts. [Supported by NIH grant RR03155.]

W-Pos99

BINDING OF SMALL BASIC PEPTIDES TO MEMBRANES CONTAINING ACIDIC LIPIDS: THEORETICAL MODELS AND EXPERIMENTAL RESULTS. ((N. Ben-Tal, B. Honig, G. Denisov, R. M. Pietzsch and S. McLaughlin)) Columbia University, New York, NY 10032 and HSC, SUNY, Stony Brook, NY 11794. (Spon. by I. Edelman)

We measured directly the binding of Lys_3, Lys_5, and Lys_7 to vesicles containing acidic phospholipids. When the vesicles contain 33% acidic lipids, and the aqueous solution contains 100mM monovalent salt, the standard Gibbs free energy for the binding of these peptides is 3, 5, and 7 kcal/mol, respectively. The binding energies decrease as the mole % of acidic lipids in the membrane decreases, and as the salt concentration increases. Several lines of evidence suggest that the binding is mainly due to electrostatic interactions. To calculate the binding energies from classical electrostatics, we applied the non-linear Poisson-Boltzmann equation to atomic models of the phospholipid bilayers and the basic peptides in aqueous solutions. The electrostatic free energy of interaction, which arises from both a long range coulombic attraction between the positively charged peptide and the negatively charged lipid bilayer, and a short range Born or image charge repulsion, has a minimum when $\sim$ 3Å (i.e. one layer of water) exists between the van der Waals surfaces of the peptide and the lipid bilayer. The calculated molar association constants, K, agree well with the measured values. These calculations are relevant to the membrane binding of a number of important proteins that contain clusters of basic residues such as Src, MARCKS, protein kinase C, K-Ras, HIV-1.

W-Pos100

RESOLUTION LIMITS OF NONSTATIONARY NOISE ANALYSIS DETERMINED WITH COMPUTER SIMULATIONS.

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Nonstationary noise analysis is a valuable tool for estimating single-channel parameters when excessive background noise (σ_b^2) obscures the resolution of single-channel currents (i) or when a preparation contains many active channels (N). The basic assumption for calculating the theoretical ensemble variance (σ^2), associated with the mean current (I), is that channels open only on a single conductance level: $\sigma^2 = i \cdot I - I^2/N + \sigma_b^2$. In order to describe the resolution limits of this analysis approach and to specify the accuracy of the parameter estimates quantitatively, ion current fluctuations were simulated for specific kinetic schemes, under consideration of experimental situations. A particular problem arises when subconductance states contribute to the mean current. If subconductance states are correlated with the activation process, as it has recently become apparent for potassium channels, the variance vs. mean plot must deviate from a parabolic function. Strategies for nonstationary noise analysis were developed which allow the discrimination of possible subconductance levels, considering data sets of different open probabilities or sequential inactivation. As a first approximation, alternative model-dependent, non-parabolic functions were fitted to the mean-variance data, in order to estimate subconductance levels. Applications of the analysis procedures to experimental current fluctuations and their comparison with computer simulations will be discussed.

K CHANNELS - PHYSIOLOGY/PHARMACOLOGY

W-Pos101

INHIBITORY EFFECT BY THROMBOXANE A_2 AGONIST ON K_{Ca} CHANNEL ACTIVITY IN HUMAN CORONARY ARTERY SMOOTH MUSCLE CELLS. ((Y. Tanaka and L. Toro)) Dept. of Anesthesiology, UCLA School of Medicine, Los Angeles, CA 90095-1778.

Large conductance calcium-activated potassium (K_{Ca}) channels have been shown to play an important role in the regulation of contractility of vascular smooth muscles including coronary arteries of many species. The inhibition of this channel was suggested to contribute to the coronary contraction elicited by thromboxane A_2 (TXA_2), a potent vasoconstrictor (Scornik, F.S. and Toro, L., *Am. J. Physiol.* **262**: C708-C713, 1992). In the present study, the effect of U46619, a TXA_2 mimetic, on K_{Ca} channel activity was examined in freshly dissociated smooth muscle cells of human coronary artery with the inside-out patch-clamp technique. When both sides of the membrane were exposed to 140 mM K^+ solution, single channel conductance was about 200 pS, whereas it was around 130 pS when the pipette was filled with 135 mM Na^+ and 5 mM K^+ solution. Open probability (P_o) of these large conductance channels exhibited both Ca^{2+}- and membrane voltage-dependency. Dehydrosoyasaponin I (DHS-I), a triterpenoid glycoside, increased the P_o of the K_{Ca} channels 2 - 3 times the control when applied to the intracellular side of the membrane at 250 nM, thus indicating that the α-subunit of the channel is functionally coupled with its β-subunit. By adding U46619 (100 - 500 nM) to the internal side of the channel, P_o was decreased between 10 - 50% of the control value. SQ 29548 (5 μM), a specific TXA_2 receptor antagonist, restored the channel activity suppressed by U46619 to near control level. These results suggest that TXA_2, through activation of its specific receptor, can inhibit the activity of K_{Ca} channel complex (α + β) expressed in human coronary artery, and that this inhibitory modulation of the channels contributes in part to its coronary constrictor activity. Supported by NIH grant HL47382.

W-Pos102

NITRIC OXIDE-INDUCED MODULATION OF BK_{Ca} CHANNELS IN RAT CEREBRAL ARTERY MYOCYTES ((M. Holland, N.B. Standen & J.P. Boyle)) Cell Physiology & Pharmacology, University of Leicester, LE1 9HN, UK

Endothelial derived nitric oxide (NO) plays a major role in the regulation of tone in large cerebral arteries such as the basilar artery, where NO-induced hyperpolarization is thought to contribute to vasorelaxation. Channels reported to have been activated by NO include single BK_{Ca} channels via activation of G-kinase (cerebral arteries[1]) and K_{ATP} channels (mesenteric arteries[2]).

We have studied the effects of NO on whole cell K^+ currents in single smooth muscle cells isolated from rat basilar artery. Steady state currents induced by NO at a holding potential of -40mV were measured using the conventional whole cell configuration in symmetrical 140 mM K^+. Following the addition of an activated NO donor, SIN-1A, in the presence of superoxide dismutase (50 U/ml), an inward current was activated. This current was unaffected by glibenclamide (5 μM) or apamin (100 nM) but was rapidly and almost completely inhibited by 100 nM iberiotoxin (IbTX). This contrasts with the extremely slow reversal observed following bath washout of SIN-1A. The unitary channel amplitude of the channel activated by NO was -11 pA, giving a calculated conductance of 270 pS. Ramps were run from -50 to +50 mV following the activation of inward current by NO. The IbTX-sensitive current, which was increased relative to control values at all ramp potentials, reversed at a potential close to the potassium equilibrium potential (0 mV). The toxin sensitivity and unitary conductance identified the channel responsible for this current as the large-conductance, calcium-activated potassium channel (BK_{Ca}).

Therefore, the activation of BK_{Ca} channels by NO in cerebral arterial myocytes could underlie the reported NO-induced hyperpolarization in large cerebral arteries.

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

W-Pos103

PREGNANCY AND K^+ CURRENTS OF FRESHLY DISSOCIATED RAT UTERINE MYOCYTES. ((S.Y.Wang, M.Yoshino, J.L.Sui and C.Y.Kao)) SUNY Downstate Med Ctr. Brooklyn, NY 11203.

By combined use of different holding potentials and selective blocking agents, cellular I_K's of non-pregnant (NP) and late-pregnant (LP) myocytes have been sorted into components that are differently affected by pregnancy. A transient outward current, half-inactivated at -77 mV, occurs in 50% of NP but not in LP myocytes. In NP myocytes, total I_K is noisy from an iberiotoxin-sensitive current, with half-activation at 39 mV ($V_{0.5}$). In LP cells, I_K is smoother; $V_{0.5}$ of the noisy component is 63 mV, agreeing with 68 mV at pCa 7 seen in V-p_o relations of single 140 pS maxi-K channels in detached inside-out patches. Intracellular release of Ca^{2+} from nitr 5-Ca complex increases I_K of all 15 NP cells by 4.8 fold (mean); in LP cells, 44% (n=16) show no change in I_K, while 56% (n=20) show an increase of 2.0 fold. A dendrotoxin-sensitive current is seen in LP but not in NP myocytes. Thus, pregnancy shifts the expression of whole-cell I_K from large-conductance Ca^{2+}-activated channels to smaller-conductance delayed rectifier channels, partly by increasing the latter, and partly by de-expressing the former by a positive shift of its activation parameters. (Aided by NIH grants HD00378 and DK 39731).

W-Pos104

K CHANNELS IN PULMONARY ARTERIAL SMOOTH MUSCLE: MOLECULAR CHARACTERISATION AND OXYGEN SENSING. ((J.L. Turner, A. S. Chang*, A.M. Brown* & R.Z. Kozlowski)) University Department of Pharmacology, Mansfield Road, Oxford, OX1 3QT and *Rammelkamp Centre for Research, MetroHealth Medical Centre, Cleveland, Ohio, 44109. (Spon. by A.F. Brading).

The identity of the K channels involved in sensing hypoxia by pulmonary arterial smooth muscle is controversial. Our experiments have revealed an inwardly rectifying K current (n=3), a voltage- and Ca^{2+}-activated K current sensitive to TEA (10mM; n=3) and a voltage-activated delayed outward K current. This current (I_{out}) appears to consist of a rapidly inactivating component, more sensitive to 4-AP (1mM; n=3) than TEA (1mM; n=4), and a slowly inactivating component sensitive to both TEA (1mM; n=4) and 4-AP (1mM; n=3). Exposure of cells to hypoxia (~20-30mmHg), under conditions which would be expected to preclude activation of K_{Ca} channels, produced a significantly different ($p<0.05$) reduction in the rapidly (15.5±3.2%; n=9) and slowly (25.8±3.2%; n=9) inactivating components of I_{out}. These results prompt the question "which K channel type is affected by hypoxia?". We have begun to address this question using RT-PCR. Specific primers based upon the conserved nucleotide sequences unique to the K_V and inward rectifier gene families were designed. 20-30 PCR products for each gene family were analysed. The majority of sequences attained represented the rat-homologue of IRK-1. For the K_V family all of the sequences represented rat K_V 1.5. In view of the inactivation kinetics of K_V 1.5, our results suggest that it may be the structural basis for the observed, hypoxia sensitive, slowly inactivating component of I_{out}.

W-Pos105

COMPONENTS OF OUTWARD CURRENT IN RAT VENTRICULAR MYOCYTES. ((H.M. Himmel, E. Wettwer, Qi Li, and U. Ravens)) Institut für Pharmakologie, Universität Essen, Germany.

Recent studies demonstrated the presence of 4 depolarization-activated K^+-channels in rat ventricle on mRNA- and protein-level. However, only 2 components of outward current could be discriminated so far, i.e. a transient and a sustained current (I_{to}, I_{sus}). We searched for functional correlates of the 4 cloned channels in rat ventricular cardiomyocytes with whole-cell voltage-clamp technique. Analysis of steady-state inactivation curves (range -140 to +20 mV, 500 ms steps to +60 mV) for I_{peak} and I_{late} (at 500 ms) yielded 3 components each, 2 inactivating (a, b) and a non-inactivating one (c). Boltzmann curve fitting resulted in:

n=54	a (%)	V.5a (mV)	ka (mV)	b (%)	V.5b (mV)	kb (mV)	c (%)
I peak	23±1	-95±1	-10±0.4	51±2	-40±1	-5±0.2	28±1
I late	26±1	-93±1	-9±0.3	9±1	-30±1	-5±0.3	29±1

While relative amplitude, $V_{.5}$ and k of the a- and c-components were identical for I_{peak} and I_{late}, amplitude and $V_{.5}$ differed significantly for the b-components ($p<0.001$), suggesting that b-I_{peak} represents I_{to} whereas a-, b- and c-I_{late} are 3 different sustained currents. This was further supported by their differential sensitivity to pharmacological agents (TEA, 4-AP, clofilium, quinidine, dendrotoxin). The 3 I_{late}-components did not show a marked transmural gradient. In conclusion, we have presented evidence that in addition to I_{to}, 3 different sustained current components contribute to outward current in rat ventricular myocytes.

W-Pos106

KINETIC ANALYSIS OF THE TRANSIENT K^+ OUTWARD CURRENT INACTIVATION IN HUMAN VENTRICULAR MYOCYTES ((R. Koopmann[1], E. Wettwer[2], G.J. Amos[2], U. Ravens[2] and K. Benndorf[1])) [1]Institut für Vegetative Physiologie, Universität zu Köln, 50924 Köln, Germany. [2]Institut für Pharmakologie, Medizinische Einrichtungen der Universität-GH Essen, 45122 Essen, Germany.

A model to describe inactivation kinetics of the transient outward current (I_{to}) in human ventricular myocytes is discussed. Most of the experimental data used were published previously (Wettwer et. al., Circulation Research 75, 1994). The main properties of inactivation are: (1.) Steady state inactivation was characterized by a half maximium value and a slope parameter of -32±4 mV and 3.9±0.3 mV, respectively, when fitted with a Boltzmann distribution. (2.) At voltages of great steady state activation (+10 mV to +60 mV) inactivation kinetics is voltage independent. (3.) Kinetics of the recovery from inactivation is steeply voltage dependent. During the recovery, I_{to} in most of the cells exceeded the control current in a voltage dependent manner (up to 22% at -60 mV, up to 40% at -100 mV).
A Markovian state model is derived which fits all data with a single set of equations. In this model, inactivation is voltage independent and partially coupled to activation. The inactivated channel needs less energy for uncoupling of activation and inactivation than for recovery from inactivation. The overshoot during the recovery is modeled by introducing a 'reservoir' state, in which the channel cannot activate, to the available system in which the channel can activate.

W-Pos107

Potassium Currents of Human Atrium Kai S. Lee, Esther W. Lee, Lucy R. Sun Carol A. Simmons. Cardiovascular Pharmacology, Upjohn Laboratories, Kalamazoo, MI 49007. (spon. by KC Chou)

The pharmacology of potassium channels in native human heart cells have not been well characterized. We isolated human atrial cells by modifying a method developed previously (Lee et al, *Nature* 278:269-271,1979). Cells were internally perfused with K^+ glutamate using the suction pipette method (Lee et al, *Nature* 265:751-753,1977), and externally, with Tyrode's solution containing 2 or 0.2 mM Ca^{2+} at 37 C. Negative voltage steps lasting 150 ms from holding potential of -40 mV elicited a hyperpolarizing activated inward rectifying current, I_{K1}, measuring about 331±83 pA at -100 mV. Positive to -30 mV, an outward rectifying, time-independent, fast activating current appeared and reached to 991±4 pA at 50 mV. This current displayed properties similar to the background K^+ current, I_{K_2}. Positive to 0 mV, a large, transient outward current, I_{to}, appeared and reached to 1720±43 pA at 50 mV. I_{to} was mostly removed upon removal of external Ca^{2+} from 2 mM to 0.2 mM or on application of 1 μM nifedipine. No I_{Kr} was detected by the 150 ms pulse at 37° C. The human K^+ currents were stable without "run-down" in 30 minutes of recording. Ibutilide, a clinically potent class III antiarrhythmic drug, at 10^{-8} to 10^{-6}M concentrations, had no significant inhibitory effect on these human K^+ currents. We conclude that the freshly isolated human atrial cell provides a stable, clinically relevant preparation for the characterization of native human K^+ channels and for the study of therapeutic drugs.

W-Pos108

ZERO $[K^+]_o$ REDUCES TRANSIENT OUTWARD CURRENT I_{to} IN RAT AND HUMAN VENTRICULAR MYOCYTES. ((E. Wettwer, H. Terlau*, I. Trebeß, H.M. Himmel and U. Ravens)) Inst. für Pharmakologie, Universität Essen, Germany. *Max-Planck Inst. für Experimentelle Medizin, Göttingen, Germany. (Spon. G. Boheim),

The availability of *shaker* K^+ -channels and their rate of C-type inactivation are regulated by external K^+ depending on a single amino acid in the pore region (López-Barneo et al., *Receptors and Channels* 1: 61, 1993). At 0 $[K^+]_o$ outward currents decrease despite an increased driving force. Current reduction in 0 $[K^+]_o$ has also been described for the native cardiac potassium currents I_{Kr} and I_{to}. We have investigated the effect of 0 $[K^+]_o$ on I_{to} of rat and human ventricular cardiomyocytes with conventional whole-cell voltage clamp technique. Outward currents were elicited with voltage steps from -80 to +40 mV (300 ms). In 0 $[K^+]_o$ the transient current component I_{to} (difference I_{peak}-I_{late}) was reduced to 26 ± 4 % (n=12) in rat and to 74 ± 6 % (n=3) in human cells. The apparent K_D of the effect was 0.1 mM in rat. After return to 5.4 $[K^+]_o$ I_{to} recovered to 89 ± 9 % in rat. The I/V-relation showed continous reduction between -30 and +60 mV. Steady-state inactivation in 0 $[K^+]_o$ was shifted by 10 mV to negative potentials. Current reduction in 0 $[K^+]_o$ was also observed upon additional withdrawal of $[Na^+]_o$. It is concluded that I_{to} in the two species is modulated by extracellular K^+ in a similar way but with different sensitivities. The experiments may help to understand structure and regulation of native channels.

W-Pos109

A PUTATIVE K CHANNEL (K_{ATP}) REGULATOR OF G1 PROGRESSION IN MCF-7 HUMAN BREAST CANCER CELLS. ((E. Klimatcheva and W.F. Wonderlin)) Dept. Pharm. & Tox., West Virginia Univ., Morgantown, WV 26506

We recently reported that tetraethylammonium (TEA), 4-aminopyridine (4-AP), quinidine, glibenclamide and linogliride all inhibit the proliferation of MCF-7 human breast cancer cells, but only quinidine, glibenclamide and linogliride, and not TEA or 4-AP, arrest the cell cycle in early G1 (*J. Cell. Phys., 162:163*). We surmised from this differential drug sensitivity that the activity of ATP-sensitive K channels (K_{ATP}) might be required for passage through early G1, and our goal now is to identify the current(s) inhibited by these drugs. Whole-cell patch recordings from MCF-7 cells revealed three primary components of the I/V relations (Table). An outwardly-rectifying current, activated at potentials positive to -20mV, was insensitive to internal ATP and external quinidine and linogliride. Two linear currents, distinguished by their reversal potentials, were both completely blocked by internal ATP and partially blocked by quinidine and linogliride; however, the depolarized linear current was also blocked by TEA. Only the negative linear current displayed the appropriate drug sensitivity to be the target of drugs that selectively arrest in G1. Single-channel recordings revealed that the negative linear current was due to a small conductance (8.5 ±0.2pS) ATP- and quinidine-sensitive K channel. The i/V was linear with an extrapolated null Vm at -86 ±4mV, near E_K (-84mV). The gating was voltage independent with long openings. The activity of this channel might be required for passage through early G1 phase. (supported by the CAMC Foundation L. Newton & Katharine S. Thomas Endowment Fund)

Current:	Linear-	Linear+	Out. Rect.
Reversal Vm	-57mV	-7mV	?
90 μM Quin	↓	↓	no effect
10mM TEA	no effect	↓↓	↓↓
770μM Lino	↓	↓	no effect
2mM ATP	↓↓	↓↓	no effect

W-Pos110

INVOLVEMENT OF α_1-ADRENOCEPTORS AND PROTEIN KINASE C IN THE INHIBITION OF THE CARDIAC INWARD RECTIFIER POTASSIUM CURRENT BY VOLATILE ANESTHETICS. ((A. Stadnicka, Z.J. Bosnjak, W.M. Kwok)) Department of Anesthesiology, Medical College of Wisconsin, Milwaukee, WI 53226.

Cellular mechanisms by which volatile anesthetics Sevoflurane, Isoflurane, and Halothane inhibit the cardiac inward rectifier potassium current (I_{KIR}) are not known. We used the whole-cell patch-clamp technique to examine the contributions of α_1-adrenoceptors (α_1AR) and protein kinase C (PKC) to the anesthetic-induced inhibition of I_{KIR} in ventricular myocytes isolated enzymatically from guinea pig hearts. All experiments (n=3 to 5 in each group) were carried out in the presence of Propranolol (1 μM) to exclude β-adrenergic input. Sevoflurane (0.89 mM), Isoflurane (0.92 mM) and Halothane (0.94 mM) decreased I_{KIR} monitored at -110 mV from a -40 mV holding potential by 9.3±1.6%, 9.9±1.9% and 8.5±2.3%, respectively. Methoxamine (1 mM), an α_1AR agonist, decreased I_{KIR} by 42±2.1%. Methoxamine in combination with either Sevoflurane or Isoflurane further decreased I_{KIR}, but the effects appeared to be additive. However, Methoxamine potentiated the inhibitory effects of Halothane, decreasing I_{KIR} by 65.6±8.4%. Prazosin (0.1 mM), an α_1AR antagonist, had no effect on the inhibitory action of Sevoflurane, but augmented (approximately 50%) the effects of Isoflurane. In contrast, Prazosin prevented the inhibition of I_{KIR} by Halothane. A selective inhibitor of PKC, bisindolylmaleimide (BIM, 100 nM) had no significant effect on I_{KIR} under control conditions, and did not affect the inhibition of I_{KIR} by either Sevoflurane or Isoflurane. However, in the sustained presence of BIM, the inhibition of I_{KIR} by Halothane was completely removed. These results suggest differential role of α_1AR in the mechanism of volatile anesthetic action on cardiac I_{KIR}, and indicate an involvement of PKC mediated pathway in the inhibition of I_{KIR} by Halothane.

W-Pos111

CAN CYTOSKELETON ACTIN STABILIZATION AND TYROSINE KINASE INHIBITION AFFECT CARDIAC K_{ATP} CHANNELS RUN-DOWN AND REACTIVATION BY MgATP IN INSIDE-OUT MEMBRANE FRAGMENTS?

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ATP hydrolysis is important for reactivation of run-down cardiac K_{ATP} channels by MgATP but serine/threonine protein kinases are not necessary for this process (Furukawa et al., J. Physiol. 479: 95-107, 1994). We tested an involvement of membrane-bound tyrosine kinase and cytoskeleton actin assembly, which may modulate ionic channels activity and utilize ATP hydrolysis energy, in maintaining K_{ATP} channels in operational state. K_{ATP} channel currents were recorded in inside-out membrane fragments from rat ventricular myocytes at 23-24 and 34-36 °C using patch-clamp technique. In every experiment channel activity, which had appeared on excising the patch in ATP-free "intracellular" solution, declined spontaneously with a time constant of 0.5-8.5 min. Application of phalloidin (5-10 μM), an actin filament stabilizer, slowed down this spontaneous run-down (4-8-fold increase of the time constant in 8 out of 11 patches) and maintained activity of K_{ATP} channels reactivated by 2 mM MgATP (in 2 out of 3 patches) due to prevention of bursts shortening and interbursts intervals prolongation without alteration of intraburst kinetics; however, it could not restore the activity of completely run-down channels. Genistein (1-10 μM), a tyrosine kinase inhibitor, added to the "intracellular" solution did not block the ability of 2 mM MgATP to reactivate K_{ATP} channels after spontaneous (5 patches) and 1 mM Ca^{2+}-induced (2 patches) run-down. These results suggest that cytoskeleton actin stability is favourable for maintaining cardiac K_{ATP} channels in operational state and that membrane-bound tyrosine kinase is not necessary for reactivation of these channels by MgATP.

Supported by MSER and RFFI grants 94-04-11876 and 95-04-12056.

W-Pos112

EFFECTS OF ISCHEMIA AND POST-ISCHEMIC REPERFUSION ON ATP SENSITIVE K^+ CHANNELS AND Ca^{2+}ACTIVATED K^+ CHANNELS OF RAT SKELETAL MUSCLE FIBERS.

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We have recently shown that reperfusion increases resting Cl^- and K^+ conductances and depresses the excitability of rat skeletal muscle fibers suggesting that alterations of ion channels occur during reperfusion. In the present work, the K^+ channels present on the surface membrane of ischemic and ischemic-reperfused rat skeletal muscle fibers were surveyed by patch clamp technique. A model of ischemia and reperfusion has been performed in rat skeletal muscle as described in Tricarico and Conte Camerino (*Pflugers Archiv.* **426**:44-50, 1994). Single fibers were obtained by enzymatic treatment of extensor digitorum longus muscle from unoperated controls, contralateral controls, and muscles that underwent 4 h of ischemia or 4 h of ischemia followed by 1 h of reperfusion. Single channel recordings were performed in inside-out configuration, at 20 °C, at constant voltage or during voltage steps (Tricarico and Conte Camerino, *Mol. Pharmacol.* **46**:754-761, 1994). The most frequent K^+ channel present on the ischemic, contralateral control and unoperated control muscles was the ATP sensitive K^+ channel (K_{ATP}) with a single channel conductance (γ) of 61 pS. No changes have been observed after ischemia in the open probability nor in the number of active channels/area (*N*); however, ischemia reduced the sensitivity of K_{ATP} channels to ATP and glybenclamide. In contrast, the most frequent K^+ channel present on the reperfused fibers was the Ca^{2+} activated K^+ channel ($K_{Ca}2^+$) with a γ of 268 pS. Reperfusion led to a 3 fold increase of *N* without altering γ. The abnormal abundance of $K_{Ca}2^+$ channels and the decline of K_{ATP} channel activity observed in the reperfused muscles can be related to the high cytosolic Ca^{2+} content and to the increase of the oxidative stress occurring during reperfusion. Supported by Italian M.U.R.S.T.(40%) 93-94.

W-Pos113

CHARACTERIZATION OF AN INWARDLY RECTIFYING K^+ CURRENT IN RAT CORTICOTROPES AND ITS REGULATION BY CORTICOTROPIN RELEASING HORMONE.

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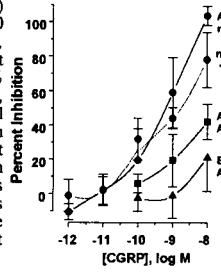
We have previously shown that stimulation of corticotropes with corticotropin releasing hormone (CRH) induces membrane depolarization and firing of Ca^{2+}-dependent action potentials. In this work on highly enriched cultured rat corticotropes, we used the amphotericin perforated patch technique to study an inwardly rectifying K^+ current (K_{ir}) and its possible role in the CRH-induced depolarization. The bathing solution contained (in mM): 150 NaCl, 5 KCl, 0.05 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose, 30 sucrose, 0.05 $LaCl_3$, 0.001 TTX, pH 7.4 (36°C). Large inward currents were recorded negative to E_K, whereas outward currents were small. The inward current appeared instantaneously and exhibited little time-dependent inactivation. The maximum conductance of the inward current increased proportionally to the square-root of $[K^+]_o$. The inwardly rectifying current was absent when $[K^+]_o$=0 and was not affected by replacement of external Na^+ with N-methyl-D-glucamine$^+$. A positive shift in the zero current potential with increasing $[K^+]_o$ was well described by the Goldman Equation with P_{Na}/P_K=0.068, indicating that the inwardly rectifying current was largely K^+ selective. K_{ir} was blocked in a voltage-dependent manner with external Cs^+ (K_d: 35 μM, 192 μM and 1245 μM at -120 mV, -90 mV and -60 mV, respectively; n=3) and a voltage-independent manner with Ba^{2+} (K_d=5.9 μM; n=4). The current was slightly attenuated (14.9±3.7%; n=7) by 5 mM 4-aminopyridine and was insensitive to 5 mM TEA (n=4). Addition of 20 nM CRH inhibited a K^+ selective current that was active at the cell resting potential in physiological saline. In 50 mM $[K^+]_o$, CRH reduced K_{ir} by 23.3±2.1% (n=5). These results suggest that CRH inhibition of K_{ir} is responsible for the CRH induced depolarization. Supported by NIH grants DK44363 and DK39553.

W-Pos114

RENAL MICROVASCULAR ACTIONS OF CALCITONIN GENE RELATED PEPTIDE (CGRP).

((Martina Reslerova, Rodger Loutzenhiser)), Smooth Muscle Research Group, Department of Pharmacology and Therapeutics, University of Calgary, Calgary, Alberta, Canada, T2N 4N1.

The effects of CGRP on the responsiveness of renal afferent (AA) and efferent (EA) arterioles to angiotensin II (Ang II) and on the myogenic reactivity of the AA to elevated pressure were investigated using the *in vitro* perfused hydronephrotic rat kidney model (Circ Res 74:861-869, 1994). Ang II (0.1 nM) reduced AA diameters from 16.7±0.7 to 6.8±0.6 μm and EA diameters from 12.4±1.4 to 5.1±0.6 μm. At 10 nM, CGRP inhibited the AA and EA responses to Ang II by only 42±10% and 21±20%, respectively (figure). In contrast to Ang II-induced vasoconstriction, pressure-induced vasoconstriction was completely abolished by 10 nM CGRP. Thus, raising perfusion pressure from 80 mm Hg to 180 mm Hg reduced AA diameters from 15.9±0.9 to 7.2±1.1 μm in controls (p=0.002) but did not alter diameters in the presence of 10 nM CGRP (16.4±0.6 and 16.6±0.5 μm, respectively). This action of CGRP was not prevented by 10 μM glibenclamide (103±5% versus 78±16% inhibition, p=0.21, GLIB, figure). Our findings suggest that the renal vasodilatory actions of CGRP are dependent on the underlying determinants of basal tone, in that myogenic reactivity is more sensitive than agonist-induced vasoconstriction. Our findings also indicate that the renal microvascular actions of CGRP do not depend on ATP-sensitive potassium channels, in that GLIB did not prevent CGRP-induced vasodilation.



W-Pos115

CA2+ AND VOLTAGE-DEPENDENCE OF K+ CHANNELS IN THE CRYPT BASOLATERAL MEMBRANE.

((Walters, R.J., Sepulveda, F.V.)) BBSRC Babraham Institute, Cambridge, U.K..

A basolateral K+ conductance modulated by carbachol dominates the resting membrane potential of small intestinal crypts. Single-channel recordings were obtained from the crypt basolateral membrane to identify the underlying ion channels. In cell-attached patches with 145mM KCl in the pipette an inwardly-rectifying K+ channel activity with a slope conductance of 32-34 pS shows time-dependent inactivation at depolarized potentials and is carbachol insensitive. Upon patch excision the inward rectifier shows no voltage-dependent inactivation and is Ca2+-dependent. A second single-channel activity is activated by carbachol at both spontaneous and hyperpolarized holding potentials. In excised patches this channel has a unitary conductance of 18-20 pS and is both Ca2+- and voltage-dependent. Ion substitution experiments reveal that this channel is cation non-selective with a PNa/Pk of 1.7. Pipette solutions containing 145mM NaGluconate were used to drive EK to more negative values and ENa and ECl to more positive values. In cell-attached patches both membrane depolarization and carbachol activate a K+ channel with a conductance of 2-4pS when fitted to the GHK equation. Although this K+ channel activity disappears upon patch excision, replacement of 'cytosolic' Na+ with K+ evokes a reversible increase in outward current across the membrane patch. This membrane-patch current exhibits time-dependent activation at depolarized potentials, is insensitive to changes in cytosolic Ca2+ and most strikingly its PK/PNa ratio decreases from 18 to 3 as K+ is increased from 10 to 145mM by equimolar substitution of K+ for Na+. The properties of this membrane patch current suggest that a high density of proteins of low unitary conductance are present in the crypt basolateral membrane. Funded by the CFRT, U.K..

W-Pos116

MOLECULAR LOCALIZATION OF THE BINDING SITE FOR QUINIDINE IN A CARDIAC K CHANNEL.

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We have previously shown that quinidine blocks the cloned hKv1.5 channel in micromolar concentrations (EC_{50}=6.2 μM) by acting as an open channel blocker and that binding is probably stabilized by hydrophobic interactions. The equivalent electrical binding distance δ=0.19 suggested a binding site in the internal mouth of the pore. To test these hypotheses we used site-directed mutagenesis of hKv1.5 followed by expression in L-cells. Because the highly charged cytoplasmic segment immediately distal to the end of S6 could fold back in the pore, we examined the charge neutralizations E524Q+D526N, E528Q+E529Q and H522G. Each yielded functional channels with wild type characteristics without significant effects on the affinity for quinidine. Deletion Δ57 (C-terminal 57 amino acids) increased the affinity 2-fold, due to a 2-fold increase of the association rate constant. No functional expression was obtained with mutations to eliminate aromatic residues in S6 (Y521N and F517V). Mutation T477S (=T441S in *Shaker*) resulted in a 2.5-fold reduction of the affinity (EC_{50}=15 μM). Mutations in the mid-section of S6 (T505I, T505S, V512A) increased the affinity to 0.7, 3.9 and 1.3 μM, respectively, without changing the binding distance. Several mutations in the S4S5 linker had minimal effects on quinidine binding. Taken together, the results are consistent with a binding site for this clinically used drug in the internal mouth of the pore. The increased affinity of Δ57 suggests an enhanced diffusional access due to deletion of about 2/3 of the C-terminal moiety, while the ~10-fold increase in affinity with T505I is consistent with hydrophobic stabilization of binding. Supported by HL47599 and HL46681.

W-Pos117

ACTIVATION AND INACTIVATION KINETICS OF AN E-4031 SENSITIVE CURRENT (I_{KR}) FROM SINGLE FERRET ATRIAL MYOCYTES.

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Ferret atrial myocytes can display an E-4031 (Esai, Japan) sensitive current I_{Kr} which is similar to that previously described for guinea pig cardiac myocytes. We examined the ferret atrial I_{Kr} as the E-4031 sensitive component of current using the amphotericin B perforated patch clamp technique. Steady state I_{Kr} during depolarizing pulses showed characteristic inward rectification. Activation time constants during a single pulse were voltage dependent, consistent with previous studies. However, for potentials positive to +30 mV I_{Kr} time course became complex and included a brief transient component. We examined the envelope of tails of the drug sensitive current for activation in the range 0 to +50 mV and found that the tail currents for I_{Kr} do not activate with the same time course as the current during the depolarizing pulse. The activation time course determined from tail currents was voltage insensitive over the range +30 to +50 mV (n=5) but was voltage sensitive for potentials between 0 to +30 mV and appeared to show some sigmoidicity in this range. These data indicate that activation of I_{Kr} occurs in at least two steps, one voltage sensitive and one voltage insensitive, the latter of which becomes rate limiting at positive potentials. We also examined the rapid time dependent inactivation process which mediates rectification at positive potentials and found that it showed a $V_{1/2}$ of -16 mV and a slope factor of 21.5 mV. The time constants for this process were only weakly voltage dependent changing from 23 to 11 ms for potentials of -10 to +50 mV, respectively. From these data we constructed a linear 4 state model which reproduces the general features of ferret I_{Kr} including the initial transient at positive potentials and the apparent discrepancy between the currents during the initial depolarizing pulse and the tail current.

W-Pos118

Ibutilide Is A Poor I_{Kr} Blocker Compared to Dofetilide in Guinea-pig Atrial Cells.

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Ibutilide and dofetilide are potent class III antiarrhythmics in clinical development. Their activities on native atrial K^+ channels are unknown. We compared the K^+ channel effects of these compounds on freshly isolated guinea-pig atrial cells using standard techniques (Lee et al, *Nature* 265:751-753, 1997, *Nature* 278:269-271,1979). Pipette solution contained K-glutamate, and the bathing Tyrode's solution contained 5 μM nifedipine. At 37°C and with cell held at -40 mV, voltage steps of 150 ms negative to -70 mV elicited I_{K1}. Neither ibutilide nor dofetilide had significant effect on I_{K1}. Positive to -30 mV, two types of outward current were elicited. One resembled the background K^+ current, $I_{K,p}$, based on its rapid kinetics and time-independence. Ibutilide, but not dofetilide, at 10^{-6} and 10^{-7}M, increased $I_{K,p}$, from 102±21 pA to 141±22, and to 153±24 pA respectively at 20 mV. The other current was I_{Kr} that displayed a mild inward rectification above 0 mV and a time-dependent tail current. Ibutilide blocked I_{Kr} concentration dependently, but only at high concentrations of 10^{-7}M and above. Dofetilide, in contrast, blocked I_{Kr} at a much lower concentration of 10^{-9}M. This study clearly differentiates ibutilide from dofetilide as being a poor I_{Kr} blocker, and further affirms that I_{Kr} can not contribute to ibutilide's APD prolongation below 10^{-7}M, especially considering ibutilide also activates $I_{K,p}$ in this concentration range.

W-Pos119

INHIBITION OF THE SLOW CARDIAC DELAYED RECTIFIER K^+ CURRENT BY HALOTHANE IS MEDIATED BY A SECOND-MESSENGER SYSTEM.

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We have investigated the effects of halothane on the slow cardiac delayed rectifier K^+ current (I_{KS}) to characterize the underlying cellular mechanism of volatile anesthetic action on cardiac ion channels. In guinea pig ventricular myocytes, under whole-cell patch clamp conditons, halothane at 0.5 and 0.8 mM reversibly decreased I_{KS} measured at the end of a 2 second test pulse to +90 mV by 53.1±5.5% (n=5) and 51.9±9.3% (n=4), respectively (holding potential=-40 mV). At both concentrations, block of I_{KS} was voltage-independent. There were no significant differences in the degree of block measured in the voltage range of +10 to +90 mV. Furthermore, halothane did not induce shifts in the activation curve. However, halothane altered the time constants of I_{KS} activation. In the presence of 0.8 mM halothane, the slow time constant, τ_s, of I_{KS} activation significantly decreased from 926±17 to 606±70 msec during depolarizing test pulses to +90 mV. The fast time constant, τ_f, was unaffected. Similar results were obtained for 0.5 mM halothane. Analysis of tail currents revealed a significant change in the time constant of deactivation, τ_d. In 0.8 mM halothane, on return to -40 mV from +90 mV, τ_d decreased from 617±54 to 396±29 msec. Under excised, outside-out patch configuration in the absence of second messenger systems, halothane at 0.5 mM had no effect on I_{KS} (n=5). In contrast, in cell-attached patches, halothane blocked I_{KS}. Under whole-cell conditions, the inhibition of I_{KS} by halothane was partially reversed by dibutyryl cAMP, a membrane permeable analogue of cAMP. These results indicate that halothane inhibits I_{KS} via a second-messenger system rather than by direct interaction with the channel protein.

W-Pos120

REVERSE USE-DEPENDENT BLOCK OF THE DELAYED RECTIFIER K^+ CURRENT (I_K) BY NIBENTAN.

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Administration of nibentan (N), a novel selective class III antiarrhythmic agent, prevented experimental ventricular arrhythmias associated with coronary artery ligation and reperfusion in dog as well as those induced by endocardial pacing in patients, and has been accompanied by lengthening of cardiac repolarization. To study the mechanisms of the N effects, the experiments were performed on isolated rat ventricular myocytes using the whole cell patch-clamp technique. In the cells, 7.5 μM N decreased I_K within 10 min by 42.9±7.0% (300-ms voltage steps from -70 mV to +50 mV with 20-ms prestep to -40 mV inactivating a fast Na current). N (2.5 to 25 μM) inhibited I_K in a concentration-dependent manner. Inhibition of I_K showed reverse use-dependence, and was greater at 0.1 than 1 Hz (Fig). In contrast, N was without significant effects on the transient outward current and on inward rectifier current. Selective block of I_K gives an explanation for the effects of N on cardiac repolarization. These effects were observed at clinically relevant concentrations reached after intravenous administration of the drug.

W-Pos121

4-AMINOPYRIDINE BINDING PREVENTS GATING CHARGE IMMOBILIZATION AND C-TYPE INACTIVATION IN THE CLONED HUMAN POTASSIUM CHANNEL KV1.5.

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4-Aminopyridine (4-AP) effects on activation and inactivation of cloned human Kv1.5 delayed rectifier channels were studied in a human embryonic kidney cell line. Ion fluxes through K^+ channels were prevented by replacing permeant monovalent cations with N-methyl-D-glucamine. Measurement of "on" gating currents (I_g) in non-conducting channels showed that 4-AP selectively immobilized a slow component of charge movement during depolarization. On repolarization, 4-AP prevented immobilization of "off" gating charge in a dose-dependent fashion. At potentials between +20 and +80 mV 4-AP increased the ratio of the integral of off-I_g/on-I_g and accelerated the time constant for recovery from the immobilized state. In conducting channels, prevented the development of slow inactivation whereas intracellular TEA induced rapid open channel block, but did not affect C-type inactivation. This suggests that prevention of C-type inactivation by 4-AP was not due to interaction at a site near the inner vestibule of the pore. 4-AP prevention of inactivation and charge immobilization resulted in a transient increase of current during the very first stages of depolarization. This increase was overcome during maintained depolarization by 4-AP block of open channels. Gating current measurements showed unequivocally for the first time, that at higher concentrations 4-AP can become trapped in deactivating channels, thus allowing for "post-repolarization" block of ionic current. The data demonstrate that C-type inactivation in human delayed rectifier K^+ channels is tightly coupled to channel activation and that removal of inactivation subsequent to 4-AP binding results in a transient increase in outward K^+ current. Rapid entry of Kv1.5 channels into the inactivated state may act as a safety factor to prevent premature repolarization during the cardiac action potential. Supported by MRC Canada and HSFO.

W-Pos122

DIHYDROPYRIDINE ACTION ON SHAKER POTASSIUM CHANNELS.

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It was shown recently that dihydropyridines (DHPs), which were primarily known as voltage-dependent Ca^{2+} channel agonists and antagonists, can also affect voltage-dependent K^+ channels. We examined effects induced by dihydropyridines on *Shaker* potassium channels expressed in *Xenopus* oocytes using the patch-clamp method.

We have tested nifedipine, nicardipine, nimodipine, and BayK8644. All of these induced a time-dependent reduction of the K^+ currents through a mutant Shaker channel with disrupted N- and C-type inactivation (ShBΔ6-46 T449V) at concentrations from 10 to 300 μM. When applied from the intracellular side, the onset of the current reduction was very rapid and the effect was readily reversible. Application from extracellular side yields much slower and less reversible action. The time course of the current decline induced by the DHPs was approximated by a single exponential. The single channel analysis showed that the DHPs reduced the mean open time in a concentration-dependent manner without markedly affecting the unitary conductance.

The efficacy of the DHPs was dependent on the residue 463 located in the S6 segment. This residue is also known to influence C-type inactivation. Mutating alanine to isoleucine at this position causes the DHP efficacy to decline. Since the DHP's induced apparent inactivation, we investigated whether they compete with N-type or C-type inactivation. The results suggest that the DHPs do not compete with either inactivation and that they may work to accelerate the intrinsic inactivation rates.

(Supported in part by Klingenstein Fellowship and McKnight award to T.H.)

W-Pos123

PHARMACOLOGICAL CHARACTERIZATION OF THE VOLTAGE-GATED POTASSIUM CHANNEL Kv1.3 AND A G380H-MUTANT. ((H. Rauer, C. Hanselmann and S. Grissmer)). Department of Applied Physiology, University of Ulm, 89081 Ulm, Germany. (Spon. by M. Cahalan)

To obtain structural and functional information about voltage-gated K^+channels we characterized the pharmacological properties of the Kv1.3 channel and a mutation in the S5/S6 linker, G380H. The whole-cell mode of the patch-clamp technique was used to examine the channel properties after injection of *in vitro* transcribed cRNA in RBL cells. All measurements were done in Ringer solution (in mM: 160 NaCl, 4.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, pH 7.4) with patch-pipettes containing KF (in mM: 145 KF, 1 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 10 EGTA, pH 7.2). The G380H mutant channels inactivate ~20 times slower than the wt channels and the peak current amplitude does not decrease significantly with repetitive pulses (200 ms, 1 Hz) unlike the wt channels. For the pharmacological characterization we used verapamil, diltiazem, and nifedipine. Kv1.3 wt currents were not affected if the compounds were applied intracellularly up to 1 mM. External application of the compounds reduced the wt current in a dose-dependent manner with K_d-values of 8, 47, and 53 μM, respectively. Current through the G380H mutant channels was less sensitive to block by the drugs with K_d-values of 209, 139, and 47 μM, respectively. The correlation of the compound size (verapamil, 20 Å > diltiazem, 15 Å > nifedipine, 11 Å) and the blocking efficiency suggests that the exchange of glycine for a larger histidine at position 380 might narrow the outer vestibule of the channel thereby obstructing the access of the larger drug verapamil to its binding site deeper in the pore. This would confirm models by other investigators using peptide toxins to probe the channel (Hidalgo *et al., Science* 268:307,1995; Miller, *Neuron* 15:5,1995; Aiyar *et al., Neuron in press*). Diltiazem and nifedipine are apparently not large enough to interact with position G380. Other mutant channels are currently under investigation to determine other binding sites for the drugs also regarding their protonation state (DeCoursey, *J. Gen. Physiol.* 106:1, 1995) and get additional information about the dimensions of the outer vestibule of Kv1.3. (Supported by a grant from Pfizer Inc, CT)

W-Pos124

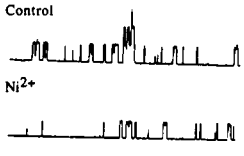
MOLECULAR DETERMINANTS FOR STEREOSELECTIVE BUPIVACAINE BINDING IN hKv1.5 CHANNELS. ((L. Franqueza, E. Delpón, P. Gay, D.J. Snyders, J. Tamargo, C. Valenzuela)). Institute of Pharmacology and Toxicology, CSIC/Univ. Complutense, 28040 Madrid, SPAIN; Vanderbilt Univ., Nashville, TN 37232, USA.

The effects of R(+)-bupivacaine (RB) and S(-)-bupivacaine (SB) on wild type hKv1.5 and two mutated channels were studied after expression in L-cells using the whole-cell configuration of the patch-clamp technique. Steady-state block of wild-type hKv1.5 channels (250 ms depolarization to +60 mV) was highly stereoselective with RB being more potent than SB (K_D=4 and 27 μM, respectively). Mutation T477S in the P-loop (internal TEA site) blunted the stereoselective difference: K_D values were 4 and 12 μM for SB and RB, respectively. Mutation T505I in the S6 segment completely abolished the stereoselectivity: the K_D value was 10 μM for both RB and SB, i.e., the affinities were affected in opposite direction. To study the energetic coupling between the ligand (B) and the amino acids at positions 477 and 505 (hKv1.5), we used the thermodynamic mutant cycle approach (Hidalgo and MacKinnon, *Science* 1995;268:307-310). In this analysis one enantiomer corresponded to "wild-type" drug (SB) and the other to "mutant" drug (RB). The cross influence of each channel mutation on the effect of each enantiomer was quantified by measuring the coupling coefficient Ω (relation between K_D values, for independent interactions Ω=1). The Ω value for the interaction between RB and T505I channels was 6.75, but only 2.25 for T477S. These results strongly suggest that threonine at position 505 is an important determinant of stereoselective bupivacaine binding to hKv1.5 channels, although an interaction at T477 cannot be ruled out. This work has been supported by Grants FIS 95/0318 and HL47599.

W-Pos125

Effects of Ni^{2+} on Ca^{2+}-Sensitive K^+ Currents in Highly Ca^{2+} Dialyzed Vascular Myocytes. Robert H. Cox and Steven Petrou, Bockus Research Institute, Graduate Hospital and Dept. of Physiology, University of Pennsylvania, Philadelphia, PA, 19146.

We previously demonstrated high activity of charybdotoxin-sensitive K^+ (K_{Ca}) currents in highly Ca^{2+} dialyzed rabbit portal vein (PV) myocytes recorded by whole cell, patch clamp methods. This study was performed to further investigate the mechanisms responsible for this high level of activity. Single myocytes were freshly dispersed from rabbit PV by collagenase and elastase treatment, and K_{Ca} currents measured using both whole cell and single channel methods. Whole cell I_K was reduced 10±3% by 0.1mM Cd^{2+} (to block I_{Ca}) and 52±9% by 5mM Ni^{2+} (to block I_{Ca} and Na:Ca exchanger). Inhibition of I_K by Ni^{2+} was associated with a shift in availability in the depolarizing direction by 12±4mV. Rapid superfusion of myocytes with a Li^+ substituted external solution (to inhibit Na:Ca exchange) had no effect on current recorded at +40mV from -60mV holding potential. High single channel K_{Ca} activity was still recorded from -40 to +40mV in both inside-out and outside-out patches exposed to 5mM EGTA on both sides of the patch. Addition of 5mM Ni^{2+} to the superfusion solution of outside-out patches decreased nP_o at 0mV (-70±18%) as well as single channel current amplitude (-11±3%). Ni^{2+} also shifted the voltage-dependence of nP_o to the right by about 8±2mV. These results suggest that PV myocytes possess high K_{Ca} activity in the absence of Ca^{2+} (i.e., Ca^{2+}-insensitive) and that Ni^{2+} inhibits $I_{K(Ca)}$ by a surface charge effect.



W-Pos126

CLASSIFICATION OF K^+CHANNEL IN HEPATOCYTE PLASMA MEMBRANE

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Recently, it has been known that various single cells possess several types of K^+channels. We have found four different types of K^+ channels in the plasma membrane of guinea-pig hepatocytes, and report properties about these K^+channels. The patch clamp technique (cell attached patch) was applied to isolated guinea-pig hepatocytes obtained by collagenase perfusion method. 150 mM KCl was used as pipette internal solution. Inward single channel currents observed at resting membrane potential were classified into the following based on their conductance and kinetics;(1) K_L: the largest conductance and fast kinetics (2)K_{MF}: moderate conductance and fast kinetics(3)K_{MS}:moderate conductance and slow kinetics (4)K_S:the smallest conductance and slow kinetics. All these channels had linear I-V relationship and showed no voltage dependency. Single channel conductances were K_L:80 pS K_{MF}:35 pS K_{MS}: 21pS K_S: 3pS respectively. Channel density of K_{MF}, K_{MS} and K_S were almost similar, but K_L had very low channel density. Application of 20 μM noradrenerine or 5 μM A23187, which increase intracellular Ca^{2+} concentration, activated K_{MF} and K_{MS} but not K_L and K_S.These observations indicate that guinea-pig hepatocytes possess at least four types of K^+channels, and K_{MF} and K_{MS} are Ca^{2+} activated K^+channels.

W-Pos127

EXTERNAL PROTONS ENHANCE THE INWARD-RECTIFYING K CHANNEL IN PLANT STOMATAL GUARD CELL PROTOPLAST. ((N. Ilan[1],A. Schwartz[1] and N. Moran[2])) [1]Dept.Bot.Fac.Agriculture,Hebrew Univ., and [2]Dept. Neurobiol., Weizmann Inst. Rehovot 76100, ISRAEL.

The plant Hyperpolarization-activated K (K_H) channels, unlike their animal couneterparts, share homologies with the K channels of the *Shaker* family, and are not blocked by intracellular Mg^{2+}. The K_H channels encounter a physiological range of extracellular pH between 7.2 and 5.1. The acidic phase coincides with an enhanced influx of K^+ into the stomatal guard cells. Using patch clamp in a whole-cell mode, we examined the effect of varying external pH (8.1-4.4) on the K_H channels conductance (G_K). Acidification from pH 5.5 to 4.4 increased G_K by 30-150%, in a voltage dependent manner. Alkalinization from 5.5 to 8.1 decreased G_K by ~15%. G_{max}, the voltage-*in*dependent maximum cell conductance, increased by ~60% between pH 5.5 and 4.4, with apparent pK_a of 5.3, most likely due to voltage-*in*dependent recruitment of voltage-responsive channels. Additionally, acidification from pH 5.5 to 4.4 shifted the voltage dependence of P_o, the open probability, by ~+25 mV. Within the framework of a CCO model, protonation causes a 25 mV increase in the electrical field sensed by the gating subunits, and a small increase in the effective charge moved during the final transition to the Open state. Using the surface-charge model, we attribute the shift of P_o to the protonation of negative surface charges near the gating subunits, with an apparent (bulk) pK_a of 4.6 and mean spacing of 30 A. The pK_a 's of the above two putative classes of protonation sites resemble those of β or γ carboxyls of glutamic or aspartic residues.

W-Pos128

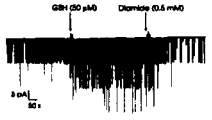
COCAINE BLOCKS THE ACETYLCHOLINE-ACTIVATED MUSCARINIC K^+ CHANNEL IN MAMMALIAN HEART CELLS. ((Y.-F. Xiao and J.P. Morgan)) The Harvard-Thorndike Laboratory and the Cardiovascular Division, Department of Medicine, Beth Israel Hospital, Harvard Medical School, Boston, MA 02215

Although cocaine cardiotoxicity, including myocardial ischemia and infarction and ventricular fibrillation, has been linked to sympathomimetic and local anesthetic effects, the mechanism(s) remains to be defined. Little is known about the effects of cocaine on cardiac muscarinic acetylcholine receptors. Therefore, in this study we assessed the effects of cocaine on the acetylcholine-activated muscarinic K^+ current ($I_{K(ACh)}$) in ferret cardiac myocytes by using the nystatin-perforated whole-cell recording technique. Extracellular application of 1 μM carbachol evoked $I_{K(ACh)}$ in both atrial and left ventricular myocytes, but the current density recorded at a holding potential of 0 mV in the atrial cells (2.9 ± 0.3 pA/pF, n = 21) was much greater than in the left ventricular myocytes (0.6 ± 0.1 pA/pF, n = 12, $p < 0.0001$). With 5 mM extracellular and 140 mM intracellular K^+ solutions $I_{K(ACh)}$ reversed its polarity at around -80 mV (n = 19). 1 μM atropine completely blocked $I_{K(ACh)}$ in both atrial and left ventricular myocytes. In atrial cells, 1 μM cocaine inhibited $I_{K(ACh)}$ by 25 ± 8% ($p < 0.05$, n = 6) and 100 μM almost completely blocked the current. The concentration of cocaine to produce 50% inhibition of $I_{K(ACh)}$ is 25 μM. Cocaine suppressed both inward and outward portions of $I_{K(ACh)}$ and did not alter the current-voltage relation. Cocaine caused similar potent suppression of $I_{K(ACh)}$ in left ventricular myocytes. It is known that the parasympathetic innervation of the heart plays an important homeostatic role with regard to the heart rate and the membrane electrical stability. The anti-muscarinic effect of cocaine may act synergistically with other actions of the drug and be an important mechanism for its arrhythmogenesis.

W-Pos129

REGULATION OF CA^{2+}-ACTIVATED K^+ CHANNEL GATING BY SULFHYDRYL REDOX AGENTS. ((Zhao-Wen Wang, and Michael I. Kotlikoff)) Department of Animal Biology, University of Pennsylvania, Philadelphia, PA 19104. (Sponsored by M.M. Civan)

The gating and inactivation of several channels have been shown to be dependent on the redox state of sulfhydryl groups of channel cysteine residues. We examined the effects of sulfhydryl redox agents on the gating of large-conductance, Ca^{2+}-activated potassium (K_{Ca}) channels. Experiments were performed on inside-out and outside-out patches from enzymatically dissociated equine tracheal myocytes. In inside-out patches, the sulfhydryl reducing agents DL-dithiothreitol, β-mercaptoethanol, and reduced glutathione (GSH) augmented K_{Ca} channel open-state probability (nP_o) in a concentration-dependent manner, whereas the sulfhydryl oxidizing agents diamide and 2,2'-dithiodipyridine exhibited the opposite effect. In the same patches, GSH (50 μM) increased nP_o from 0.022 ± 0.002 to 0.100 ± 0.020, and addition of diamide (0.5 mM) reversed the effect (0.008 ± 0.002, n = 5).



Application of the membrane-impermeant reducing agent GSH (170 μM) to bath solution significantly increased nP_o in inside-out (control = 0.048 ± 0.024, GSH = 0.556 ± 0.130, n = 4), but not outside-out (control = 0.035 ± 0.022, GSH = 0.049 ± 0.037, n = 4) patches. These results suggest that: 1) the redox state of a critical sulfhydryl group(s) on K_{Ca} channels or a closely related protein regulates channel gating; 2) reduction of the sulfhydryl group(s) markedly increases channel open-state probability at fixed calcium concentration and voltages, whereas oxidation of the group(s) has the opposite effect; 3) the sulfhydryl group(s) responsible for this regulation is accessible from the intracellular side of the membrane; and 4) K_{Ca} channels are generally in a mixed redox state under initial experimental conditions.

W-Pos130

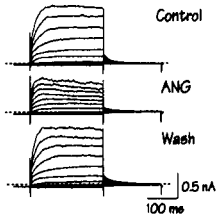
ION CHANNELS IN HUMAN AORTA ENDOTHELIAL CELLS AND HUMAN CAPILLARY ENDOTHELIAL CELLS. ((Flora Jow, Randy Numann, and Thomas Colatsky)) Wyeth-Ayerst Research, Princeton, NJ.

Endothelial cells play a critical role in modulating vascular tone through the release of vasoactive molecules such as NO, endothelin and prostacyclin. We present data characterizing the ionic currents found in human aorta (HAEC) and capillary (HCEC) endothelial cells and their response to perfusion-induced stress. Whole cell currents from HAEC and HCEC were N-shaped, consisting of a Ba sensitive inward rectifier and several outward currents. HAEC but not HCEC responded to bath perfusion with a large outward current composed of an outwardly rectifying Cl^- current and a non-selective cation current. Extracellular Ca^{2+} was required for the response to flow (n=8). Both HAEC and HCEC displayed a progressive increase in outward current (340% n= 55), due to the dialysis of an unknown intracellular component. This outward current was 25% Cl^- and 50% TEA sensitive (20mM), with an unknown residual current accounting for the rest. HAEC but not HCEC contain an IbTX sensitive outward current. These results indicate that human endothelial cells contain a rich variety of ionic currents that differ considerably from those of endothelial cells of other species. The unique ion channels that comprise these currents may play important roles in modulating human endothelial cells function.

W-Pos131

ANGIOTENSIN II ACTIVATION OF PROTEIN KINASE C DECREASES WHOLE-CELL DELAYED RECTIFIER K^+ CURRENT IN RABBIT VASCULAR MYOCYTES ((O. Clément-Chomienne, M.P. Walsh, and W.C. Cole)) Smooth Muscle Research Group, University of Calgary, Calgary, Alberta, Canada, T2N 4N1.

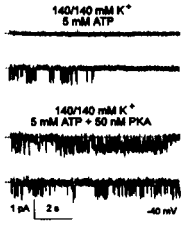
The effect of angiotensin II (ANG) on 4-aminopyridine (4AP)-sensitive delayed rectifier K^+ current (K_{dr}) in rabbit portal vein myocytes was studied by whole-cell patch clamp. Myocytes dialysed with 10 mM BAPTA and 5 mM ATP were exposed to 100 nM ANG and the effect on end-pulse and tail current amplitude during 250 ms steps from -50 mV to between -30 and +30 mV followed by a step to -40 mV was determined. At room temperature, 9 of 14 myocytes exposed to ANG showed a decline in K_{dr} which was reversed upon washout (see fig.). The reduction in K_{dr} by ANG was blocked by losartan (1 μM). Replacement of external Ca^{2+} with Mg^{2+} did not affect inhibition of K_{dr} by ANG. At 30°C, all myocytes (17 of 17) exposed to ANG showed a decrease in K_{dr}. Dialysis with calphostin C (1 μM) or chelerythrine (50 μM) prevented the inhibition of K_{dr} by ANG (30°C). PKC isoenzymes expressed in rabbit portal veins were identified by Western blotting using isoenzyme-specific antibodies. α, ζ, and ε isoenzymes were demonstrated to be present. The lack of a requirement for Ca^{2+}, and sensitivity of the ANG response to chelerythrine, suggest the involvement of the Ca^{2+}-independent PKC isoenzyme ε in the signal transduction pathway responsible for K_{dr} inhibition by ANG. (Support: MRC (WC), AHSF (MW), HSFC / AHFMR (OCC)).



W-Pos132

PROTEIN KINASE A AND ISOPROTERENOL INCREASE THE ACTIVITY OF 15 pS DELAYED RECTIFIER K^+ CHANNELS IN RABBIT VASCULAR MYOCYTES ((E.A. Aiello[1], M.P. Walsh, and W.C. Cole)) [1]Centro de Investigaciones Cardiovasculares, UNLP, LaPlata, Argentina and Smooth Muscle Research Group, University of Calgary, Calgary, AB, Canada, T2N 4N1.

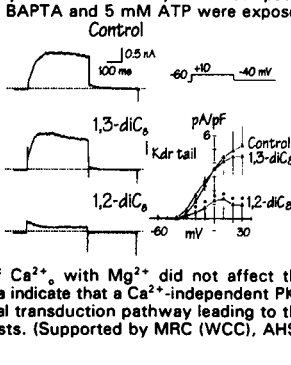
Isoproterenol (ISO) increases delayed rectifier K^+ current (K_{dr}) in rabbit portal vein myocytes by a mechanism sensitive to the peptide inhibitor of cAMP-dependent protein kinase (PKA) (AJP 268:H926,1995). The present study sought to identify the properties of the K_{dr} channel affected by ISO (1 μM) and whether constitutively active PKA (50 nM) affects the open probability (P_o) of single K_{dr} channels. Excised patch recordings were made with bath and pipette solutions containing (in mM) 140 KCl, 5 ATP, 0.5 $MgCl_2$, 10 Hepes, 5.5 glucose and 140 KCl, 1 $MgCl_2$, 5.5 glucose, 1 $CaCl_2$, 0.0001 iberiotoxin, 10 Hepes, respectively (pH 7.2). Cell-attached patches were studied with a similar pipette solution and a high K^+/Ca^{2+}-free bath solution. Single channels of ~15pS in excised and cell-attached patches displayed inactivation at positive potentials, sensitivity to 4-aminopyridine (10 mM), and rundown after patch excision. Upon exposure to PKA, P_o in excised patches increased from 0.018 ± 0.005 to 0.192 ± 0.04 (n = 6, see figure). ISO increased P_o from 0.02 ± 0.01 to 0.05 ± 0.01 (n = 5) in cell-attached patches. These results indicate that a 15 pS K_{dr} channel in vascular myocytes is up-regulated by a phosphotranserase reaction involving PKA in response to ß-adrenoceptor activation. Support: MRC (WCC) & AHSF (MPW).



W-Pos133

PROTEIN KINASE C ACTIVATION DECREASES WHOLE-CELL DELAYED RECTIFIER K^+ CURRENT IN RABBIT VASCULAR MYOCYTES ((W.C. Cole, E.A. Aiello[1], O. Clément-Chomienne, D.P. Sontag, and M.P. Walsh,)) [1]Centro de Investigaciones Cardiovasculares, UNLP, LaPlata, Argentina and Smooth Muscle Research Group, University of Calgary, Canada, T2N 4N1.

The effect of protein kinase C (PKC) activation on delayed rectifier K^+ current (K_{dr}) in rabbit portal vein myocytes was studied by whole-cell patch clamp. Myocytes dialysed with 10 mM BAPTA and 5 mM ATP were exposed to diacylglycerol (DAG) analogs or a phorbol ester, PdBu. The active DAG analog, 1,2-diC_8 (10 μM) caused an ~ 85 % inhibition of K_{dr} but was without affect on residual current following treatment with 20 mM 4-aminopyridine. The inactive DAG analog, 1,3-diC_8 (10 μM), did not affect K_{dr}. PdBu (100 nM) also caused a reduction in K_{dr}. Dialysis for 20 min with the specific inhibitors of PKC, calphostin C (1 μM) and chelerythrine (50 μM), reduced the inhibition of K_{dr} by 1,2-diC_8, supporting the involvement of PKC. Substitution of Ca^{2+}_o with Mg^{2+} did not affect the suppression of K_{dr} by 1,2-diC_8. The data indicate that a Ca^{2+}-independent PKC isoenzyme, ε, may play a role in a signal transduction pathway leading to the supression of K_{dr} by contractile agonists. (Supported by MRC (WCC), AHSF (MPW), HSFC and AHFMR (OCC)).



W-Pos134

DUAL EFFECT OF FATTY ACIDS ON THE DELAYED RECTIFIER K^+ CURRENT IN RAT PULMONARY MYOCYTES: STRUCTURAL RELATIONS AND INVOLVEMENT OF PKC. ((S.V. Smirnov and P.I. Aaronson)) Department of Pharmacology, UMDS, St Thomas's Hospital, London SE1 7EH, UK.

Externally applied arachidonic acid (AA) caused a dual effect on the delayed rectifier K^+ current (I_K) in single rat pulmonary arterial myocytes: potentiation of the initial I_K peak and simultaneous inhibition of the current at the end of the depolarizing pulse. The effect of AA was not was not associated with a shift in either the current-voltage relationship or the steady-state inactivation dependency for I_K; half-inactivation potential for I_K peak was -32±3 and -34±3 mV, and for I_K at 120 ms was -24±3 and -28±3 (n=5), for control and 10 μM AA respectively. The inhibitory effect of I_K was correlated with an increase of the number of double bonds in the fatty acid molecule (from 0 to 6, all in *cis* configuration). Changes in the length of the fatty acid (between 14 and 22 carbons) did not, however, significantly alter the inhibitory effect. Both arachidonyl alcohol, which is uncharged, and arachidonyl coenzyme A, which is suggested not to "flip" across the plasmalemmal bilayer, had much smaller (P<0.002) inhibitory effects on I_K than did AA (35±2 %, n=5, 36±7%, n=4, and 78±2%, n=7, respectively, all at 50 μM). 50 μM linolelaidic acid (18:2, *trans*) produced markedly less inhibition than did linoleic acid (18:2, *cis*) (22±2%, n=4, and 61±10 % n=5, P<0.006), respectively. The potentiation of the I_K peak was observed over a wide range of test potentials (-30 to +100 mV), but was more prominent near the current "threshold" than at very positive voltages. This potentiation measured at 0 mV, but not the subsequent inhibition of I_K, was abolished by protein kinase C inhibitors chelerythrine (10μM) and staurosporine (100 nM). These results suggest that two different mechanisms may take part in the bidirectional effect of fatty acids on I_K in rat pulmonary myocytes. The block of I_K by long chain fatty acids is enhanced by the presence of multiple double bonds, negative charge, and a *cis* configuration. The enhancement of the I_K peak appears to involve the activation of protein kinase C.

This work was supported by the BHF grant BS/95001.

W-Pos135

INWARD RECTIFICATION OF THE LARGE CONDUCTANCE POTASSIUM CHANNELS IN SMOOTH MUSCLE CELLS FROM RABBIT PULMONARY ARTERY. ((O.Osipenko*, V.A.Snetkov**, J.P.T.Ward** and A.M.Gurney*)) *Divisions of Pharmacology & **Allergy & Respiratory Medicine, UMDS, St.Thomas's Hospital, London & *Physiology & Pharmacology, University of Strathclyde, Glasgow, UK.

Large conductance Ca^{2+}-dependent K^+ channels (BK_{Ca}) were studied in smooth muscle cells enzymatically dissociated from rabbit pulmonary artery using the patch-clamp method. Current-voltage relationships for the single channel currents recorded in cell-attached patches revealed strong inward rectification, which disappeared after patch excision. Permeabilization of the membrane at the opposite end of the cell from the patch electrode, carried out using saponin, β-escin, equinatoxin, nystatin or amphotericin also removed rectification in cell-attached patches. A variety of nucleotide phosphates, as well as alkaline phosphatase, protein kinase A α-subunit and cytosolic extract from pulmonary artery smooth muscle, applied to the cytosolic side of inside-out patches, did not mimic the inward rectification seen in cell-attached patches. However Mg^{2+} ions and polyamines (spermine and spermidine but not putrescine) induced concentration- and voltage-dependent block of the BK_{Ca} channels, resulting in inward rectification. Effects of Mg^{2+} and polyamines were not additive, suggesting a similar site of action. Co-application of these agents at the highest tested concentrations was, however, unable to reproduce the degree of inward rectification that was usually observed during the cell-attached mode of recording. These results suggest the existence in the cytoplasm of the smooth muscle cell of an unidentified endogenous potassium channel blocker. (Supported by the *British Heart Foundation and the **Wellcome Trust).

W-Pos136

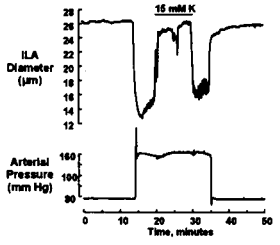
ECONAZOLE INHIBITS ENDOTHELIUM-DEPENDENT RELAXATION, IN PART, BY INHIBITING SMOOTH MUSCLE K^+CHANNELS. ((D.K. Bowles, G.M. Dick and M. Sturek)) Vascular Biology Lab, Dalton Cardiovascular Research Center and Department of Physiology, University of Missouri, Columbia, MO 65211.

Bradykinin stimulation of porcine coronary artery endothelial cells results in the release of at least three relaxing factors: nitric oxide (NO), prostacyclin (PGI_2), and endothelium-derived hyperpolarizing factor (EDHF). In the porcine coronary artery there is a NO/PGI_2-independent relaxation presumably due to EDHF, which is thought to be a cytochrome P450 metabolite of arachidonic acid. Econazole inhibits cytochrome P450 and has been used to examine the role of EDHF in relaxation. Econazole has been reported to inhibit K^+ channels in other cell types; therefore, we hypothesized that econazole attenuates the NO/PGI_2-independent relaxation by inhibiting K^+ channels in the underlying smooth muscle. Isometric tension recording techniques were used to show that the inhibition of the NO/PGI_2-resistant relaxation (75% relaxation) by econazole (49% relaxation) was mimicked by 4-AP (41% relaxation), whereas TEA was without effect (69% relaxation). Whole-cell voltage-clamp techniques demonstrated that econazole is an extremely potent inhibitor of smooth muscle K^+ currents (98%, 97% and 89% inhibition at 0, +20 and +50 mV, respectively), including that which is sensitive to 4-AP. Our data clearly indicate that econazole inhibits EDHF action; therefore, caution must be used when interpreting results obtained with this putative inhibitor of EDHF production. (Supported by NIH HL09086, HL52490, RCDA HL02872, and AHA predoctoral fellowship)

W-Pos137

POTASSIUM-INDUCED VASODILATION IN RENAL MICROVESSELS: FUNCTIONAL AND PATCH-CLAMP STUDIES. ((G.J. Waldron, K. Loutzenhiser, L. Chilton, W.C. Cole, R. Loutzenhiser)), Smooth Muscle Research Group, University of Calgary, Calgary, Alberta, Canada, T2N 4N1.

The mechanism(s) of K-induced vasodilation were investigated using the *in vitro* perfused hydronephrotic rat kidney (HK) and microvascular smooth muscle cells (VSM) isolated from single renal interlobular (ILA) and afferent arterioles. In the HK, increasing renal perfusion pressure from 80 to 160 mm Hg reduced ILA diameters from 27.1±2.5 to 17.5±3.0 μm ($p<0.001$, n=6). Elevating $[K]_o$ from 5 to 15 mM, returned diameters to 27.6±2.7 μm (figure). These vasodilatory effects of $[K]_o$ were inhibited by 10-300 μM barium. Individual ILAs were obtained from normal rat kidneys by a collagenase-digestion procedure. Single VSM from individually isolated ILAs were then dispersed directly into microscope-stage mounted chambers for patch-clamp studies. Whole-cell studies indicated single cell capacitances of 12.1±1.9 pF (n=6). Voltage ramp (-120 to +20 mV) protocols suggested an inwardly rectifying current at membrane potentials negative to -60 mV. Single channel currents were recorded from cell-attached patches under symmetrical K (140/140) conditions. At a transmembrane potential of -55 mV, three channel types were observed. These channels displayed unitary conductances of approximately 9, 15, and 60 pS. Studies are underway to characterize the properties of these channels and to determine their involvement in the renal microvascular response to elevated $[K]_o$.



CELLULAR ELECTROPHYSIOLOGY

W-Pos138

ELECTROPHYSIOLOGICAL CHARACTERIZATION OF HUMAN OSTEOBLASTS AND A HUMAN OSTEOBLAST PRECURSOR CELL LINE. ((M. Steinert, C. Hanselmann and S. Grissmer)). Department of Applied Physiology, University of Ulm, 89081 Ulm , Germany.

Mechanical stimulation by uniform and cyclic elongation has been shown to influence proliferation and differentiation of human osteoblasts (Neidlinger-Wilke *et al., J. Orthop. Res.* **12**:70, 1994). To see whether ionic channels are involved in this process, we used the whole-cell mode of the patch-clamp technique to characterize the electrophysiological properties of human osteoblasts. These cells were obtained from patients treated for bone fractures. Cells were cultured in DMEM (10 % FCS) and after the second passage used for the electrophysiological experiments by plating the cells onto cover slips. After adherence to the cover slip ion channel expression was characterized using different external and internal solutions. With normal Ringer solution in the bath (in mM: 160 NaCl, 4.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, pH 7.4) and K-Asp (in mM: 160 K-aspartate, 1 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 10 EGTA, pH 7.2) in the patch pipette we recorded inward current at potentials more negative than -80 mV. These inward currents were elicited by continuously ramping the voltage from -160 to 40 mV within 400 ms every 10 s (holding potential -80 mV). Replacing Na^+ by K^+ in the external solution resulted in inward current at potentials more negative than 0 mV indicating that human osteoblasts express inward rectifier channels as has been shown for osteoclasts from rat (Sims *et al., J. Am. Physiol.* **256**:C1277, 1989) and chicken (Ravesloot *et al., PNAS* **86**:6821, 1989). The other recorded current was a volume-sensitive Cl^- current. Swelling was induced by exposing the cells to hypotonic external solution (67 % Ringer). Replacing external Cl^- by aspartate reduced the current and shifted the reversal potential towards 0 mV. In addition, the current was reduced by the application of 100 μM SITS. This kind of volume-sensitive Cl^- current has been described in a variety of other cell types including a rat osteoblastic cell line (Gosling *et al., J. Physiol.* **485**.3:671, 1995). Further studies are planned to address the question whether these or other channel types might be involved in the response of bone cells to mechanical stress.

W-Pos139

DIFFERENTIAL EFFECTS OF PINACIDIL, STROPHANTHIDIN, AND NOREPINEPHRINE ON DELAYED RECTIFIER AND HYPERPOLARIZATION INDUCED INWARD CURRENTS IN CULTURED ATRIAL TUMOR CELLS (AT-1 CELLS). ((M. L. Bhattacharyya, S. Sarker and K.P. Mull)) Dept. of Physiology, Meharry Medical College, Nashville TN 37208.

Pinacidil (pin) a K^+ channel opener has been shown to prevent reperfusion arrhythmias and calcium overload induced arrhythmias due to strophanthidin (Na^+-K^+ ATPase inhibitor). We tested the effects of pin, (3μM),strophanthidin (stph. 10^{-6}M) and norepinephrine (NE, 10^{-6} M; which acts synergystically with stph.) on I_{kr} and hyperpolarization induced inward current (I_{hi}) in atrial tumor cells derived from transgenic mice (AT-1 cell). Overlapping inward currents were eliminated by holding the cells at -40mV and using $CdCl_2$. I-V relationship in I_{kr} type current shows that they peak at 10-20 mV. Stph., NE, and pin.blocked the peak current for a +40mV depolarization (1s pulse) by 28.2 ± 5.3%, 47.8 ± 15.3% and 21.1 ± 13.8% and the steady state current at the end of 1s pulse by 44.8 ± 16.8%, 46.2 ± 20.7% and 22.7 ± 5.1% respectively (n=8). Tail currents were not altered significantly. The I_{hi} current due to a step to -170 mV did not change in the presence of stph., or pin., but was increased in the presence of NE by 22.1 ± 0.6% (n=4). Reduction in I_{kr} by stph., is consistent with its effect on APD, while the increased I_{hi} by NE attest to its effect on increasing heart rate. Pin's effects on I_{kr} remain unclear. Supported by NIH grant 2 SO6 GMO8037-24.

W-Pos140

PRESSURE MODIFIES THE EXCITABILITY OF PYRAMIDAL NEURONES IN THE RAT HIPPOCAMPUS. ((K.T. WANN and A.P. SOUTHAN*)) Pharmacology Dept., Welsh School of Pharmacy, Redwood Building, King Edward VII Avenue, Cardiff CF1 3XF, UK and * Dept. of Biochemistry, Imperial College of Science and Technology and Medicine, London SW7 2AZ, UK.

High pressure produces hyperexcitability and convulsive episodes *in vivo*. Here we have attempted to define the cellular basis of the excitability increase induced by high pressure in CA1 pyramidal neurones in hippocampal slices. In field potential studies the orthodromic population spike was increased at 10 MPa (helium pressure) and a second spike was recruited at 5-10 MPa. The antidromic population spike was also increased at 10 MPa. Paired pulse potentiation was unaltered at pressure. Intracellular measurements showed that for much of the stay at pressure there was no significant change in the steady-state membrane potential, input resistance, threshold potential and action potential characteristics. In contrast high pressure (>5 MPa) produced episodically spontaneous transient depolarising (20 mV) swings in membrane potential accompanied by repetitive action potential firing and marked increases in excitability. In addition, pressure (5-10 MPa) also reduced the accommodation of action potential discharge and decreased, in parallel, the AHP following a single spike or train of action potentials. It is tempting to conclude that such effects may contribute to the well known convulsive effects of pressure observed *in vivo*. The actions of anaesthetics ketamine and methohexitone which ameliorate and exacerbate respectively the effects of high pressure *in vivo* were also examined at 5-10 MPa. The activity of these agents was unaffected by high pressure.

W-Pos141

SWELLING-INDUCED CHLORIDE CURRENT DEPOLARIZES DOG ATRIAL CELLS. ((X. Du and S. Sorota)) Dept. of Pharmacology, Columbia University, New York, NY 10032.

In whole cell patch clamp studies, the resting potential (RP) of dog atrial cells was stabilized near the K^+ equilibrium potential by including GTPγS in the pipette solution to activate the ACh-induced K^+ current ($I_{K\text{-}ACh}$). Positive pressure was applied to patch electrodes to inflate cells and activate the swelling-induced chloride current ($I_{Cl\text{-}swelling}$). This maneuver prevented an apparent dilution of intracellular K^+ that occurs when cells are osmotically swollen. Glybenclamide (10 μM) was present in the bath solution. Inflation induced and outwardly-rectifying current; the apparent reversal potential was -35 ± 4 mV ($[Cl^-]_{pipette}$ = 42 mM; n=7) or -47 ± 1 mV ($[Cl^-]_{pipette}$ = 17 mM; n=11). With either $[Cl^-]_{pipette}$, inflation resulted in a statistically significant ($p<0.05$, Student-Newman-Keuls test) depolarization of the resting membrane (see table). Niflumic acid (NA) (100 μM) blocked $I_{Cl\text{-}swelling}$ and evoked a significant repolarization (see table). NA partially inhibited $I_{K\text{-}ACh}$. A fast flow system was used to determine the effect of anion substitution on $I_{Cl\text{-}swelling}$. Relative permeabilities and conductance were determined under bi-ionic conditions (except for I^-). The selectivity series was determined to be I^- (2.02) > NO_3^- (1.30) ≥ Br^- (1.27) > Cl^- (1.0) > methanesulfonate (0.40) ≈ F^- (0.38). The conductance of most test anions were lower than chloride: NO_3^- (1.02) ≈ Cl^- (1.0) > Br^- (0.85) > F^- (0.63) ≥ I^- (0.58) > methanesulfonate (0.40) >> aspartate (impermeant). Preliminary ion substitution experiments revealed that I^- substitution could partially repolarize inflated cells. Results demonstrate that $I_{Cl\text{-}swelling}$ can depolarize dog atrium with physiological $[Cl^-]_{pipette}$.

$[Cl^-]_{pipette}$	Cont. RP	Inflate RP	NA RP
42 mM	-90 ± 1 mV	-69 ± 4 mV	-86 ± 1 mV
17 mM	-89 ± 1 mV	-71 ± 2 mV	-83 ± 2 mV

W-Pos142

ROLE OF L-TYPE CALCIUM CURRENT IN MAINTAINING DISCONTINUOUS CARDIAC ACTION POTENTIAL CONDUCTION. ((David Golod, Rajiv Kumar, Ronald Wilders, Habo J. Jongsma, Ronald W. Joyner Eric E. Verheijck, Antonni C.G. van Ginneken, Mary Wagner, and William N. Goolsby)) Emory University, Atlanta, GA; Utrecht University, and University of Amsterdam, The Netherlands. (Spon. by J. Pooler)

Action Potential conduction in cardiac tissue varies in different regions, and under different pathological circumstances, from being nearly continuous to being discontinuous with significant delays at localized regions. Our objective was to determine the role of L-type Calcium current (I_{Ca}) in discontinuous conduction with controlled values of coupling conductance (G_c). We used a mathematical model of a Guinea Pig (GP) Ventricular cell (Luo and Rudy, *Circ. Res.* 74:1071-1096;1994), solved in real-time on a computer with variable electrical coupling to simultaneously recorded real GP ventricular cells to investigate effects of Nifedipine (1 μM, 'NIF'), Isoproterenol (20 nM, 'ISO'), and BayK8644 (1 μM, 'BAY') on action potential conduction from the real GP cell to the LR Model cell. For each real cell, we adjusted the effective size of the cell, by scaling coupling current, to match that of the LR Model cell to produce a current threshold of 2.6 nA for a 2 mSec pulse. Conduction time varied from 1-2 mSec, with G_c = 50 nS, to 20-30 mSec at a critically low G_c which varied from 7-9 nS. NIF raised critical G_c and produced longer delays for G_c < 15 nS. ISO and BAY decreased critical G_c and shortened conduction times for G_c < 15 nS. During conduction, there was a significant partial repolarization of the real cell, which was increased by NIF and decreased by ISO and BAY. With fixed G_c (15 nS) varying LR Model cell size from 0.2 to 2.0 varied conduction time from 1-2 mSec (size 0.2) to 20-30 mSec at a critical size of 1.4 - 1.6. NIF lowered critical size of the LR Model, while ISO and BAY raised the critical size. We conclude that I_{Ca} plays a significant role in maintaining depolarization in the leader cell of a cell pair to allow coupling current to activate the follower cell. Modulation of I_{Ca}, either pharmacologically or by sympathetic tone, may significantly modulate discontinuous conduction in cardiac tissue.

W-Pos143

INTERACTIONS BETWEEN A SPONTANEOUSLY PACING CARDIAC CELL MODEL AND ISOLATED RABBIT VENTRICULAR CELLS. ((Mary B. Wagner, David Golod, Rajiv Kumar, Ronald Wilders, Habo J. Jongsma, Ronald W. Joyner, Eric E. Verheijck, Antonni C.G. van Ginneken, , and William N. Goolsby)) U. C. San Francisco; Emory University, Atlanta, GA; Utrecht University, and The University of Amsterdam, The Netherlands.

The heart contains groups of cells with spontaneous activity coupled to cells which are excitable but quiescent. To understand the role of geometrical asymmetry and coupling conductance (G_c) on this type of system, we studied pairs of coupled heart cells in which one cell was an isolated rabbit ventricular cell and the other cell was the Wilders *et al.* (Biophys.J. 60:1202-1216;1991) SA Node cell model (SAN model), using real-time computer simulation of the model cell coupled to the real cell by computed coupling currents. Using a standard size SAN model cell, there was no value of G_c that allowed the model cell to drive the real cell. For a larger model size (x 5), as G_c increased, there was a progression through three conditions: 1) pacing of the model but not driving of the real cell (PND), 2) pacing with driving (PD) and 3) the model not pacing (NP) due to the electrotonic load of the real cell. Critical G_c for transition from PND to PD varied from 5-8 nS and for transition from PD to NP varied from 13-23 nS. As G_c increased from low values the cycle length (CL) of the model cell also increased, but as G_c increased to greater than the critical value for the transition from PND to PD the CL of the model cell abruptly decreased. Continued increases in G_c produced further increases in CL of the model cell. Elevation of $[K^+]$ from 4 to 8 mM depolarized the real cell and increased critical values of G_c for the transitions from PND to PD and from PD to NP. Thus, elevated K^+ abolished driving of the real cell for lower values of G_c but created driving of the real cell for higher values of G_c. We conclude that there is a critical range of G_c for which the SAN model cell of sufficient size can successfully drive a real cell and that elevation of K^+ alters this range of G_c which may convert conditions of pacing but not driving to pacing and driving of the real cell and may convert conditions of not pacing to successful pacing and driving of the real cell, depending on the value of G_c.

W-Pos144

USE-DEPENDENT SENSITIVITY OF MICRO CARBON FIBER ELECTRODES. ((Zhuan Zhou)) Dept. of Physiology, Loyola Univ. Med. Center, Maywood, IL 60153 and Dept. of Medicine (Jewish Hospital), Washington Univ. Med. Center, St. Louis, MO 63110.

Micro carbon fiber electrode (CFE) has been used for electrochemical detection of quantal neurotransmitter release from endocrine cells, immune cells and neurons, during the last 5 years. Factors which influence the sensitivity of the polypropylene insulated CFE (ProCFE) have been studied. As measured by the half-height-duration ($t_1/2$) of individual amperometric spikes from rat chromaffin cells, the sensitivity is highest (or $t_1/2$ is smallest), if the tip of the CFE touches the cell (CTC) membrane directly. With the CTC configuration the major statistical results are: (i) Within 6 months, $t_1/2$ is not dependent on the length of time between cutting and using a CFE tip. (ii) The sensitivity of a given CFE is not dependent on the recording time, or number of recorded events. (iii) For a given a CFE, $t_1/2$ is dependent on the number of CTCs; If all CTCs are gentle, with CTC number ≤ 4, there is no apparent increase in $t_1/2$; however, after an average of 5-8 CTCs, CFE-sensitivity is reduced by half. (iv) $t_1/2$ is same when Hg, 3 M KCl or 4 M NaCl is the CFE back-filling solution. (v) $t_1/2$ is not affected by the slow background "puffer artifact". (vi) The sensitivity of a used CFE can be fully recovered after properly recutting the CFE tip.

W-Pos145

CELL-ATTACHED RECORDING OF MEMBRANE POTENTIAL OF VENTRICULAR MYOCYTES AND ENDOTHELIAL CELLS. ((R. Ochi, M. Watanabe and M. Tateyama)) Department of Physiology, Juntendo University School of Medicine, Hongo, Tokyo 113, Japan.

We evaluated the suitability of the current clamp mode of the cell-attached patch clamp technique (CA) for measurement of the membrane potential (Em) of cardiac myocytes using both the CA and whole-cell recording technique (WC), and applied this non-invasive method for estimating the Em of endothelial cells. Glass pipettes filled with high K^+, low Ca^{2+} solution with a resistance of 2-5 MΩ were used. In guinea-pig ventricular myocytes, Em measured by CA (Em_{CA}) was -70.5±6.5 mV (n=14) and Em measured by WC (Em_{WC}) was -71.5±5.3 mV (n=4) by WC at 5.4 mM $[K^+]_o$, values decreased with increasing $[K^+]_o$ between 5.4 and 140 mM with slopes of 53.4 mV/decade for Em_{CA} and 54.5 mV/decade for Em_{WC}. Em_{CA} was independent of input resistance between 1.5 and 11GΩ, indicating that patch membrane resistance is much lower than seal resistance. In cultured human aortic endothelial cells, Em_{CA} was -23.5 ±6.5 mV (n=26) in normal Tyrode , -6.3±5.9 (n=26) at 140 mM $[K^+]_o$, -12.2±6.6 (n=26) at 20 mM $[Cl^-]_o$, -44.4±12.6 (n=15) at 0 mM $[Na^+]_o$ and -47.4±13.3 mV (n=6) in the presence of 10 μM ATP. These Em_{CA} values were well fitted by the GHK equation; P_{Na}/P_K was 0.4 and P_{Cl}/P_K was 1.0 in the resting membrane, and ATP caused a 5-fold increase of P_K.

W-Pos146

DESENSITIZATION PROPERTIES OF RODENT RECOMBINANT 5HT3 RECEPTORS. ((David D. Mott and Stephen F. Traynelis)) Department of Pharmacology, Emory University, Atlanta, GA 30322.

Type 3 serotonin receptors (5HT3) are the target for several clinically useful compounds. In an effort to better understand the biophysical properties of this receptor, we have studied desensitization of rat and mouse 5HT3 receptors. Experiments were performed at room temperature (23°C) on 5HT3 receptors stably expressed in the mammalian cell line HEK 293. 5HT3 receptors desensitized markedly when exposed to a maximal concentration of either serotonin (10 μM) or the selective 5HT3 agonist, 1-(meta-chlorophenyl)-biguanide (mCPBG; 10 μM). Peak to steady state current ratios were 16.9 for serotonin and 11.8 for mCPBG (n=18). At a membrane potential of -120 mV, 5HT3 receptors typically desensitized with a single exponential time course when exposed to either 5HT (τ_{5HT} =590ms, n=17) or mCPBG (τ_{mCPBG}=222 ms, n=5) and this desensitization showed voltage-dependence, being slower at more depolarized potentials.
For both 5HT and mCPBG, the timecourse of recovery from desensitization, measured at a membrane potential of -120 mV using a double pulse protocol, could be fit with a single exponential component. However, receptors activated with mCPBG (τ_{mCPBG}= 155s) recovered about 10 fold more slowly than those activated by 5HT (τ_{5HT}= 11s). The rate of recovery from desensitization for 5HT-activated receptors (0.09 s^{-1}, n=9) agreed well with the value predicted (0.10 s^{-1}) for a linear model with a single desensitized state using our measurements of peak to steady state ratio and tau(decay). Interestingly, the predicted recovery rate for mCPBG (0.38 s^{-1}) was approximately 50-fold faster than the measured recovery rate (0.007 s^{-1}, n=6), suggesting that there may exist other routes into and out of the desensitized state. Considering a simple cyclic model (Katz and Thesleff, 1957), these data are consistent with the idea that mCPBG has a higher affinity for the desensitized state than does 5HT. Supported by the Markey Center for Neurological Sciences.

W-Pos147

THE EFFECTS OF SLOW GATING PROCESSES IN ION CHANNELS ON EXCITABILITY ((Amir Toib, Erez Braun, Vladimir Lyakhov and Shimon Marom)) Departments of Physiology and Physics, Technion, P.O.Box 9697, Haifa 31096, Israel.

Ion channels of excitable membranes are complex proteins that operate in time scales ranging from less then a millisecond to many seconds. How does this wide microscopic range of time scales show up at the macroscopic behavior of point excitable systems, beyond the envelope of a single action potential? To explore this question we have co-expressed NaII channels, together with different types of Shaker related potassium channels, at high densities in *Xenopus* oocyte membranes. The temporal dynamics were studied in a detached patch configuration, devoid of intracellular and spatial complications. Under current clamp conditions, these point excitable units demonstrate rich dynamics, including bistability and activity-dependent potentiation and depression of excitability over time scales of many seconds, phenomena which are usually attributed to extended systems. The mechanism of these dynamics was explored under voltage clamp conditions, showing that activity-dependent interactions between slow gating processes in sodium and potassium channels dictate the system's index of excitability on the many seconds time scale.

W-Pos148

MODULATION OF THE I_H CONDUCTANCE BY EXTERNAL POTASSIUM, SODIUM AND INTERNAL POTASSIUM IN MOUSE DRG NEURONS. **((A. Raes, P.P. van Bogaert))** Laboratory for Electrobiology. University of Antwerp (RUCA) B-2020 Antwerp, Belgium.

The hyperpolarization activated current I_h (inwardly directed and carried by sodium and potassium) has been demonstrated in many different neural tissues. The similar current I_f present in cardiac conductive tissue shows conductance characteristics which deviates in some aspects from the Goldman-Hodgkin-Katz equation. We evaluated some of these characteristics in mouse DRG cells which were isolated from embryonic tissue and kept in culture for 4-6 weeks. The slope conductance in the presence of different concentrations of Na^+ (replacement by N-methyl glucamine; 5mM K^+ outside) and K^+ on the outside of the cell was determined. In both cases the normalized slope conductance showed a steep relation with the outside ion concentration and fitted well with a hill equation ($k½$=21.7 mM; n=0.86 for Na^+ and $k½$=17.7mM; n=0.76 for K^+ outside). When Cs^+ was present in the pipette to replace K^+, partially or completely, a shift of the conductance-Na^+ relation to higher Na concentrations was observed. The conductance in relation to outside K^+ with Cs in the pipette decreases also by lowering outside K^+ but does not fit well the hill equation.

W-Pos149

SINGLE- AND MULTI-CHANNEL STUDIES OF POTASSIUM CURRENTS IN CIRCADIAN PACEMAKER NEURONS OF *BULLA*. **((K. Manivannan,* S. Michel,** A. Pokorny and G.D.Block))** NSF Center for Biological Timing, University of Virginia, Charlottesville, VA 22903. Present address *Dept. of Chemistry and Physics, Southeastern Louisiana University, Hammond LA 70402; **Institut fuer Zoologie, Universitaet Leipzig, 04103 Leipzig, Germany.

The eye of the cloudy bubble snail *Bulla* Gouldiana expresses a circadian (24-hour) rhythm in spontaneous compound action potentials recorded *in vitro* from the optic nerve. A group of approximately 100 electrically coupled neurons at the base of the retina known as the basal retinal neurons (BRNs) generates this circadian rhythm. It has been shown that cultured BRNs continue to exhibit the circadian modulation in membrane conductance. Recently, using whole-cell patch clamp recordings, we have identified a calcium-independent K^+ current that is modulated by the circadian pacemaker.

In this study, we perform cell-attached patch clamp recordings to characterize the "clock-controlled" conductance at the single-channel level. We present single- and multi-channel recordings from cultured BRNs before dawn (ZT 19-21) and after dawn (ZT 3-5). The observation of at least two non-inactivating channels with artificial sea water (10 mM K^+) in the bath and pipette is consistent with the whole-cell measurements. We utilized single- and multi-channel analysis to investigate the channel conductances, open probabilities and dwell times during pre-dawn (ZT 19-21) and post-dawn (ZT 3-5). For further characterization, we will also employ outside-out patches to study the pharmacology of the channels. Our goal is to identify the type of modulation used by the circadian pacemaker to change the membrane conductance at subjective dawn and dusk. *Supported by NSF CBT Fellowship and Louisiana Education Quality Support Fund (1995-98)-RD-A-21 to KM, and NSF NS15264 to GDB.*

AXONAL TRANSMISSION

W-Pos150

NERVE AXON EXCITABILITY REVISITED. ((J.R. Clay)) NIH, Bethesda, MD 20892.

Surprisingly, a sustained depolarizing current pulse elicits only a single action potential from squid giant axons, regardless of pulse amplitude. In contrast, the Hodgkin and Huxley model (HH) of this preparation predicts a steady train of AP's over a broad range of suprathreshold pulse amplitudes. The primary reason for this discrepancy concerns the potassium current, I_K. The threshold of I_K in HH is -60 mV, which is consistent with experiment. However, 90% activation occurs at +60 mV in the model. No native or wild type I_K channel has an activation curve as broad as this, including squid I_K's expressed in *Xenopus* oocytes (Patton, Perozo, and Bezanilla 1995, *Biophys. J.*, 68:A269). HH mismeasured I_K activation, in my view, because they normalized their I_K records by a linear dependence upon $(V-E_K)$. The I_K I-V relation is a non-linear function of $(V-E_K)$ which is well-described by the GHK equation (Clay, 1991, *J. Physiol.*,444:499). Records of I_K normalized by GHK give an activation curve with 90% activation at -10 mV, and a steep rising phase in the -50 to -40 mV range which acts as an impedance shunt during sustained depolarization, thereby shortcircuiting I_{Na} as the membrane potential approaches threshold a 2nd time. The position of the I_K activation curve on the voltage axis determines the response type of the axon - tonic firing in response to a sustained stimulus for a relatively positive midpoint of I_K; or rapid accomodation in the case of squid axons with a relatively negative midpoint of I_K activation on the voltage axis.

W-Pos151

CHANNEL FUNCTIONING BASED ON AN OCTAGONAL STRUCTURE MODEL AND NEURONAL SPECIFICITY OF SQUID PUTATIVE SODIUM CHANNEL SQSC1 mRNA. ((C. Sato, O. Shouno, T. Kimura, K. Hirota and G. Matsumoto)) ETL, Supermolecular Sci. Div., Tsukuba, Ibaraki 305, JAPAN

On the basis of the sequence of squid putative sodium channel SQSC1(D14525) and in its comparison with those of vertebrate, we have proposed a tertiary structure model where the transmembrane segments are octagonally aligned and the four linkers between segments S5 and S6 play a crucial role in the activation gate, voltage sensor and ion selective pore, which can slide, depending on membrane potentials, along inner walls consisting of S2 and S4 alternately. The octagonal structure model can explain the function of the channel and the action mechanisms of both tetrodotoxin (TTX) and α-scorpion toxin (ScTX), and be applied to Na, Ca, K channel and cGMP gated channel and further to inward rectifier K channel.
The SQSC1 mRNA was detected in all the nervous tissues examined; optic lobes, cerebral ganglia and giant stellate ganglia. Developmentally it was detected at least from the hatch of the squid.

W-Pos152

ED_{50} PREDICTIONS FOR A.P. BLOCK FOR ALIPHATIC SOLUTES. ((R. Hahin, J.J. Larsen and K. Gasser)) Northern Illinois Univ., DeKalb, IL 60115

50% effective doses (ED_{50}) to block propagated compound action potentials (A.P.'s) were obtained by characterizing the dose-response relation for each solute. A series of 10 solutes exhibiting successively greater octanol/water partition coefficents (K_{ow}) were used: DMSO, DMF, DMA, acetone, HMPA, MEK, cyclohexanone, dichloromethane, chloroform, and triethylamine. The K_{ow}'s for these test substances formed a sequence that showed a greater than 600-fold increase from DMSO to TriEA. A.P.'s were recorded from frog sciatic nerves using the sucrose-gap technique; test solutes in Ringer were applied to the nerve. The ED_{50}'s of all solutes were found to be nonlinearly related to log K_{ow}. ED_{50}'s for all solutes could be predicted as a function of the molar volume, dV/dn, polarity, P, and the hydrogen bond acceptor basicity, β by the following equation:

$$ED_{50} = 3.3/(10^{[2.9(dV/dn)-0.09P-3.6\beta]} + 0.08)^{0.6}$$

Voltage-clamp experiments using the vaseline-gap technique and single muscle fibers showed that each solute reduced Na currents with virtually no change in kinetic properties. Experiments conducted using a rat pancreatic secretory granule based assay for detecting chloride channel sensitive membrane fluidity changes, suggest that each solute acts to increase membrane fluidity at doses above ED_{50} values; however, fluidity changes can not solely explain the ED_{50} values. Light microscopic observations of fixed thick sections of whole nerves exposed to water soluble solutes show structural changes; however, ED_{50} values also can not be explained by osmotic effects on nerve structure. Similarly, ED_{50}'s for lipid soluble solutes can not be explained solely by Na channel block. These results suggest that the ED_{50}'s for A.P. block for aliphatic solutes are produced by a combination of effects including osmotically-induced nerve structural changes, ion channel block, and fluidity changes.

W-Pos153

VIBRATIONAL SPECTROSCOPY OF SINGLE CELL MEMBRANES USING MID-IR-TRANSMITTING WAVEGUIDES ((Mark S. Braiman, Susan E. Plunkett, and Roy E. Jonas)) Biochemistry Department, University of Virginia Health Sciences Center, Charlottesville, VA 22908.

We have been developing miniature IR evanescent-wave sensors with the aim of detecting IR difference spectra corresponding to voltage-induced structural changes of membrane channel proteins in single cells. For a given surface concentration of membrane protein in contact with the sensor, the sensitivity of the IR absorption measurement is predicted to reach a maximum when the waveguide thickness is nearly as small as the wavelength of the measuring light, requiring the use of a small-area HgCdTe detector and focusing optics with a large numerical aperture (e.g., an IR microscope). We have utilized these principles to obtain evanescent-wave IR absorption spectra from plasma membranes of single cells, specifically 1-mm-dia frog oocytes, a popular system among electrophysiologists for studying voltage-gated ion channels. Thin (<50-μm) waveguides were made by tapering and flattening chalcogenide optical fibers, or by polishing thin films of Ge between ZnS layers. We have successfully observed guided transmission of IR light through both types of waveguide, as demonstrated by the observation of strong evanescent-wave absorptions from small (mm-size) samples in contact with the surface. We obtain adequate IR throughput to detect both lipid and protein absorptions from plasma membranes of individual defolliculated and devitellinized *Xenopus laevis* oocytes.

Supported by NSF grant MCB9406681 to MSB.

W-Pos154

OPTICAL RESPONSE TO OSMOTIC STRESS AND CORTICAL DEPOLARIZATION IN ANIMAL BRAIN. Chance, B., Robertson, C.*, Gopinath, S.* Liu, H., Zhang, Y. and A. Mayevsky, A.** University of Pennsylvania, Department of Biochemistry and Biophysics, Philadelphia, PA; *Baylor Medical College, Houston TX; **Bar Ilan University, Ramat, Israel.

Light scattering consequences of a number of osmotically active substances *in vitro* in cells and in perfused liver have been reported (1,2,3). The possibilities that the brain would show physiologically and biophysically important changes have been explored in a rat brain model and in adult humans. A 200 MHz phase modulation measures optical pathlength increases concomitant with tail vein injection of various amounts of solutes (3,4). The increase of optical pathlength is measured at 816 nm and the difference responses, as measured by millimeters, measure increase of optical pathlength per milligram of solute per gm body wt which are maximal for mannitol and acetate and minimal for abmunin. Particular kinetics, for example, transient responses with mannitol are duplicated in human brain measuring scattered light changes with multiple input-output distances and continuous light systems (3). These extrinsic perturbations of the interstitial space can be compared with intrinsic perturbation due to potassium release during cortical depolarization followed euthansia in the rat brain (5). Here, congruent optical and potassium electrode responses are obtained (6), supporting the idea that the potassium deploarization can be measured in the animal brain by the optical method, an important result for localizing dysfunctional activties of the brain. Supported in part by NIH NS27346.

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W-Pos155

FERROELECTRIC LIQUID CRYSTAL MODELS OF TRANSITIONS IN VOLTAGE-DEPENDENT ION CHANNELS.
((Vladimir S. Bystrov and H. Richard Leuchtag[†]))
Pushchino State University, Pushchino, Moscow Region, 142292, Russia, and [†]Department of Biology, Texas Southern University, Houston, TX 77004. (Spon. by M. Hillar.)

Ferroelectric-superionic transition models have been previously shown to explain a number of features of the closed-open conformational transition in voltage-dependent ion channels. It was proposed that the channel acts as a chiral smectic (C^*) liquid crystal in its closed configuration, transforming to the A^* phase when open. Ferroelectric liquid crystals containing amino acids with branched alkyl groups (val, leu, ile) were found to have large spontaneous polarizations (K. Yoshino and T. Sakurai, in J. W. Goodby et al., *Ferroelectric Liquid Crystals*, Gordon & Breach, 1991). The peak of the dielectric permittivity was observed at the phase transition temperature, and this point shifts to a higher temperature with increasing applied electric field, in agreement with the thermodynamic Landau-Devonshire theory for ferroelectrics. The electric-field dependence of the channel transition has been ascribed to such a shift in the transition temperature, identified as the heat-block temperature. A sharp rise in capacitance observed in squid-axon membrane near heat block obeys the ferroelectric Curie-Weiss law (Biophys. Chem. 53:197-205, 1995).

W-Pos156

GATING EFFECTS OF Ca^{++} WHEN Na^+ CAN PERMEATE FREELY.
((Kamran Khodakhah and Clay M. Armstrong)) University of Pennsylvania, Department of Physiology, PA 19104.

We previously reported the small changes in gating current of Na channels (squid axon) when $[Ca^{++}]_o$ is changed in the presence of STX which blocks permeation through the pore. Here we report that the effect of Ca on gating is larger when permeation is intense. NMG as a sodium substitute in the external solution strongly reduces inward I_{Na} (100 mM Na & NMG are the monovalent cations) relative to TMA (100 mM Na & TMA) by a factor of 10 or more. The gating effects of Ca are quite different with the two Na substitutes. Changing Ca from 10 to 100 mM in NMG shifts gating 10 to 12 mV, while in TMA the shift (assayed from the g-V curve, ON kinetics, or closing kinetics) is about twice as large. An associated fact is that Ca block of inward current is very prominent in TMA, but not demonstrable in NMG. This suggests, in accord with previous results (Cota and Armstrong) that Ca block facilitates channel closing, and the large gating effects in TMA are associated with Ca block. Can Na channels close in the absence of Ca? On switching from 100 mM to nominally 0 Ca, I_{Na} tails after a short activating pulse initially increase by as much as 10 fold, and then over perhaps 100 s slow dramatically and decrease in size (reversible when Ca is restored). In these circumstances, the rate constant of the tails gradually approaches the inactivation rate; and with a two pulse protocol it can be shown that most of the channels are inactivating rather than closing. These experiments suggest that the closing rate of Na channels is very small in the absence of Ca.

W-Pos157

THE OPEN CONFORMATION IN THE VOLTAGE-DEPENDENT EAG K^+ CHANNEL IS DRAMATICALLY STABILIZED IN A CHIMERA CONTAINING A PORTION OF THE VOLTAGE-INDEPENDENT RAT OLFACTORY CHANNEL ((C.-Y. Tang, and D.M. Papazian)) Department of Physiology, UCLA School of Medicine, Los Angeles, CA 90095-1751. (Spon. by B. Yazejian)

Drosophila ether-a-go-go (eag) K^+ channels and rat olfactory channels (Rolf) belong to the superfamily of voltage-gated channels. Although both channels have an S4 segment, which comprises part of the voltage sensor in other voltage-gated channels, eag is voltage-dependent whereas Rolf is not. We have previously reported that, by replacing the S4 region of eag with that of Rolf, the Rolf S4 segment can support the voltage-dependent activation of eag , which suggests the voltage-independence of Rolf is not due to the charge content in the S4 segment. To identify regions of the protein outside the S4 segment that contribute to the voltage-dependent activation of eag , we have replaced the putative transmembrane segment, S3, and the following extracellular loop of eag with that of Rolf. This chimera shifts the voltage-dependence of activation by more than 100 mV in the hyperpolarized direction. To identify the minimum number of mutations that lead to this shift, we mutated an alanine that precedes S4 in eag to a glutamate, the residue at the corresponding position in Rolf. This point mutation shifts the voltage-dependence of activation by more than 60 mV in the hyperpolarized direction. These shifts in the voltage-dependence of the activation in the chimera and the point mutation cannot be explained by an electrostatic field effect on eag's voltage sensor. Rather, the open conformation of eag has been significantly stabilized. Based on these results, we propose that Rolf is voltage-independent in the physiological voltage range because the open conformation of Rolf is stabilized compared to eag and other voltage-dependent channels. We are currently working on other chimeras & mutations to test this hypothesis. (Supported by a grant from the Pew Charitable Trust to DMP.)

W-Pos158

GATING CURRENTS IN EAG K^+ CHANNELS ((C.-Y. Tang, D. Sigg, F. Bezanilla, and D.M. Papazian)) Department of Physiology, UCLA School of Medicine, Los Angeles, CA 90095-1751.

Drosophila ether-a-go-go channel (eag) is a voltage-gated K^+ channel that can be modulated by cyclic AMP (Bruggemann et al., Nature 365:445, 1993). We have recorded gating currents from eag channels expressed in *Xenopus* oocytes using a cut-open oocyte voltage clamp. Ionic currents were blocked by TEA, applied intra- and extracellularly. TEA did not appreciably immobilize the gating charge; similar results have been reported for rKv2.1 K^+ channels (Taglialatela and Stefani, Proc. Natn. Acad. Sci. USA 90:4758, 1993). In contrast, internal TEA immobilizes the off gating charge in Shaker K^+ channels (Perozo et al., Biophys. J. 62:160, 1992). Gating currents amplitudes were correlated with the level of expression of eag ionic currents. Gating currents became evident at about -80 mV, and the gating charge (Q) movement saturated at about 0 mV. Eag Q-V curves were fit with a simple Boltzmann distribution, resulting in a slope parameter similar to that obtained for the activation of the ionic current. The midpoint potential for gating charge movement was shifted to hyperpolarized potentials compared to the midpoint for the activation of ionic conductance. As in Shaker and rKv2.1 channels, the time course of activation of eag ionic currents was delayed by hyperpolarizing prepulses, suggesting the existence of transitions between a series of closed states before opening. A similar delay in gating currents was seen after hyperpolarizing prepulses. The off gating currents and deactivation of the ionic conductance had similar time courses at potentials more negative than -60 mV. Gating currents have also been recorded in excised, inside-out patches. We are currently determining the number of gating charges per channel in eag using nonstationary noise analysis. (Supported by grants from the Pew Charitable Trust to DMP and the NIH to FB.)

W-Pos159

ESTIMATION OF THE TOTAL GATING CHARGE MOVEMENT REQUIRED FOR THE ACTIVATION OF SHAKER POTASSIUM CHANNEL. ((A.Melishchuk and C.M.Armstrong)) Univ. of Pennsylvania, Department of Physiology, Philadelphia, Pa, 19104. (Spon. by P. Drain)

Using very high level of expression of Shaker H4 potassium channels in 293 cells (I_K up to 25 nA in excised patches) we have studied open probability (p_o) as a function of membrane potential (V_m) over a wide range of p_o. At very negative potentials plots of $\ln p_o$ vs V_m reach a limiting slope, which according to Hodgkin and Huxley is a measure of the gating charge (Q_g). Open probability was calculated 1) from the amplitude of the outward current (5-100 ms test pulses) with $[K^+]_o$ =0 mM; or 2) as the peak amplitude of an exponential fit to the inward tail currents (V_m = -100 mV) with $[K^+]_o$ between 10 and 150 mM. Both methods have hazards which will be discussed. $[K^+]_i$ was 150 mM in all experiments. The steepness of the $\ln p_o$ vs V_m reached a limiting value for p_o<~.01. In measurements with 0 mM $[K^+]_o$ we could resolve current for p_o<10^{-4}, and calculated $Q_g \approx 7.9\ e_o$. With $[K^+]_o$<25 mM we have resolved to p_o<.002 and obtained $Q_g \approx 9.3\ e_o$. This value may be an overestimate, due to the accumulation of K^+, which increases the amplitude of the tails as p_o increases. The effect of accumulation could be reduced by high $[K^+]_o$, but this had a special danger: some patches showed potassium action potentials in a poorly clamped area of the patch. Our present estimate is that Q_g is between 7 and 9 e_o.

W-Pos160

VOLTAGE-SENSING RESIDUES MOVE ALMOST ENTIRE DISTANCE FROM INSIDE TO OUTSIDE WHEN *SHAKER* CHANNELS OPEN. ((H.P. Larsson, O.S. Baker, D. S. Dhillon, and E.Y. Isacoff)) Group in Biophysics and Dept. of Molecular & Cell Biology, University of California, Berkeley

The approximately 20 amino-acid S4 region of voltage-gated ion channels has been predicted to span the membrane bilayer and to function as the voltage sensor of these channels. Neither transmembrane topology nor transmembrane motion have been directly demonstrated for this putative domain, however. We have probed internal and external accessibility of S4 residues to the thiol reagent methanethiosulfonate ethyltrimethylammonium (MTSET) in both open and closed, cysteine-substituted *Shaker* channels. We assayed access functionally in *Xenopus* oocytes that had been injected with channel-encoding cRNA. We find that S4 traverses the membrane with no more than 11 amino acids in the resting state, and that the distribution of buried residues changes when channels open. This change suggests an outward movement of S4 through the plane of the membrane, in which an initially intracellular residue moves to within three amino acids of the extracellular solution. The direction and extent of this motion is consistent with the idea that S4 forms a unified domain whose function is to sense voltage. The observation that S4 buries no more than two charges (within the sequence of 11 buried residues) suggests that the candidate counter charges of S2 and S3 may be sufficient to account for the stability of S4 in the membrane. Burial of 11 residues constrains the possibilities for channel structure, since the number is fewer the 18-20 necessary for an alpha-helix to span the core of a bilayer.

W-Pos161

S4 MOVEMENT CORRELATES WITH GATING CHARGE DISPLACEMENT DURING *SHAKER* CHANNEL GATING. ((L.M. Mannuzzu[1], M.M. Moronne[2], and E.Y. Isacoff[1])) [1]Molecular and Cell Biology Dept. and [2]Lawrence Berkeley Laboratory, University of California, Berkeley.

The S4 segment has been proposed to contain the gating charge, and to function as the voltage-sensor of voltage-gated ion channels. To test this model, we developed a fluorescence technique that provides a real-time measurement of the conformational rearrangements of specific channel segments during gating. Cysteine-substituted *Shaker* channels were expressed in *Xenopus* oocytes, conjugated with a fluorophore maleimide, and the fluorescence of labeled oocytes was measured in parallel with whole-cell gating currents. Positions in the N-terminal region of S4 (356C, 359C, and 365C) showed a graded, saturating fluorescence decrease with depolarization that followed the kinetics of the gating currents. The fluorescence-voltage relation correlated closely with the charge-voltage relation, but not with the voltage-dependence of channel opening. Three main findings indicate that the decrease in fluorescence is the consequence of the movement of the labeled sites into the extracellular fluid from a less polar, buried position: 1) increasing the solvent polarity from ethanol to water decreased the emission of the fluorophore in solution by 33%; 2) conjugation of the fluorophore to 359C and 365C was increased by depolarization; and 3) iodide, a membrane impermeant collisional quencher, enhanced the fluorescence change of 356C and 359C by 1.5 to 3 fold. These results indicate that activation extrudes a stretch of at least seven amino acids of S4 into the extracellular fluid during outward movement of the gating charge. The data provides physical evidence in support of the hypothesis that S4 is the voltage-sensor of voltage-gated channels.

HORMONE-RECEPTOR COUPLING

W-Pos162

THE INVOLVEMENT OF TWO Ca^{2+}-INTRACELLULAR POOLS IN SPONTANEOUS AND ATP-EVOKED Ca^{2+} RELEASES IN BOVINE AORTIC ENDOTHELIAL CELLS. ((M. G. Mozhayeva, G. N. Mozhayeva)) Sechenov Institute of Evolutionary Physiology and Biochemistry and Institute of Cytology, Russian Academy of Sciences,St. Petersburg, Russia.

In single bovine aortic endothelial (BAE) cells loaded with Fura-2, spontaneous Ca^{2+} transients in the Ca^{2+}-free medium have been revealed. In cells with resting $[Ca^{2+}]_i$ in the range from about 50 to 110 nM the biphasic dependence of Ca^{2+} transients on resting $[Ca^{2+}]_i$ was shown and spontaneous baseline Ca^{2+} oscillations were observed. At $[Ca^{2+}]_i$ exceeding 110 nM pronounced rise in Ca^{2+} transients occurred and only single transients were seen. Ryanodine (10 μM) produced Ca^{2+} responses in BAE cells. The results indicate that both IP_3-sensitive (IICR) and calcium-sensitive Ca^{2+} release (CICR) take place in BAE cells. The only IICR seems to be sufficient for generating baseline Ca^{2+} oscillations in BAE cells, whereas the development of ATP (5-100 μM)-induced Ca^{2+} response involves the CICR set in motion by oscillatory IICR of high frequency. The termination of both the spontaneous and ATP-induced calcium transients was associated with a $[Ca^{2+}]_i$ decrease to a level below the initial resting $[Ca^{2+}]_i$ (undershoot). Its depth biphasically depended on resting $[Ca^{2+}]_i$ from 50 to 110 nM, suggesting that the lack of Ca^{2+} leak from IP_3-sensitive stores can be responsible for the undershoot in this range, so the Ca^{2+} leak plays a key role in the mechanisms of initiation and termination of regenerative IICR both in spontaneous oscillations and in ATP-induced transients.

W-Pos163

FUNCTIONAL EXPRESSION OF HUMAN SPINOBULBAR ATROPHY GENE IN NSC-34 CELLS CHANGES THE VOLTAGE DEPENDENCE OF ACTIVATION OF T-TYPE CA CHANNEL. (H. Abramovici[1], A Abdullah[5], AM. Trifiro[4], L. Pinsky[3] & A. Sculptoreanu[1,2]). Dept. of Biol.[5], Dept. of Med.[4], Dept. of Hum. Gen.[3] and Depts. of Exptl. Med.[2] and Surgery[2], Lady Davis Institute for Med. Res. of SMBD- Jewish General Hospital, McGill University, Montreal, Qc, H3T 1E2 **(Sponsor A. Sculptoreanu)**.

Spinobulbar muscular atrophy (SBMA) is motor neuron degenerative disease in humans which is caused by an expanded triplet (CAG, coding for glutamine) repeat in the androgen receptor gene. Expansion of glutamines causes selective motor-neuron degeneration and mild androgen resistance, however, nothing is known about the functional consequences preceding the motor neuron degeneration and death. We tested the hypothesis that the mutation in SBMA may lead to a "gain of function" in neurons with the consequence at the level of functional expression of ion channels or their modulation by second messenger systems. Lipofection technique was used to transfect DNA encoding for the androgen receptor in NSC-34 cell line (a hybrid generated by fusion of neuroblastoma cells and embryonic spinal cord motor neurons). Three groups of cells were used: control mock transfection, normal androgen receptor and polyglutamine expanded androgen receptors. Transfection had at least 50% efficiency and gradually declined with time in culture. Electrophysiological experiments were done within the first week of transfection. We compared the effect of exposure to androgen agonist (5 nM, mibolerone) on Ca currents in the three groups of cell lines. NSC-34 cells expressed a unique low threshold, transient type of Ca current which was not affected by L-type blockers (PN 200-110), N-type Ca channel blocker (ω-conotoxin GVIA) or P-type Ca channel blocker (Agatoxin) but blocked by either 200 μM Cd^{2+} or Ni^{2+}. Exposure to androgen agonist had no effect in control cell lines or cells transfected with normal androgen receptor but significantly changed the steady-state activation in cells transfected with expanded androgen receptor. The observed negative shift (>-10 mV) in steady-state activation would result in a large increase in the transient Ca-channel window current. We suggest that the added Ca overload to the motor-neurons due to abnormal voltage dependence of transient Ca channel activation may be one mechanism responsible for motor-neuron degeneration and death in SBMA. *(Supported by grants from MRC and HFQ to AS., PN200-110 was a gift from Sandoz Canada.)*

W-Pos164

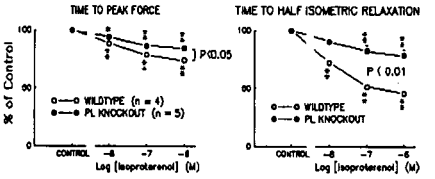
ANGIOTENSIN II STIMULATES LOW VOLTAGE-ACTIVATED, T-TYPE, Ca CHANNELS VIA A G PROTEIN, G_i. ((H-K. Lu, R.J. Fern, D. Luthin, L-P Liu, C.J. Cohen$, P.Q. Barrett)) Depts. of Pharmacology and Medicine University of Virginia, Charlottesville, VA 22908 and Merck Research Laboratories$, Rahway, NJ 07065.

Ang II is known to modulate voltage-gated Ca channels. In adrenal glomerulosa cells, Ang II stimulates Ca/CaM-dependent protein kinase II (CaMKII). Activation of CaMKII enhances T-type Ca current by inducing a hyperpolarizing shift in the voltage dependence of activation ($\Delta V_{1/2}$, the shift in the midpoint of a Boltzmann distribution; Lu et. al., Am J Physiol, 1994). We sought to determine if Ang II utilizes more than a single mechanism to control T-channel activation. Activation of whole cell Ca currents was determined isochronally by tail current measurements fixing Ca_i at 150 nM. Ang II (10 nM) shifts activation ($\Delta V_{1/2}$ = -11 mV; n=17) as does high Ca_i (500 nM; $\Delta V_{1/2}$ = -9 mV; n=6). Yet, CaMKII(281-302), a peptide inhibitor of the kinase, abolishes the effect of high Ca_i ($\Delta V_{1/2}$ = 0 mV; n=12, n.s.), but not of Ang II ($\Delta V_{1/2}$ = -7 mV; n=11, $p<0.05$). In contrast, the Ang II-induced $\Delta V_{1/2}$ is prevented by preincubation with an AT_1-receptor antagonist (1 μM Dup 753), pretreatment with pertussis toxin (300 ng/ml; 18 hours) or cell dialysis with a monoclonal antibody (mAb; 1:100 dilution, 12 μg/ml) that selectively recognizes recombinant $Gi\alpha 1,2,3$ but not $G_{o\alpha}$. Neither heat-treated mAb nor ascites fluid is inhibitory. Finally, the effect of Ang II on activation is not blocked by adding 10 μM cAMP to the pipette solution. We conclude that G_i transduces a cAMP-independent stimulation of T-channel current by Ang II. Thus, activation of T-type Ca channels is controlled via multiple mechanisms (G_i and CaMKII).

W-Pos165

CONTRACTION AND RELAXATION TIME COURSE OF WILD TYPE AND PHOSPHOLAMBAN-LESS MOUSE VENTRICULAR MYOCARDIUM AFTER BETA-ADRENOCEPTOR AGONISM. P.R. Housmans*, Gary C. Sieck*, James D. Hannon*, E.G. Kranias#, James D. Potter+. *Mayo Foundation, Rochester, MN 55905, #University of Cincinnati, Cincinnati, OH 45267, +University of Miami School of Medicine, Miami, FL 33101

We studied the effects of stimulation of the β-adrenoceptor on contractility of ventricular myocardium both in wild-type CF/1 mice and transgenic CF/1 mice made deficient in phospholamban (PL) with gene targeting methodology. Right ventricular strips were electrically stimulated to contract isometrically at 4 second intervals at 25°C in a physiological salt solution. Time to peak force (TPT) and time to half relaxation (RT 1/2) were recorded in steady state conditions of control and isoproterenol 10^{-8} to 10^{-6} M in 1 log increments. Isoproterenol decreased TPT and RT1/2 in both wild type and PL-less mouse ventricular myocardium, although significantly less so in PL-deficient mice. These differences most likely account for the effect of β-adrenoceptor agonism on the sarcoplasmic reticulum. The remaining abbreviation of contraction and relaxation in PL-less ventricular myocardium could reflect the effect expression of phosphorylation of troponin I after β-adrenoceptor agonism. Support: HL26057,HL42325 and GM36365. * $P<0.05$ ** $P<0.01$



W-Pos166

S-ACYLATION OF CYSTEINYL-CONTAINING LIPOPEPTIDES BY MAMMALIAN CELLS ((Hans Schroeder, Rania Leventis and John R. Silvius)), Dept. of Biochemistry, McGill University, Montréal, Québec, Canada H3G 1Y6

Several cysteine-containing lipopeptides, with structures modeled on the S-acylation sites of intracellular regulatory proteins such as nonreceptor tyrosine kinases and heterotrimeric G-protein α-subunits, are efficiently S-acylated by CV-1 and other mammalian cell lines. S-Acylation of fluorescent lipopeptides such as myristoyl-GlyCysGly-EDNBD (-EDNBD = ethylenediamine-NBD) was demonstrated both by chromatographic analysis of the fluorescent products and by incorporation of radiolabeled palmitic acid into the S-acylated peptides. The acylation reaction is moderately tolerant of variations in the amino acid sequence flanking the modified cysteinyl residue and in the nature of the coupled lipophilic group. However, the reaction is absolutely specific for modification of cysteine vs. serine residues and of peptides incorporating a lipophilic residue (which presumably serves to promote membrane association). Comparison of the kinetics and efficiencies of S-acylation of exogenous cysteinyl-lipopeptides vs. cellular proteins suggests that the process of lipopeptide S-acylation is enzymatically mediated. Fluorescence microscopy reveals that the product S-acylated lipopeptides accumulate specifically at the plasma membrane (where they become kinetically trapped upon acylation), suggesting that this membrane is the primary locus of the S-acylation reaction. These properties closely resemble those predicted for a putative enzymatic activity that S-acylates intracellular signaling proteins associating with the plasma membrane. (Supported by the MRC).

W-Pos167

STUDY BY FTIR SPECTROSCOPY OF THE INTERACTION OF DEHYDROEPIANDROSTERONE WITH PHOSPHOLIPID MEMBRANES. ((A. Dicko[1,2], T. Di Paolo[2] and M. Pézolet[1])) (1) Department of Chemistry and CERSIM, (2) School of Pharmacy and CHUL Research Center, Université Laval, Québec, Canada.

Dehydroepiandrosterone (DHEA) is a steroid which serves as a precursor for the formation of more potent androgens and estrogens. It is also considered as a neurosteroid and has been shown to be synthesized in the brain. In vitro experimental evidence of a direct effect of DHEA on $GABA_A$ receptor complex has been well documented. Its beneficial effect on brain functions and on many diseases was observed. However, the mechanism of its interaction with biomembranes is still unclear. In the present work, FTIR spectroscopy was used to investigate the effect of DHEA on the thermotropic behavior of phospholipids. Preliminary data on the interaction of DHEA with 1,2-dimyristoylphosphatidylcholine (DMPC) indicate that DHEA induces some conformational disorder along the acyl chain of this lipid in the gel phase. Furthermore, spectra of the ester carbonyl stretching region show that DHEA decreases the level of hydration of the glycerol moiety of the lipid molecules for both the gel and the liquid crystalline phases. These results show clear effects of DHEA on phospholipids organization; further studies with other phospholipids and their mixtures will be performed to assess the physiological relevance of our preliminary findings.

W-Pos168

CHARACTERIZATION OF THE CGRP RECEPTOR USING ELECTROPHYSIOLOGICAL AND CYTOCHEMICAL APPROACHES. (A.E. Luebke, G.P. Dahl, I.M. Dickerson) Univeristy of Miami School of Medicine, Dept. of Physiology and Biophysics, Miami, FL 33101. (Spon. by W. Nonner)

Calcitonin Gene-Related Peptide (CGRP) is one of the most potent vasodilators known, and can also serve as a cotransmitter with acetylcholine in the cochlea (organ of Corti) and at the neuromuscular junction. We have used a cloning strategy using the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) as a sensor for changes in cAMP levels to identify the CGRP receptor from the guinea pig cochlea. In this assay, receptors coupled to G_S activate adenylate cyclase, resulting in elevated intracellular cAMP levels, which in turn activate Protein Kinase A (PKA). The CFTR is a chloride channel that is activated by phosphorylation by PKA, resulting in a ligand-induced membrane current. A guinea pig organ of Corti cDNA library was screened by dividing the library into pools, transcribing the individual pools *in vitro*, and co-injecting the library cRNA with CFTR cRNA into *Xenopus laevis* oocytes until a candidate receptor was identified. The sequence for the cochlear CGRP receptor was determined, and it does not appear to be a member of the family of receptors containing seven membrane-spanning helices. Instead, the CGRP receptor belongs to a new class of receptor. We have generated antibodies to this new receptor, and using immunohistochemical techniques have begun to map the tissue distribution of the CGRP receptor. Supported in part by the American Heart Association, Florida Affiliate [9401236 (I.M.D.), 9501340 (A.E.L.)], and by NIH [GM48610 (G.P.D.), NS07044 (A.E.L.)].

W-Pos169

ENDOSOMES ARE INVOLVED IN COUPLING Ca^{2+} REGULATION AND GLYCOLYSIS IN A7R5 SMOOTH MUSCLE CELLS (SMC). (R.M. Lynch[1], L.E. Laughrey[1], M.W.Gurule[1] and R. Martinez-Zaguilan[2]) [1]Univ. Arizona, Tucson 85724; and [2]Texas Tech Univ., HSC, Lubbock, TX

Release of Ca^{2+} from intracellular stores is important for coupling receptor activation to contraction in SMC. Regions of endoplasmic reticulum are thought to act as Ca^{2+} compartments. Based on initial studies, we tested if endosomes regulate Ca^{2+} in SMC by loading cells with fluorescent dextran-conjugated Ca^{2+} dyes. Signal from single vesicles was analyzed by spectral imaging microscopy. Initiation of glycolysis appears to activate several ion transporters in SMC. In the present work, addition of glucose elicited cytosolic acidification, reduced cytosolic Ca^{2+} and increased endosomal Ca^{2+}. Drugs that block SR/ER Ca^{2+} ATPase (SERCA) suppressed glucose-induced Ca^{2+} increases in these vesicles. Most vesicles containing Ca^{2+} dyes were identified as endosomes/lysosomes, while there was little co-labeling with either mitochondria (rhod 123), or Golgi (NBD-ceramide). In contrast, co-labelling of cells with antibodies to either SERCA2, calreticulin, or Vacuolar(V) type-H^+-ATPase-antibodies, showed that there was ca. 70-80% co-localization. Measurements of pH in single vesicles indicated they were acidic, consistent with endosomes. These findings suggest: (a) endosomes are important in regulating Ca^{2+} in this SMC model; and (b) glucose metabolism can specifically modulate cell Ca^{2+} by stimulating Ca^{2+} sequestration into endosomes via SERCA. Moreover, calibration of the probes in these compartments must take into account their acidic nature.

W-Pos170

LOSS OF BRAIN WEIGHT WITH AGING: THE CONFOUNDING VARIABLE OF SECULAR TREND. ((R.P. Spencer)) University of Connecticut Health Center, Farmington, CT 06030.

Imaging studies and autopsies have documented decreased brain weight/volume with advancing age. Efforts to quantify this are complicated by the increasing body height/weight during the 20th century in many populations (secular trend). One approach is to evaluate human brain weight at ages 25 and 75 years in 9 autopsy studies from 1892 to 1994, a period of 102 years (catalogued by Hartmann et al, Pathologie 15:1561, 1994). Let 1892 be year (Y) zero. Brain weight (B in gm) increased in the 9 autopsy series.

Males	Age 25	B = 1350 + 1.35 Y (r = 0.81)
	Age 75	B = 1227 + 0.83 Y (r = 0.69)
Females	Age 25	B = 1228 + 0.98 Y (r = 0.75)
	Age 75	B = 1152 + 0.68 Y (r = 0.68)

For the 9 reported series, brain weight at age 75 (S) could be described as a function of the weight at age 25 (T).

Males	S = 403 + 0.65 T (r = 0.89)
Females	S = 456 + 0.56 T (r = 0.85)

There is a close parallelism between the male and female results over the various time intervals, as shown by the ratio of brain weight at 25 years/brain weight at 75 years; mean reporting date,

Mean year 1908	Male ratio 1.050	Female ratio 1.059
1941	1.094	1.098
1982	1.079	1.075

Still needed are data on brain/body weights over the years, or preferably allometric relationship of brain to body weights.

W-Pos171

PORE FORMATION BY NEUROTOXIC FRAGMENT OF THE PRION PROTEIN. (([1]Meng-chin Lin, [1]Tajib A. Mirzabekov, [1,2]Bruce L. Kagan)) (Spons. by Bruce A. Harayama) [1]Neuropsychiatric Institute, UCLA School of Medicine and [2]West Los Angeles Veterans Administration Medical Center, Los Angeles, CA 90024.

Prions are proteinaceous infectious agents which cause transmissible and genetic neurodegenerative diseases characterized by amyloid deposition in human and animals. A fragment of the prion protein comprising residues 106-126 (PrP 106-126) which forms beta sheet and aggregates into amyloid fibrils in vitro has been previously shown to be neurotoxic. We report here that PrP 106-126 forms ion permeable channels in planar lipid bilayer membranes. Several single channel conductances are observed, perhaps indicating varying states of aggregation of the peptide. These channels are freely permeable to common physiologic ions. The channels are voltage independent and relatively non-selective amongst ions. Channel formation is significantly enhanced by "aging" of the peptide and by acidic pH. In addition, acidic pH causes a substantial increase in single channel conductance, possible due to peptide aggregation. We propose that channel formation may be the cytotoxic mechanism of action of prions as well as other amyloid forming peptides. (supported by grants from NIH, Alzheimer's Association, and the University of California AIDS Research Program)

W-Pos172

PURIFICATION, CLONING AND EXPRESSION OF THE "SIGMA1-RECEPTOR" ((M. Hanner, F.F. Moebius, A. Flandorfer, H.-G. Knaus, J. Striessnig and H. Glossmann)) Institut für Biochemische Pharmakologie, Universität Innsbruck, A-6020 Innsbruck, AUSTRIA.

In membranes from liver, brain and other tissues [^{3}H]azidopamil specifically labels two polypeptides with apparent molecular masses of 22 and 27 kDa, respectively. Pharmacological studies suggest that the 27 kDa polypeptide carries the binding site of the "sigma$_1$-receptor". Sigma ligands (e.g. pentazocine, haloperidol, DTG and (+)-SKF10,047) inhibited specific photolabeling of the 27 kDa polypeptide. This polypeptide has been purified from guinea pig liver microsomes. Reversible [^{3}H]pentazocine binding activity was enriched about 370-fold after solubilization with digitonin and stepwise chromatography. The final preparation contained a 30 kDa polypeptide which was specifically labeled by [^{3}H]azidopamil, and with the same pharmacological properties as intact membranes. Amino acid sequence of a peptide obtained by tryptic digestion was used to raise site-directed antibodies, that specifically immunoprecipitated the photolabeled polypeptide. Degenerate primers were designed on the basis of this sequence and used to amplify a 59-base pair (bp) fragment. A specific oligonucleotide allowed the amplification of a partial cDNA which was used to screen a guinea-pig liver cDNA lGT10 library. The open reading frame of the full length clone coded for a 223 amino acid protein with a molecular mass of 25.3 kDa. Analysis of the amino acid sequence of the "sigma$_1$ receptor" revealed homology to fungal sterol isomerases. Heterologous gene expression of the cDNA in *Saccharomyces cerevisiae* leads to the formation of a high affinity pentazocine binding site that has the pharmacological properties of "sigma$_1$-receptor".

This work was supported by a Boehringer-Ingelheim fellowship (F.F.M.), the Österreichische Nationalbank (to H.G.) and grants by the Fonds zur Förderung der wissenschaftlichen Forschung, Austria (S6601 to H.G., P9351 to J.S.).

W-Pos173

CONTRASTING THE LOCALIZATION OF RI AND RIII SODIUM CHANNEL SUBTYPES IN ADULT RAT BRAIN. ((A.M. Corbett and M. Jarnot)) Department of Biochemistry & Molecular Biology, Wright State University, Dayton, OH 45435

Sodium channel subtypes RI and RIII were localized in 50 micron adult rat brain slices, using horseradish peroxidase labelled secondary antibody and DAB substrate. RI subtype specific staining was observed in cell bodies throughout the cortex, in the dentate gyrus and CA1, CA2 and CA3 layers of the hippocampus, in the Purkinje cells of the cerebellum, in the caudate putamen and hypothalamus. This staining always appeared as homogeneous staining of the entire cell body, with some staining found occasionally at the beginning of the distal processes. In contrast, RIII staining, while it also appeared in cell bodies, was punctate and localized to discrete areas of the soma. Throughout most of the adult rat brain, cells which displayed RI staining also displayed the punctate RIII staining, although the intensity of RIII staining was approximately 1/20th that of RI. Interestingly, however, the cerebellum displayed differential localization of the two subtypes: RI staining is only found in the Purkinje cells, while RIII staining is prominent in both the granular layer and the molecular layer. If this subtype expression remains following primary dissociation and culturing, then cerebellar granular cells may offer an unique opportunity to examine RIII sodium channel function. Supported by NIH NS28377.

W-Pos174

EFFECTS OF SITE-SPECIFIC MUTATIONS IN A PHYSICAL MODEL OF SODIUM CHANNEL ACTIVATION. ((C.C. CHANCEY)) Physics Dept., Purdue University Calumet, Hammond, IN 46323-2094. ((S.A. GEORGE)) Neuroscience Program and Biology Dept., Amherst College, Amherst, MA 01002.

We have modelled the effects of site-specific mutations on sodium channel voltage sensing, starting from a simple physical model of the S4 α-helix portion of the channel molecule. The interaction between the S4 α-helix segment and its environment was modelled by 1) nearest neighbor Coulombic forces, 2) the electric force due to and external electric field, and 3) static mechanical and electrostatic terms arising during the segment's resting state. These terms collectively describe a depolarization-dependent effective potential within which the S4 segment moves. Within this model, we have 1) replaced the positive residue at the extra cellular boundary of the membrane with a neutral residue, and 2) replaced the positive residue at the cytoplasmic boundary with a neutral residue. The model predicts an asymmetry in the shift in polarization needed to activate the channel, with the shift direction dependent on whether the neutralized amino acid is closer to the extracellular or cytoplasmic side of the membrane.

W-Pos175

INFLUENCE OF TOLUENE AND MEMBRANE LIPID CHAIN PACKING ON T_2-WEIGHTED MRI OF BRAIN ((Jumi Lee & Judith A. Barry)) LDDR & NIAAA, NIH, Bethesda, MD.

The intensity changes observed in T_2-weighted magnetic resonance images of brain in toluene abusers can be explained by changes in acyl chain packing due to toluene intercalation into central nervous system membranes. Incorporation of toluene into membranes has been modeled using phospholipid bilayers with varying unsaturation. In the presence of 20 to 100 mM toluene at 40 °C, transverse relaxation (T_2) values at 0.47 T in dipalmitoyl-phosphatidylcholine (DPPC) bilayers decreased up to 40%, those in 1-palmitoyl-2-oleolyPC (POPC) and dimyristoylPC (DMPC) *increased* by about 60%, while T_2 in dioleoylPC (DOPC) responded little to toluene. The longitudinal relaxation time (T_1) varied less than 5% for all lipids. ^{31}P and ^{2}H NMR powder patterns provided evidence of toluene incorporation into the bilayer. The results demonstrate that chain packing in lipid bilayers makes a significant contribution to the T_2 of free water protons, suggesting that packing influences water bound in the lipid-water interface. This data could explain the simultaneous occurrence of hyper- and hypointensity in T_2-weighted images.

W-Pos176

BRAIN VOLTAGE-DEPENDENT SODIUM CHANNELS SHOWED REVERSAL CHANGES IN THEIR LIDOCAINE/QX-314 APPARENT BINDING AFFINITY. ((Blanca C. Salazar, Cecilia Castillo, Mary E. Días, Esperanza Recio-Pinto.)) Anesthesiology Dept.,Cornell University,NY,NY10021. Instituto Internacional de Estudios Avanzados (IDEA), Apt. 17606, Caracas 1015-A, Venezuela.

Local anesthetics (LAs) block the open state of various voltage-dependent sodium channels. In muscle-derived channels lidocaine (LI) and the permanently charged lidocaine analog QX-314(QX) produced a constant level of open channel block. In brain-derived channels, we found that the level of the LI/QX-induced open channel block showed periods in which it was lower. In fact, in brain channels the level of open channel block showed two dominant modes a high- and a low-affinity mode (low-K_d and high-K_d).Increasing the QX concentration increased the lifetime of the low-K_d mode while it decreased the lifetime of the high-K_d mode. Survival plots of the lifetimes of the low- and high-K_d periods could be fitted with one exponential. However, it became apparent that there was more than one low-K_d mode and probably more than one high-K_d mode. The level of the LI/QX-induced open channel block increased with depolarization and the magnitude of this voltage-dependence was the same for the various K_d modes. Then the distance between the LA binding site and the intracellular channel aspect does not change with K_d changes. The various K_d modes suggest the presence of various open channel conformations. Noise analysis showed that the various K_d modes displayed the same k_{off} but different k_{on} rates. Therefore the channel open conformational changes most likely involve changes in the hydrophilic pathway leading to the LA binding site, rather than changes at the LA binding site itself.

W-Pos177

SENSITIVITY OF T-TYPE Ca CHANNELS TO VOLATILE ANESTHETICS ((J.J. Pancrazio[1], T.S. McDowell[1], P.Q. Barrett[2] and C. Lynch III[1])) Depts. [1]Anesthesiology and [2]Pharmacology, Univ. Virginia, Charlottesville, VA 29908. (Spon. by J.J. Pancrazio).

T-type Ca channels have prominent roles in initiating pacemaker activity in excitable tissue. Using the whole-cell patch-clamp method, we examined the effects of clinically relevant concentrations of inhalational anesthetics on T-type Ca current ($I_{Ca,T}$) expressed in cardiac and neuroendocrine cell types. While the $I_{Ca,T}$ in guinea pig ventricular myocytes exhibited little sensitivity to high concentrations of halothane (1.4 mM) or isoflurane (1.2 mM), ~20% reversible depression with each, $I_{Ca,T}$ in thyroid C cells was reversibly diminished by 33 ± 6% (mean ± SEM, n=6 cells) and 22 ± 4% (n=6) by isoflurane (0.7 mM) and halothane (0.7 mM), respectively. An equipotent concentration of enflurane (1.2 mM) proved most effective; $I_{Ca,T}$ decreased by 46 ± 6% (n=6). No changes in inactivation or activation of $I_{Ca,T}$ were apparent with anesthetic administration. $I_{Ca,T}$ of adrenal glomerulosa cells exhibited a similar differential sensitivity to the inhalational agents. Whereas halothane (0.7 mM) depressed $I_{Ca,T}$ by 24 ± 1% (n=5), isoflurane (0.7 mM) and enflurane (1.2 mM) caused reductions of 47 ± 4% (n=6) and 53 ± 3% (n=4), respectively. Slight shifts in voltage-dependent activation to hyperpolarized potentials were observed with isoflurane and more so with enflurane. We conclude that: 1) anesthetics may discriminate between different T-channel subtypes; 2) anesthetics differ in their ability to block neuroendocrine $I_{Ca,T}$, and assuming neuronal and neuroendocrine T-channel similarity, these differential anesthetic effects may contribute the clinically observed differences in electroencephalogram background activity. (This work supported by NIH grant GM31144 to C.L.)

W-Pos178

HALOTHANE EFFECTS ON HUMAN NEURONAL VOLTAGE-DEPENDENT L-TYPE CALCIUM CHANNELS ((I.Nikonorov, E.Recio-Pinto and T.J.J.Blanck)) Department of Anesthesiology, Cornell University Medical College and Hospital for Special Surgery, NY, 10021

One of the mechanisms by which halothane reduces the level of intracellular Ca may be through a reduction in the number of functional voltage-dependent L-type calcium channels. We studied the influence of halothane on calcium channel currents in neuronally differentiated human neuroblastoma cells (SH-SY5Y) in the presence of 2 μM Bay K at 23° C. Single channel calcium currents (cell-attached patch) were recorded in response to 1000 msec test pulses to -20mV applied at 0.5Hz from a -40mV holding potential. Bath solution in mM: 120 KGluconate, 25 KCl, 2 $MgCl_2$, 2 EGTA, 2 HEPES. Pipette solution in mM: 15 $BaCl_2$, 110 Sucrose, 2 EGTA, 2 HEPES, 10 TEA and 0.001 TTX. Bay K was added to both the bath and pipette solutions. It was found that halothane did not affect the single channel conductance, but decreased the rate of activation (latency) and the mean-open lifetime of L-calcium channels in a dose-dependent manner. At 0.2mM, halothane decreased the rate of activation but did not affect the mean open lifetime and at 1.6 mM halothane decreased both the rate of activation and the mean open lifetime. These effects were reversible. However the level of recovery showed variability, indicating the presence of rundown of the channel.

W-Pos179

VOLATILE ANESTHETICS DECREASE Ca^{2+} TRANSIENTS IN CEREBELLAR GRANULE CELLS. (Ning Miao and Carl Lynch III)
Dept. of Anesthesiology, University of Virginia, Charlottesville, VA 22908

In isolated cerebellar granule cells, depolarization activated Ca^{2+} influx through a various classes of voltage-gated Ca channels (VGCC) leads to an elevation of cytosolic [Ca^{2+}]. Anesthesia may be in part mediated by inhibi tion of VGCC and the consequent decreases in neurotransmitter release.

Methods: Cerebellar granule cells were isolated from 7-day old rat pups, cultured on glass cover slips in growth medium with 25 mM K^+, and used at days 7-10 of culture. After 20 min incubation in 3 μM fura-2 AM, cover slips were placed in a spectroflurometer cuvette at 37°C; [Ca^{2+}] was estimated by the ratiometric method. In control, 1 or 2 MAC(minimum alveolar conc) anesthetic studies, Ca^{2+} influx was initiated by depolarization with 50 mMKCl.

Results: Halothane (0.75, 1.5%), isoflurane (1.3, 2.5%), and enflurane (1.7, 3.5%) depressed the KCl-evoked peak and plateau increases above basal levels (typically 70-80 nM). Increases in [Ca^{2+}] (in nM) were:

Ca^{2+}:	peak	plateau		peak	plateau		peak	plateau
Control	672±70	231±20	**Control**	640±70	287±36	**Control**	682±56	251±22
.75% halo	399±23	171±17	**1.3% isof**	390±35	146±11	**1.3% enf**	316±20	116± 7
1.5% halo	183±21	77± 8	**2.5% isof**	136±25	52±10	**2.5% enf**	137±21	62± 7

In these cells, 1 μM nicardipine also decreased Ca^{2+} transients by 70%.

Discussion: Equivalent 1 and 2 MAC of the three commonly used volatile anesthetics reduced by ~50 and ~75% the peak and plateau [Ca^{2+}] influx. The similar effect of the L-type VGCC blocker nicardipine suggests that volatile anesthetics may reduce the Ca^{2+} transient by blocking L-type VGCC in these granule cells cultured in 25 mM KCl. (Support by NIH grant GM31144)

W-Pos180

INFRARED CHEMICAL IMAGING OF DRUG-INDUCED NEUROTOXICITY IN THE RAT CEREBELLUM ((E. Neil Lewis[1], Ira W. Levin[1], Joseph P. Hanig[2], and David S. Lester[2])) [1]Laboratory of Chemical Physics, NIDDK, National Institutes of Health, Bethesda MD 20892. [2]Center for Drug Evaluation and Research, Food and Drug Administration, Laurel, MD 20708

The newly developed technique of high definition infrared micro-spectroscopic imaging has the capability of visualizing complex intrinsic chemical distributions in biological materials and the potential for performing rapid histological examinations. We have applied this approach, which simultaneously collects infrared images and spectra, to study cerebellar tissue from Sprague Dawley rats that were treated with cytarabine. The antineoplastic drug, cytarabine, has been shown to induce neurotoxic responses in the rat, morphologically expressed as significant Purkinje cell death in a dose-dependent manner. The samples were prepared as 10 μm thick, unstained sections from frozen cerebella of rats that were exposed to either defined doses of cytarabine or control saline. For data collection the sections are layered onto calcium fluoride windows. Using a step-scan Fourier transform infrared spectrometer in conjunction with a high sensitivity infrared focal-plane array detector, spectra and images, containing 128x128 pixels, were collected from a series of treated and untreated animals at a spectral resolution of 16 cm^{-1} and a spatial resolution of approximately 14 μms. Infrared absorbances of both the lipid and protein fractions of the control and treated samples were particularly strong in the granule layer relative to the rest of the sample. In addition, in many of the treated samples, the individual Purkinje cell bodies could also be distinguished. Data will be presented showing detailed spectral and image analyses performed on these samples. Spectral differences observed between discrete regions within individual samples will be highlighted.

W-Pos181

DETERMINISTIC CHAOS IN PACEMAKER NEURONS OF SNAIL: EXPERIMENT, MODEL AND SIMULATION. ((A.O.Komendantov@# and N.I.Kononenko#)) @Glushkov Institute of Cybernetics and #Bogomoletz Institute of Physiology, Natl. Acad. Sci., GSP 252601, Kiev, Ukraine. (Spon. by T.R.Chay)

Different regimes of pacemaker activity in bursting neuron of a snail have been investigated both in intact cells and in mathematical model by using computer simulation. The model includes spike-generating mechanism, slow-wave generating mechanism, inward potential-dependent Ca current, Ca-inhibited Ca-current, buffering of intracellular Ca ions. The model demonstrates the majority of known experimental phenomena and allows one to investigate the role of separate components in generation of different kinds of pacemaker activity: beating, bursting and chaotic modes. The bifurcation parameters employed are the chemosensitive conductances responsible for slow-wave generation. They are established to be modulated by neuropeptide which is released by interneuron(s). Quantitative and qualitative diagnoses show that routes to chaos were generated. Bifurcations demonstrate some sudden and intermittent transitions from periodic to aperiodic behavior. Sensitive dependence upon initial conditions has been observed in the model. The positive largest Lyapunov exponents have been estimated for chaotic discharges. Phase diagram of initial conditions has a delicate fractal-like structure. Parameter-independent mode transitions from periodicity to aperiodicity and vice versa have been induced by both short-term shifts of membrane potential and concentration of intracellular Ca ions.

W-Pos182

CHARACTERIZATION OF THE FIRING BEHAVIOUR OF RAT CEREBELLAR GRANULE CELLS: A CELL-ATTACHED PATCH-CLAMP STUDY ((M.-L. Linne[1], S.S. Oja[1] and T.O. Jalonen[2])) [1]Univ. of Tampere, Medical School, 33101 Tampere, Finland, and [2]Albany Medical College, Div. of Neurosurgery, Albany, NY 12208, USA.

The extracellular patch-clamp technique is the most reliable method for measuring cell membrane currents and potential changes in small cells, such as cerebellar granule cells. The cell-attached voltage-clamp configuration allows simultaneous detection of single ion channel openings and action potential current waveforms (APCs, capacitive transients across the cell membrane due to action potential generation). In this work cerebellar granule cells obtained from 7-day-old rats and cultured for 6-8 days were used. Spontaneous APCs occurred without any external stimuli. Both mono- and biphasic APCs were detected and their amplitudes (positive peak) were typically 50-60 pA. In one set of experiments 23 % (61/262) monophasic and 77 % (201/262) biphasic APCs were propagated with a typical frequency of 1-2 Hz. 92 % of the APCs were associated with instantaneous outward channel openings (presumably K^+) after the spike, and 21 % of these also with small inward channels (presumably Na^+) immediately before the spike. As we have found that taurine enhances the firing frequency, we have also used neurotransmitters GABA and glycine to modulate the firing behaviour.

W-Pos183

DISTRIBUTION OF HIGH-CONDUCTANCE Ca^{2+}-ACTIVATED K^+ CHANNELS IN RAT BRAIN: TARGETING TO A PRESYNAPTIC COMPARTMENT ((H.-G. Knaus[1¶], C. Schwarzer[2], R.O.A. Koch[1], A. Eberhart[1], G.J. Kaczorowski[3], H. Glossmann[1], F. Wunder[4], O. Pongs[5], M.L. Garcia[3] & G. Sperk[2])) [1]Inst. Biochem. Pharmacol., University Innsbruck, Austria, [2]Inst. Pharmacol., Neuropharmacol. Unit., University Innsbruck, Austria, [3]Dept. Membrane. Biochem. & Biophys., Merck Res. Labs. Rahway, NJ, 07065, U.S.A., 4Inst. Cardiovasc. & Arteriosclerosis Res., Pharma Res.Centre; BAYER AG, Germany, 5Center for.Mol.Neurobiology, Hamburg, Germany

Tissue expression and distribution of the high-conductance Ca^{2+}-activated K^+ channel (*Slo*) was investigated in rat brain by immunocytochemistry, *in situ* hybridization and radioligand binding employing the novel high-affinity (K_d 22 pM) ligand [^{3}H]iberiotoxin-D19C. A sequence-directed antibody directed against *Slo* revealed the expression of a 125 kDa polypeptide in rat brain by Western blotting, and precipitated bound [^{3}H]iberiotoxin-D19C in solubilized brain membranes. *Slo* immunoreactivity was highly concentrated in terminal areas of prominent fiber tracts; the substantia nigra *pars reticulata*, globus pallidus, olfactory system, interpeduncular nucleus, as well as cerebellar Purkinje cells. *In situ* hybridization data supported the expression pattern of *Slo*. The distribution of *Slo* was confirmed by [^{3}H]iberiotoxin-D19C autoradiography and in microdissected brain areas by Western blotting and binding studies. The latter studies also established the pharmacological profile of neuronal *Slo* channels. The expression pattern of *Slo* is consistent with targeting into a presynaptic compartment which implies an important role in neural transmission. (¶supported by the APART program and FWF grants [S6611-MED and P-11187])

W-Pos184

REGULATION OF MAMMALIAN HIGH CONDUCTANCE, CALCIUM-ACTIVATED POTASSIUM (MAXI-K) CHANNELS. ((A.P. Braun, E.K. Heist, and H. Schulman)) Dept. of Neurobiology, Stanford University School of Medicine, Stanford, CA 94305

Maxi-K channels participate in a diverse range of physiological processes and represent a unique class of ion channel whose gating depends both on membrane voltage and cytosolic calcium. Maxi-K channels appear to be heteromultimers, comprised of a pore-forming α subunit and smaller β subunit. To study maxi-K channel function, the mouse brain α subunit (m*Slo*, Pallanck and Ganetzky, 1994) and bovine β subunit (Knaus et al., 1994) have been transiently expressed in HEK 293 cells, using green fluorescent protein as a transfection marker. In excised inside/out membrane patches, expressed channels display voltage- and calcium-dependent gating properties similar to maxi-K channels expressed in *Xenopus* oocytes. Co-expression of the β subunit with α increases the calcium sensitivity of maxi-K channels ~ 10 fold compared with expression of the α subunit alone. Addition of cytosolic ATP (0.2 mM) to membrane patches upregulates maxi-K channel activity, whereas the non-hydrolyzable ATP analogue, App(NH)p, is ineffective. This finding is consistent with results from reconstituted native maxi-K channels and indicates that recombinant expressed maxi-K channels are also associated with membrane regulatory elements, as reported for neuronal channels. The roles of such regulatory elements on maxi-K channel function are currently being investigated.

W-Pos185

THEORY OF FREQUENCY ENCODING AND MODULATION WITH APPLICATION TO THE HODGKIN-HUXLEY SYSTEM. ((Yuanhua Tang)) Department of Physiology, Cornell University Medical College, New York, NY10021 and ((Hans G. Othmer)) Department of Mathematics, University of Utah, Salt Lake City, UT84112

Sensory neurons often respond to stimulation with periodic excursions of the membrane potential whose frequency is proportional to the intensity of stimulation, thereby frequency encoding the stimulus (Tang and Othmer, *Proc. Natl. Acad. Sci., USA*, 92:7869-7873). We have developed a theory of forced oscillatory systems described by singularly-perturbed differential equations which shows that an important mechanism for achieving frequency encoding is selective regulation of the slow dynamics in the recovery phase. We develop analytical relationships that predict the firing frequency associated with a given analog signal, and the original analog signal can be predicted by the observed firing rate. We apply the theory to a model oscillatory neuron described by modified Hodgkin-Huxley equations. It is shown that selective regulation of the on- and off-rates of the slow kinetics of the potassium channel during the recovery phase is more effective than injection of a current for control of the firing frequency. One possible biochemical mechanism for such regulation is through phosphorylation of the delayed rectifier potassium channel (Perozo et al., *J. Gen. Physiol.*, 98:18-34).

The theory is also applied to excitable systems of Hodgkin-Huxley type under stimulation by injected current. For this system the input-output relationship can only be computed numerically using bifurcation techniques. We find that the bandwidth for frequency-encoding of a current input signal can be increased significantly by modulating the kinetic rates of the channels, for example by protein phosphorylation. The predicted firing pattern of individual neurons in response to glutamate stimulus with or without phosphorylation simulates the experimental results of Madison and Nicoll on hippocampal neurons well (*J. Physiol.* 372:38-43).

W-Pos186

(Moved to M-Pos419a)

W-Pos187

CHARACTERIZATION OF GLYCINE EFFLUX AND VOLUME REDUCTION MECHANISMS IN BARNACLE MUSCLE CELLS ((K.McGruder, C.Peña-Rasgado, & H. Rasgado-Flores)) Dept. Physiol. & Biophys. FUHS/Chicago Med. Sch. N.Chicago, IL 60064

Glycine (gly) is the most abundant intracellular amino acid in barnacle muscle cells (~ 90 mM). Under isosmotic or hyposmotic conditions, an increase in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) induces the synthesis of cAMP which promotes in turn the efflux of osmolytes (e.g., gly) leading to water loss and volume reduction. The present work had two aims: i) to identify the mechanism(s) by which gly exits barnacle muscle cells in response to volume reduction effectors (i.e. exposure of the cells to hyposmotic conditions in the presence of extracellular Ca^{2+} or exposure to an isosmotic increase in $[Ca^{2+}]_i$ or cAMP); and ii) to assess if all gly efflux induces volume reduction. The experimental strategy consisted of comparing the gly efflux accompanying volume reduction with the efflux expected from the measured volume reduction assuming that this reduction was entirely due to the gly efflux. Gly efflux was measured radiometrically from cells perfused with solutions containing gly (100 mM) as the predominant osmolyte able to exit the cell in response to the presence of volume reduction effectors. Calculation of the expected increase in gly efflux from a given measured volume loss was accomplished using the following equation: gly efflux = ([gly]$_i$xR)/surface area, where R is the measured rate of volume change (μl/sec) in response to the volume loss effector. The results show that when the cells were exposed to hyposmotic conditions in the presence of Ca_o, the ratio of measured/expected gly efflux was very close to unity (i.e. 1.09). When 1 mM cAMP was used as the volume reduction effector the ratio was 1.77. However, if extracellular Cl^- (Cl_o) was replaced with methanesulfonate this ratio was reduced to 1.1. Finally, when 20 μM $[Ca^{2+}]_i$ was used as the effector the ratio was 2.4 in the presence of Cl_o but only 1.1 in its absence. This indicates that an increase in $[Ca^{2+}]_i$ and cAMP induces gly efflux accompanied with water but also a gly efflux exchanged with Cl_o. Interestingly, an increase in $[Ca^{2+}]_i$ also promoted the exchange of intracellular gly for extracellular gly or taurine without promoting volume reduction. Furthermore the anion channel blockers SITS (0.2 mM) and niflumate (0.3 mM) inhibited gly efflux. It is suggested that gly efflux is mediated *via* anion channels.

W-Pos188

SINGLE AND DOUBLE LIGAND OCCUPANCY OF MULTI-CONFORMATIONAL CHANNELS EXPLAIN ANOMALOUS KINETICS OF FACILITATIVE SUGAR TRANSPORTERS. ((J.A. Hernández, J. Fischbarg, J.P. Koniarek, and J.C. Vera)). Dept. Physiol. & Cell Biophys., Columbia U., NY, and Prog. Molec. Pharmacol., Mem. Sloan Kettering, N.Y.

The four-state simple carrier model (SCM) is applied to diverse membrane transporters. However, in facilitative sugar transporters (GLUTs), while unidirectional fluxes under zero-trans and equilibrium-exchange fit a SCM, fluxes under infinite-cis and infinite-trans experiments do not to fit the same SCM. This "anomalous" behavior of GLUTs remains unexplained. We propose instead that GLUTs are channels in which experiments under zero-trans or equilibrium-exchange conditions and trans-effects correspond to a single-occupancy regime, whereas experiments under infinite-cis or infinite-trans conditions correspond to higher occupancies. We model a two-site channel with two conformational states. At single-occupancy (zero-trans or equilibrium-exchange), the model behaves as a SCM with trans-stimulations. At higher occupancy (infinite-cis or infinite-trans), the same channel model behaves as an SCM with kinetic parameters *different* from those for single-occupancy. Numerical results from our model are consistent with facilitative glucose transport across erythrocyte membranes. Hence, if GLUTs are multi-conformational channels, their particular kinetic properties can result from transitions between single and double channel occupancies. Supported by EY08918 and Sloan Kettering Institutional Funds.

W-Pos189

RESPONSES OF INTERNAL PH TO ADDITIONS OF FATTY ACID TO PANCREATIC ß-CELLS(HIT): SINGLE CELLS VS. POPULATIONS. ((Frank Caserta, Jr., J. A. Hamilton, V. Civelek and B. E. Corkey)) Boston University School of Medicine, Boston, Ma. 02118

It has been established that populations of pancreatic ß-cells in suspension show an immediate decrease in pH when fatty acid is added to the suspension. These data support the idea of fatty acid transport by "flip-flop" (diffusion) across the cell membrane. Until now, there has been no data about the behavior of single ß-cells during and after the addition of fatty acid. When single cells in a petri dish were observed after addition of oleic acid, the intracellular pH fell immediately as it did when fatty acid was added to a cell suspension. The pH remained low for the entire time until we added BSA and then immediately rose to a value close to the original value as it did in cell populations. In contrast to the above results, when single cells were subjected to puffer microprofusion with oleic acid the cells returned to their original pH while the fatty acid was still being added.

W-Pos190

DETERGENT SOLUBILIZATION AND RECONSTITUTION OF LYSOLIPID TRANSPORT ACTIVITY FROM INTESTINAL BRUSH-BORDER MEMBRANES ((Cesar A. Angeletti and J. Wylie Nichols)) Emory University School of Medicine, Atlanta GA 30322

Normally, large amounts of phospholipids enter the small intestine by ingestion or bile secretion and are degraded into lysophospholipids and fatty acids. Even though spontaneous transmembrane transfer of lysophosphatidylcholine across phospholipid bilayers is very slow, a significant fraction is absorbed intact from the intestine suggesting that lysophospholipid transport across the brush border membrane of enterocytes is protein-mediated. This transport activity was recently demonstrated using NBD (7-nitrobenz-2-oxa-1,3-diazol-4-yl)-labeled lysophospholipids and short chain diacyl phospholipids to assay transport from the across purified brush-border membrane vesicles (BBMV) from rabbit intestine (Zhang & Nichols, *AJP, 267*, G80, 1994). As a first step toward the purification of the protein(s) responsible for this activity, we developed a protocol to solubilize BBMV with Triton X-100 and reconstitute the lysolipid transport activity in phospholipid vesicles. The reconstituted proteoliposomes transported NBD-lysophospholipid from the outer to inner leaflet whereas control liposomes without protein did not. DEAE sephadex bound the transport activity from Triton X-100-solubilized BBMVs at pH 7 and above. These data suggest that Triton X-100 solubilization, separation, and reconstitution is a feasible approach to the purification of lysolipid transport activity from the rabbit intestine. Supported by ACS grant BE-148.

W-Pos191

ARGININES, BUT NOT LYSINES, FOUND IN THE MEMBRANE-SPANNING REGIONS OF UNCOUPLING PROTEIN ARE ESSENTIAL FOR NUCLEOTIDE INHIBITION. ((Martin Modriansky and Keith D. Garlid)) Dept. of Chemistry, Biochemistry, and Molecular Biology, Oregon Graduate Institute of Science & Technology, Portland, OR 97291-1000.

Mitochondrial uncoupling protein (UcP) facilitates proton backflux across the inner membrane to produce heat. The activity of the protein is allosterically inhibited by purine di/trinucleotides. We have shown that this inhibition is abolished by mutating Arg276 [Murdza-Inglis et al. (1994) J. Biol. Chem. 269, 7435]. The presence of other positively charged residues within the transmembrane regions raises the question of their involvement in the inhibition process, mainly because of the large negative charge carried by the ligands. We designed single mutations of Arg182 to Thr, Arg83 to Gln, Lys268 to Gln, and Lys72 to Gln and assayed the mutant proteins for H^+ transport activity and its GDP sensitivity using UcP reconstituted into liposomes. Arg182 and Arg83 mutants each abolished the GDP inhibition, without affecting H^+ transport. Lys72 and Lys268 mutants each retained GDP sensitivity, but showed partial loss of H^+ transport activity. Thus, each of the arginines, but not the lysines, are essential for GDP inhibition. (Supported by NIH grant GM 31086.)

W-Pos192

Mg^{2+} TRANSPORT ACROSS LIPOSOMES CONTAINING ADENINE NUCLEOTIDE TRANSLOCASE
((C. Marfella, A. Romani, and A. Scarpa))
Case Western Reserve University, Cleveland, Ohio, 44106

We previously reported that nanomolar concentrations of cAMP induce a time dependent Mg^{2+} efflux from isolated rat heart and liver mitochondria. The efflux is prevented by atractyloside, a specific inhibitor of Adenine Nucleotide Translocase (AdNT). Subsequent studies showed that binding, rather than cAMP-dependent phosphorylation, of AdNT, was responsible for Mg^{2+} efflux from isolated rat mitochondria. In this work this observation has been further investigated and characterized using a far simpler system: a liposome preparation containing purified AdNT and where Mg^{2+} or Mg^{2+} and ATP were trapped in the intravesicular space. AdNT freshly purified from beef heart mitochondria was reconstituted in liposomes composed of phosphatidyl-choline, phosphatidyl-ethanolamine and cardiolipin (ratio 4:4:1) and have a homogeneous size of 200 nm. Liposomes were added to media without Mg^{2+} and the rate of intravesicular Mg^{2+} efflux was kinetically and quantitatively measured using the Mg^{2+} metallochromic indicator eriochrome blue and dual wavelenght spectrophotometry. Using this system a time dependent efflux of Mg^{2+} was observed which was inhibited by atractyloside. Addition of ADP greatly enhances the rate of Mg^{2+} efflux and pretreatment with cAMP further enhances Mg^{2+} efflux. Binding studies using labelled cAMP indicate that there is a saturable specific cAMP binding with a Km of approximately 35 nM and with a stoichiometry of 2 cAMP binding/1 AdNT molecule incorporated in the liposomes. (supported by NIH HL 18708)

W-Pos193

MAGNESIUM EXTRUSION IN LIVER PLASMA MEMBRANE: EVIDENCE FOR TWO DISTINCT MECHANISMS. ((C. Luca, A. Romani and A. Scarpa)) Dept. Physiol. & Biophys., CWRU, Cleveland, OH, 44106-4970. USA.

Preliminary evidence indicates that liver cells regulates Mg^{2+} transport across the plasma membrane through an efflux mechanism inhibited by amiloride and influx mechanism inhibited by Ca^{2+}-channel blockers. The operation of these two transport pathways was investigated in purified liver plasma membranes. The vesicles were loaded with 10 mM Mg^{2+} during the isolation procedure, or with 20 mM Mg^{2+}, in the absence or in the presence of ATP, ADP or GTP, immediately after the purification procedure. The plasma membranes were incubated in a medium devoided of permeant ions, and the Mg^{2+} efflux was measured by atomic absorbance spectrophotometry, following the addition of increasing concentrations of different cations and the rapid sedimentation of plasma membranes. Under all these experimental conditions, approx. 15-30 nmol Mg^{2+}/mg protein were extruded from the vesicles following the addition of Na^+ or K^+, or Ca^{2+} and Mn^{2+}. Na^+ (or K^+) and Ca^{2+} had an additive effect on Mg^{2+} extrusion. Both the Na^+ and the Ca^{2+}-induced extrusion of Mg^{2+} were blocked by amiloride, while Ca^{2+} channel blockers were ineffective. The vesicles loaded with ATP, ADP, GTP, but not with ATPγS, extruded larger amounts of Mg^{2+} than the vesicles loaded only with Mg^{2+}. A qualitatively similar increase in Mg^{2+} extrusion was also observed in vesicles loaded with Tris-Pi or NH4-Pi, suggesting a role of Pi in enlarging the amount of Mg^{2+} trapped in the vesicles. Also in this case amiloride, and not Ca^{2+}-channel blockers, inhibited Mg^{2+} efflux. These data are consistent with the presence of two distinct Mg^{2+} efflux mechanisms, the first being activated by Na^+ or monovalent cations, and the second being activated by Ca^{2+} or divalent cations. Although the inhibition by amiloride might suggest a non-specific effect on both transporters, it may alternatively support the presence of a single mechanism whose transport capacity is potentiated by the co-presence of monovalent and divalent cations. (Supported by NIH HL 18708).

W-Pos194

MODULATION OF CFTR CHLORIDE CHANNELS BY TYROSINE KINASE INHIBITORS. ((I. C.-H. YANG, F. Wang, E.M. Price* and T.-C. Hwang)) Departments of Physiology and *Veterinary Biomedical Sciences, Dalton Cardiovascular Research Center, University of Missouri-Columbia, Columbia, MO, USA.

Using the cell-attached patch-clamp technique, we investigated the mechanism of CFTR modulation by tyrosine kinase inhibitors. Wild-type CFTR channels were expressed in Hi-5 insect cells by infecting the cells with baculovirus containing recombinant CFTR. Genistein (50 μM), a tyrosine kinase inhibitor, increased the CFTR chloride channel activity in the presence of a maximal concentration of forskolin (10 μM) by 44.92 ± 7.31 (n=4) fold, while having no effect by itself. Tyrphostin, a structurally different tyrosine kinase inhibitor, also potentiated forskolin's effect. Daidzein (50 μM), an inactive analog of genistein, could not potentiate the effect of forskolin on CFTR channel activity. Calyculin A (50 nM), a membrane permeant phosphatase 1 and 2A inhibitor, stimulated forskolin-induced CFTR chloride channel activity by 17.22 ± 2.04 fold (n=6). Genistein still enhanced CFTR activity induced by saturating concentrations of forskolin (10 μM) and calyculin A (50 nM). Calyculin A similarly potentiated CFTR activity induced by saturating concentrations of forskolin (10 μM) and genistein (50 μM). In addition, partial activity of CFTR channels was observed after forskolin was withdrawn in the continuous presence of calyculin A; whereas genistein (50 μM) failed to prevent complete deactivation. Noise analysis of the CFTR activity in patches containing multiple channels revealed a significant difference in the corner frequency of the noise spectra: 0.4690 ± 0.2943 Hz (n=4) for genistein plus forskolin and 0.7118 ± 0.3353 Hz (n=5) for calyculin A plus forskolin ($p<0.05$). Our data suggest that the regulatory mechanism of genistein is through an inhibition of phosphatases other than phosphatases 1 and 2A and that CFTR channels in different phorsphorylation states have different patterns of gating. Supported by the AHA, Missouri Affiliate.

W-Pos195

TRANSPORT OF ^{22}NA AND $^{32}PO_4$ ON THE HUMAN RENAL NAPI-3 COTRANSPORTER EXPRESSED IN HEK-293 CELLS ((R.T. Timmer, P.M. Smith, J.D. Hill, Y. Yang and R.B. Gunn)) Department of Physiology, Emory University School of Medicine, Atlanta, GA, 30322-3110.

The human renal Na-Pi cotransporter is non-obligatory for phosphate. The expression of human renal NaPi-3 cDNA in human embryonic kidney cells causes an increase in both ^{22}Na influx and Na_o-dependent $^{32}PO_4$ influx. This demonstrates for the first time that NaPi-3 also cotransports sodium. The Na_o-independent $^{32}PO_4$ influx is small and not increased by transfection/expression of the NaPi-3 gene. In sham transfected cells the ^{22}Na influx is 60 times the $^{32}PO_4$ flux and in expressing cells it is 25-30 times the $^{32}PO_4$ influx under the same conditions [mM: 10Na, 0.1 PO_4, pH 7.6, 21°C]. The $K_{½}^{Na}$ for activation of the $^{32}PO_4$ influx was 128mM Na, the $K_{½}^{PO4}$ for activation of $^{32}PO_4$ influx was 84μM, the $K_{½}^{Na}$ for ^{22}Na influx in low Na cells was about 23mM. Extracellular PO_4 inhibited ^{22}Na influx. The simultaneous activation of ^{22}Na and $^{32}PO_4$ influx by expression of NaPi-3 cannot be quantitatively described by either: 1) a mechanism with a constant ^{22}Na background flux and all new ^{22}Na and $^{32}PO_4$ transport having a fixed and reasonable (≤ 5Na:1PO_4) stoichiometry; or 2) a mechanism in which native and new transporters are the only pathway but ^{22}Na is not obligated to be transported with phosphate. The simplest model to describe quantitatively the effect of adding new transporters has 1) a constant ^{22}Na background flux and 2) non-obligatory ^{22}Na transport that is competitively reduced as PO_4 forms coplexes for cotransport. Amiloride is not an inhibitor but related compounds are: Benzamil < EIPA < MIA. Ouabain does not inhibit directly, but does increase steady state cell Na, which inhibits $^{32}PO_4$ influx. AsO_4 and VO_4 inhibit with K_i values of 0.4 and 1mM, respectively.
(Supported in part by NIH grants HL28674 to RBG and F32HL08989 to RTT)

W-Pos196

MEMBRANE POTENTIAL AND MECHANICAL PROPERTIES OF RAT HEPATOCYTES DURING TOXIC HEPATITIS. ((A.Y. Kabakov[1], L.A. Radkevich[2])) [1]Inst. of Developmental Biology, [2]Inst. of Biol. Tests of Chem. Compounds, Moscow, Russia. ([1]Present address: Dept. of Physiology, UTSW Med. Center at Dallas, Texas 75235-9040).

Effect of poisoning with CCl_4 (0.2mg/250g) on membrane potential (MP), on its stability during ischaemia (MPS), and on mechanical strength of rat hepatocytes (MSH) and intercellular junction (MSJ) were studied. Depolarization of MP (4-7 mV) and increase of MPS during 1-6 day were caused by decrease of intracellular ATP and total ATPase activity that had effect on Na^+,K^+ pump activity (Biophysics, **33**, 890-896). Hyperpolarization on 7th day were related with partial recovering of Na^+,K^+ pump activity (J.Theor. Biol., **169**, 51-64). Decrease of MPS on 11th day were caused by increase of total ATP activity. The MSH falls during first 3 days; MSJ falls in period from 2nd 5th days. Reconstitution of MSH and MSJ were in the same order.

W-Pos197

A RETINAL GLUTAMATE TRANSPORTER CLONE FROM SALAMANDER MIMICS PROPERTIES OF NATIVE RETINAL CONE TRANSPORTER. ((S. Eliasof, J.L. Arriza, C.E. Jahr, S.G. Amara, and M.P. Kavanaugh. Vollum Institute. O.H.S.U., Portland OR 97201. (Spon. by M. Forte)

Glutamate-elicited currents measured under whole cell patch clamp in retinal cones isolated from the tiger salamander (ambystoma tigrinum) display properties that have been attributed to either a glutamate receptor-gated chloride channel (Sarantis et al., Nature 332:451, 1988), or a glutamate transporter associated with a chloride conductance (Eliasof & Werblin, J. Neurosci. 13:402, 1993). We have cloned 4 transporters from the salamander retina which mediate uptake of 3H-glutamate and studied their properties using two electrode voltage clamp recordings in Xenopus oocytes expressing these transporters. All four clones mediate a glutamate-activated anionic conductance, which is revealed as an outward current at positive potentials when external chloride is substituted for more permeant anions such as nitrate (Wadiche et al., Neuron 15:721, 1995). However, one clone, SR30, has a significant glutamate-elicited outward current at positive potentials even when chloride is the principal external anion. Both the inward and outward currents are sodium dependent, and both are reduced by 23 ± 6 % by the glutamate transport blocker dihydrokainate at 1 mM. Furthermore, the current can be elicited by several amino acids, with an Imax of L-glutamate ≈ L-aspartate ≈ D-aspartate > D-glutamate. These properties are similar to the currents previously described in isolated retinal cones, suggesting that SR30 may represent a major component of the glutamate reuptake mechanism in cones. Consistent with this, SR30 RNA was detected in isolated retinal cones using single cell PCR. The sequence of SR30 indicates that it is a novel subtype distinct from the four previously described basic subtypes of glutamate transporters, EAAT1 through EAAT4. Further work is underway to verify the location of SR30 transporter protein in the salamander retina using fluorescently labeled antibodies directed toward the carboxyl terminal region of SR30.

W-Pos198

THE PHENOTYPE OF hu MDR 1 TRANSFECTANTS NOT PREVIOUSLY EXPOSED TO CHEMOTHERAPEUTIC DRUGS. ((M.M.Hoffman, L.-Y. Wei and P.D. Roepe)) Molecular Pharmacology & Therapeutics Program at the Raymond & Beverly Sackler Foundation Laboratory, Sloan - Kettering Institute, and Graduate Program, Cornell University Medical College, 1275 York Ave. New York, NY 10021.

Multidrug resistance (MDR) mediated by overexpression of MDR protein (P-glycoprotein) has been associated with intracellular alkalinization and membrane depolarization. Virtually all MDR cell lines have been created via protocols that at some stage involve growth on chemotherapeutic drugs, which are potent and complex and alter cells in many ways. To precisely define the MDR phenotype mediated by hu MDR 1 protein, and to test whether pH_i / $\Delta\Psi$ alterations are directly due to MDR protein overexpression and sufficient to cause the MDR phenotype mediated by MDR protein, we have co - transfected hu MDR 1 cDNA and a neomycin resistance marker into LR73 fibroblasts and have selected G418 resistant stable transfectants. A variety of clones expressing different levels of hu MDR 1 protein were isolated, however, unlike all previous work with hu MDR 1 transfectants the clones were not further selected with or maintained on chemotherapeutic drugs. Clones were analyzed for drug resistance, intracellular pH (pH_i), plasma membrane electrical potential ($\Delta\Psi$), and stability of MDR 1 protein overexpression. LR73/hu MDR 1 clones exhibit elevated pH_i and decreased $\Delta\Psi$, consistent with previous work with mu MDR 1 clones (*Biochemistry 33*, 7239). The extent of these perturbations is related to the level of hu MDR 1 protein expression. "Pulse - elevating" pH_i of the untransfected parental cell line via growth on alternating % CO_2 shows that the magnitude of the observed pH_i perturbations is almost sufficient to cause the level of drug resistance that is measured. Along with earlier work (*Biophys.J 69*, 883) that measured drug resistance for depolarized and acid CFTR clones, these data support the contention that the drug resistance exhibited by MDR cells that is unequivocally due to MDR protein expression can be explained by perturbations in $\Delta\Psi$ and pH_i. *Supported by grants from the Cystic Fibrosis Foundation &Wendy Will Case Fund, and the NIH (RO1 GM54516, GM55349, & NCI-P30-CA-08748). P.D.R. is a Sackler Scholar at MSKCC.*

W-Pos199

SODIUM AND POTASSIUM FLUXES DURING ENDOSOMAL ACIDIFICATION. ((R. Damion Hardison, Jeff S. Weeks, Tanica D. Moore, Taletha C. Moore, Emily J. Besser, J. Abra Watkins, C-Y. Li, and Jonathan Glass)) Center for Excellence in Cancer Research, Treatment, and Education, LSUMC-S, Shreveport, LA, 71130.

The regulation and control of endosomal acidification is a rate limiting process involved in iron adsorption via transferrin processing during endocytosis. Rabbit reticulocyte endosomes provide a relatively simple model for investigating detailed relationships among ionic fluxes, membrane potential generation ($\Delta\Psi$), and pH gradient formation (ΔpH). Under well defined buffer conditions, sodium and potassium fluxes are observed using SBFI or PBFI membrane impermeant fluorescent probes while ΔpH and $\Delta\Psi$ generation are monitored with fluorescein labeled differic transferrin or Oxonol VI respectively. Upon the addition of 1 mM ATP, the kinetics of acidification are biphasic with a rapid phase for influx ($2.3\pm0.4 \times 10^{-2}$ s^{-1}) and a slower phase for gradient dissipation or proton leak ($4.1\pm0.9 \times 10^{-4}$ s^{-1}). In contrast, apparent $\Delta\Psi$ generation is triphasic with observed rate constants of >10 s^{-1}, $3.5\pm0.5 \times 10^{-3}$ s^{-1}, and a dissipation rate of >1 $\times 10^{-4}$ s^{-1}. Apparent $\Delta\Psi$ generation proceeds to about +80±10 mV and a ΔpH of about 0.9±0.1 is obtained. Sodium and potassium fluxes show kinetics similar to the heterogeneity observed for $\Delta\Psi$ and ΔpH generation. The origin of the fast event (>10 s^{-1}) is not clear and is likely to be a spectral artifact. However, the efflux phase and slower influx phase are consistent with $\Delta\Psi$ generation and ΔpH dissipation respectively. Selective use of ionophores and inhibitors of Na^+, K^+, and/or H^+ transport indicate that K^+ and Na^+ effluxes are qualitatively and quantitatively similar. These observations provide the first evidence of K^+ fluxes in reticulocyte endosomes and suggest that both Na^+ and K^+ efflux occurs, coupled to or concomitant, with H^+ and Cl^- influxes.

W-Pos200

THE MEMBRANE DOMAIN OF THE HUMAN ERYTHROCYTE ANION EXCHANGER REVEALED BY SPECIFIC SUBSTRATE INDUCED PHOTOSENSITIZED LABELING.

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Specific labeling of transport proteins and their microdomains in the cell plasma membrane can be achieved by site directed photosensitization of the photoreactive hydrophobic membrane probe,[^{125}I]-5-Iodonaphthyl-1-azide (^{125}INA).In human erythrocytes, the anion exchanger (Band 3) could be specifically labeled and studied pharmacologically in situ using it's fluorescent substrate 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-Taurine as the photosensitizer. Labeling occured within the real time of translocation of the substrate across the membrane. Moreover, within the same time frame we could label, and thus identify in situ, the constituents of the membrane microdomain surrounding the erythrocyte anion exchanger.Labeling patterns of protein and lipids thus obtained, reveal that proteins of the erythrocyte membrane are unevenly distributed in the lateral plane of the membrane. Most membrane proteins are segregated in clusters organized around Band 3 which are surrounded by a microdomain of specific lipid composition. These clusters are separated by wide segments of protein free lipid domains.

W-Pos201

REGULATORY VOLUME DECREASE IS INDEPENDENT OF INTERNAL CALCIUM CHANGES IN N1E115 and NG108 CELLS. ((J. Altamirano, M.S. Brodwick, J.F. Alvarez-Leefmans)) . Dept. Physiology and Biophysics, UTMB, Galveston, TX 77555-0641; Depto. de Farmacologia CINESTAV-IPN, Ap. Postal 14-740, MEXICO 07000 D.F.; and Depto. de Neurobiologia, Inst. Mex. de Psiquiatria, Calzada Mexico-Xochimilco 101 MEXICO 14370, D.F.

Previous studies have implicated changes of internal Ca^{2+} as the trigger signal for regulatory volume decrease (RVD) in many cell types. Consistent with these studies was the observation that increasing Ca_i with ionomycin caused a volume decrease in isosmotic conditions. We therefore examined the volume and Ca_i changes simultaneously using Fura-2 optical techniques. Ca^{2+} was measured ratiometrically at 358 and 380 nm, whereas volume was measured at the intracellular isosbestic point of 358 nm. When challenged with a 40% hyposmotic solution, N1E115 and NG108 cells responded with a transient increase in cell volume which relaxed back toward control values in 23.2 ± 1.9 (SEM) min at an average rate of -1.61 % per min ± 0.35. The average maximal RVD was 42.2% ± 4. Accompanying the volume response was a transient increase in Ca_i signal. When the cells were bathed in Ca-free EGTA solutions and loaded with BAPTA-AM, the Ca_i signal was abolished but the RVD persisted. For cells not pretreated with BAPTA, a calcium pulse applied during the RVD relaxation did not affect the rate or extent of the RVD. When a second hyposmotic challenge was applied about 30 min after the end of the first challenge, RVD and the Ca_i transient were absent. Similar volume responses were measured with calcein, a fluorescent probe with properties different from Fura. The desensitization persisted in 0Ca-EGTA externalsolutions. We conclude that increased Ca_i is not required for RVD or desensitization. (supported by NINDS grant NS29227 and CONACyT Mexico grant F-285-N9209)

W-Pos202

CALCIUM INFLOW MECHANISM IN GUINEA-PIG HEPATOCYTES PLASMA MEMBRANE. ((H.Matsuda, *R.Sato, T.Nagano, **I.Hisatome, T.Aramaki)) Nippon Medical School, Tokyo, Japan, *Kinki University , Osaka, Japan, **Tottori university, Tottori ,Japan

Calcium mobilizing agonists ,for example, vasopressin and angiotensinII induce a rise of free intracellular Ca^{2+} concentration in hepatocytes by means of Ca^{2+} release from intracellular stores and Ca^{2+} inflow across the plasma membrane. However, the mechanism by which agonists stimulate plasma membrane Ca^{2+} inflow is not well understood. Therefore, we investigated whether Ca^{2+} permeable channels exist or not in the plasma membrane of guinea-pig isolated hepatocytes using the patch clamp technique. In cell attached patches, inward channel activities were observed at resting potential with a pipette containing 110 mM $BaCl_2$ solution. The opening amplitude was about 0.6 pA and open probability was 0.004. This channel showed no voltage dependency and I-V relationship was linear with slope conductance of 20 pS. The open time distribution was fitted with one exponential, while closed time distribution was fitted with the sum of two exponentials. 5μM Bay K 8644 in the internal pipette solution did not affect the channel activity. These observations suggest that Ca^{2+} permeable channels exist in the plasma membrane of guinea-pig hepatocytes and may be a pathway for Ca^{2+} inflow in the basal state and hormone stimulated state. Moreover, this channel was considered to be different from voltage-operated Ca^{2+} channels in excitable cells.

W-Pos203

NUCLEAR Ca^{2+} STORE REGULATES DIFFUSION ACROSS THE NUCLEAR ENVELOPE. ((C Perez-Terzic, L. Stehno-Bittel and D.E. Clapham)) Mayo Foundation, Rochester, MN 55905.

Using isolated nuclei and intact nuclear envelopes (nuclear ghosts) from *Xenopus laevis* oocytes, we studied the movement of small molecules across the nuclear membrane. In contrast to models that assume unregulated diffusion of molecules <70 kD through the nuclear pore complex, we found that transmembrane diffusion in nuclei was regulated by Ca^{2+} stores localized in the nuclear envelope. This conclusion is based on the observation that following depletion of Ca^{2+} from the nuclear store using either $InsP_3$ or Ca^{2+} chelators, diffusion of 10 kD dextran dyes into the nucleus was abolished. This block was not dependent on the nucleoplasm since it occurred in nuclear ghost preparations which lack nucleoplasm. Smaller molecules and ions diffused freely into nuclear ghosts and intact nuclei even following Ca^{2+} store depletion. Our results demonstrate that the nuclear Ca^{2+} store regulates the diffusion of intermediate-sized molecules, but not of molecules smaller than 500 D.

W-Pos204

FHUA, A TRANSPORTER OF THE *ESCHERICHIA COLI* OUTER MEMBRANE, IS CONVERTED INTO A CHANNEL UPON BINDING OF BACTERIOPHAGE T5. ((BONHIVERS, M., GHAZI, A., BOULANGER, P. and L. LETELLIER)) Laboratoire des Biomembranes CNRS URA1116, Université Paris-Sud Bat.432, 91405 Orsay cedex, France. (Spon. by A. Coulombe)

The *Escherichia coli* outer membrane protein FhuA catalyzes the transport of Fe^{3+}-ferrichrome and is the receptor of phage T5 and Φ80. The purified protein inserted into planar lipid bilayers showed no channel activity. Binding of phage T5 to FhuA resulted in appearance of high conductance ion channels. The electrophysiological characteristics of the channels (conductance, kinetic behaviour, substates, ion selectivity including the effect of ferrichrome) showed similarities with those of the channel formed by a FhuA derivative of which the "gating loop" (Δ322-355) had been removed (Killmann *et al.*, EMBO J., 1993, 12, 3007-3016). Binding of phage T5 to FhuA in *Escherichia coli* cells conferred SDS sensitivity to the bacteria suggesting that such channels also exist *in vivo*. These data suggest that binding of T5 to loop 322-355 of FhuA, which constitutes the T5 binding site, unmasks an inner channel in FhuA. Both T5 and ferrichrome bind to the closed state of the channel but only T5 can trigger its opening.

W-Pos205

ELECTRICAL CURRENTS ASSOCIATED WITH THE NUCLEOTIDE TRANSPORT BY THE RECONSTITUTED MITOCHONDRIAL CARRIER

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The electrophoretic export of ATP against the import of ADP in mitochondria bridges the intra versus extra mitochondrial ATP potential gap. Here we report that the electrical nature of the ADP/ATP exchange by the mitochondrial ADP/ATP carrier (AAC) can directly be studied by measuring the electrical currents via capacitive coupling of AAC-containing vesicles on a planar lipid membrane. The currents were induced by the rapid liberation of ATP or ADP with UV flash-photolysis from caged nucleotides. Six different transport modes of the AAC were studied: hetero exchange either with ADP or ATP inside the vesicles, initiated by the photolysis of caged ATP or ADP; homo exchange ADP_{in}/ADP_{in} or ATP_{in}/ATP_{in}; and caged ADP or ATP with unloaded vesicles. The hetero exchange produced the highest currents and with longest duration in line with the electrical charge difference ATP^{4-} versus ADP^{3-}. Surprisingly, also in the homo exchange and with unloaded vesicles small currents were measured with shorter duration. In all three modes with caged ATP a negative charge moved into and with caged ADP out of the vesicles. All currents were completely inhibited by a mixture of the inhibitors of the AAC, carboxyatractyloside and bongkrekate, which proves that the currents are exclusively due to AAC function. The observed charge movements in the hetero-exchange system agree with the prediction from transport studies in mitochondria and reconstituted vesicles. The unexpected charge movements in the homo-exchange or unloaded systems are interpreted to reveal transmembrane rearrangements of charged sites in the AAC when occupied with ADP or ATP. The results also indicate that not only ATP^{4-} but also ADP^{3-} contribute, albeit in opposite direction, to the electrical nature of the ADP/ATP exchange, which is at variance with former conclusions from biochemical transport studies. These measurements open up new avenues of studying the electrical interaction of ADP and ATP with the AAC.

W-Pos206

Identification of the epitope for monoclonal antibody 4B1 which uncouples lactose and proton translocation in the lactose permease of *Escherichia coli*.

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Monoclonal antibody 4B1 binds to a conformational epitope on the periplasmic surface of the lactose permease of *Escherichia coli*, uncoupling lactose and H^+ translocation in a manner indicating that it blocks deprotonation [Carrasco et al. (1984) *Biochemistry 23*, 3681]. By using surface plasmon resonance, 4B1 binding to purified lactose permease is shown to exhibit a K_D of about 5×10^{-10} M. The combined use of mutants containing 6 contiguous His residues in each periplasmic loop in the permease and Cys-scanning mutagenesis in conjunction with chemical labeling demonstrates that 4B1 binds specifically to the periplasmic loop between helices VII and VIII and that Phe247 and Gly254 are the primary determinants. Remarkably, although 4B1 binding uncouples lactose and H^+ translocation, none of the amino acid residues in periplasmic loops, particularly Phe247 or Gly254, play an important role in the transport mechanism, and binding of avidin to the loop containing the epitope has no effect on transport activity. Furthermore, 4B1 binding markedly alters the reactivity of V331C permease [Wu et al. (1994) *Protein Sci 3*, 2294], as well as the fluorescence of the mutant protein after labeling with 2-(4'-maleimidylanilino)-naphthalene-6-sulfonic acid. Therefore the uncoupling effect of 4B1 involves highly specific interactions which in all likelihood exert a torsional effect on the loop, resulting in a conformational change in helix VII and/or VIII that alters the pK_as of residues involved in lactose-coupled H^+ translocation.

W-Pos207

FUNCTIONAL CONSEQUENCES OF MUTATING TWO CYSTEINE RESIDUES IN THE YEAST MITOCHONDRIAL CITRATE TRANSPORT PROTEIN.

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In order to probe the role of native cysteine residues in the function of the yeast mitochondrial citrate transport protein (CTP), we have embarked upon a program, using oligonucleotide-directed, site-specific mutagenesis, to replace each Cys alone and in combination with Ser. To date, we have constructed and functionally characterized the single C28S replacement and the double C28S/C73S replacement. The mutated CTPs were overexpressed in *E. coli*, purified, and functionally reconstituted in a liposomal system. We observed that the above mutations caused little change in the K_m or the substrate specificity of the reconstituted CTP. Furthermore, V_{max} values were minimally affected (i.e., single and double mutant values were approximately 90% and 57% of the wild-type values). Experiments with membrane impermeable, hydrophilic sulfhydryl reagents (i.e., p-chloromercuribenzo-sulfonate, methanethiosulfonate (MTS) ethyltrimethylammonium, and MTS ethylsulfonate) indicate that the wild-type and the single Cys mutant are considerably more sensitive to inhibition by these reagents than is the double Cys mutant. In combination, the above results suggest that: i) neither Cys28 nor Cys73 plays an essential role in the CTP transport mechanism; and ii) Cys73, which resides in a putative membrane-spanning domain, is accessible to hydrophilic sulfhydryl reagents and confers considerable sensitivity upon the CTP to these agents. We are proceeding to mutate the remaining two native cysteines in order to complete construction of a Cys-less CTP which will provide a template for reintroduction of Cys at specific sites that can then be tagged with chemical and biophysical probes. Supported by NSF grant MCB-9219387 and NIH grant DK44993 to R.S.K.

W-Pos208

MITOCHONDRIAL RETENTION OF NUCLEIC ACIDS.

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Isolated rat liver mitochondria were tested for their capacity to transport or release DNA under conditions that alter the membrane permeability transition pore. Mitochondria were incubated for 10 min in a sucrose medium containing a 10 Kb plasmid, with salmon sperm DNA subsequently added as a trap to minimize degradation. Following incubation and centrifugation in a microfuge, DNA in the pelleted mitochondria and the supernatant was purified and analyzed for the presence of the plasmid and mitochondrial DNA (mtDNA) by dot blotting and probing. Addition of Ca^{2+} and phosphate increased the level of plasmid DNA in mitochondria; this level was markedly higher with cyclosporine A (CSA) present and was not decreased by the uncoupler FCCP. Treatment of pelleted mitochondria with DNAse markedly decreased the amount of plasmid retained, indicating that much of it was bound to the outer mitochondrial membrane; the presence of salmon sperm DNA attenuated this degradation of plasmid DNA. A similar pattern of retention was also observed for the added salmon sperm DNA, with a marked stimulation of binding by CSA and degradation of bound DNA by DNAse. MtDNA recovered in the pellet was also increased by CSA while the amount present in the supernatant was decreased. These data indicate that CSA, perhaps acting through the mitochondrial permeability transition pore, can alter binding of foreign DNA to and release of mtDNA from rat liver mitochondria.

W-Pos209

THE EFFECT OF Cd^{2+} ON THE FUNCTIONAL RELATIONSHIP BETWEEN THE NADH-LINKED ELECTRON TRANSFER AND THE H^+-ATPase IN PLANT ROOT PLASMA MEMBRANE VESICLES.

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Plasma membranes from corn root cells have been found to contain a NADH-linked electron transfer system and a H^+-ATPase, which are believed to contribute to the transport of nutrients and toxins. However, no effort has been attempted to identify the possible functional relationship between these two energy releasing processes. In this study, we investigated the influence of the activation of one enzyme to the activities of the other. Control experiments showed that the hydrolysis and proton pumping activities of the H^+-ATPase were not significantly affected by the activation of NADH-linked electron transfer. Similarly, the activation of the ATPase showed little effect to the electron transfer. The e^- transfer but not the ATPase induced a movement of Cd^{2+} which could be followed by measuring turbidity change of the membrane vesicles. This Cd^{2+} induced light scattering was observed in the presence of 0.1 mM ferricyanide, 10 μg of antimycin A, 50 μM cadium chloride, and 0.2 mM NADH. The addition of 2 mM ATP substantially slowed and delayed the increase in light scattering. The addition of Cd^{2+} in the absence of NADH-linked e^--transfer had no effect on the H^+-ATPase activities. We have shown that the light scattering increase is related to Cd^{2+} uptake by the plasma membrane vesicles. Thus, the rate decrease and the delay in the uptake suggest that the ATPase activity may be involved in the extrusion of Cd^{2+} from the vesicles.

W-Pos210

CYTOPLASMIC pH IN YEAST: EFFECTS OF INORGANIC CATIONS.

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The yeast plasma membrane H^+-ATPase, which generates a membrane potential difference, and a pH gradient, negative and alkaline inside the cell, respectively, can be stimulated by ion transport or the increased pH of the medium, and this stimulation increases the metabolic rate of the cell. The internal pH of the cells is not constant, and can be measured (1-2). Pyranine, introduced by electroporation has been found a useful indicator of the cytoplasmic yeast pH (1), and was used to measure the effects of inorganic cations on this parameter in yeast. First, the method published (1) was improved by alkalinizing the dye with NH_4OH, instead of tris base. The results indicated a selective increase of the internal pH of the cells, which was more selective, and of a higher range at low than at high pH. Also, by the addition of a substrate, changes of the distribution of the dye between the cytoplasm and the vacuole were found, which indicated also an active role of this organelle on the regulation of the internal pH of the cell, and indicating that this organelle is not necessarily always acid.
Also, divalent and trivalent cations were found to produce an acidification of the internal pH of the cell, probably due to the role of the exchange of divalent cations for protons, between the cytoplasm and the vacuole of the cell.

1. Peña *et al.*, J. Bacteriol. 177:1017-1022 (1995).
2. Cimprich *et al.* FEMS Microbiol. Lett. 130:245-252 (1995)

W-Pos211

GATING OF THE *ESCHERICHIA COLI* OUTER MEMBRANE PORINS BY HYDROSTATIC PRESSURE.

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Porins are water-filled pores in the outer membrane of Gram-negative bacteria. Although existing primarily in the open state, these channels may be gated by voltage (1) and periplasmic constituents (2). Here, we report the finding that porins can also be gated by hydrostatic pressure, supporting the idea of porin plasticity (3). Membrane vesicles of *E coli* carrying a plasmid expressing either OmpC or PhoE porins were reconstituted in artificial liposomes and examined with the patch-clamp technique. At positive and negative voltages (-70mV to +70mV), neither OmpC nor PhoE were affected by application of negative hydrostatic pressure (suction) up to -40mmHg. However, at negative voltages (up to -100mV) OmpC, but not PhoE, exhibited a marked increase in gating activity in response to the application of positive hydrostatic pressure (+10 to +30mmHg). The relative increase in standard deviation of the mean current (at -40mV) in the presence of positive pressure, for PhoE was (1.009±0.047, n=4 patches) and for OmpC was (4.173± 0.325, n=4), indicating a marked increase in gating of these channels. In addition, PhoE/OmpC chimeric porins showed a greater tendency to exhibit pressure sensitivity as the proportion of OmpC in the construct increased. Furthermore, examination of PhoE porins mutated in the loop L3 region suggest a possible role of this domain in conferring sensitivity to pressure. These data provide further evidence that regulation of porin behaviour may be more complex and that control of the molecular sieve function of the outer membrane may be more dynamic.

1. Lakey, J.H. & Pattus, F. (1989) *Eur. J. Biochem.* **186,** 303–308.
2. Delcour, AH *et al.* (1992) *FEBS Letters.* **304,** 216-220.
3. Buehler, LK *et al.* (1991) *J. Biol. Chem.* **266,** 24446–24450.

W-Pos212

SELECTED CYSTEINE POINT MUTATIONS CONFER MERCURIAL SENSITIVITY TO THE MERCURIAL-INSENSITIVE WATER CHANNEL MIWC.

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The mercurial-insensitive water channel (MIWC) is a 30-32 kDa integral membrane protein expressed widely in fluid-transporting epithelia (Hasegawa et. al. J. Biol. Chem. 269:5497-5500, 1994). To investigate the mercurial insensitivity and key residues involved in MIWC-mediated water transport, amino acids just proximal to the conserved NPA motifs (residues 69-74 and 187-190) were mutated individually to cysteine. Complementary RNAs were expressed in *Xenopus* oocytes for assay of osmotic water permeability (P_f) and $HgCl_2$ inhibition dose-response. Oocytes expressing the cysteine mutants were highly water permeable with P_f values (24-33 x 10^{-3} cm/s) not different from wild-type (WT) MIWC. P_f was reversibly inhibited by $HgCl_2$ in mutants S70C, G71C, G72C, H73C, and S189C, but insensitive to $HgCl_2$ in the other mutants. $K_{1/2}$ values for 50% inhibition of P_f by $HgCl_2$ were (in mM): 0.40 (S70C), 0.36 (G71C), 0.14 (G72C), 0.45 (H73C), 0.24 (S189C), and >1 for WT MIWC and the other mutants. To test the hypothesis that these residues are near the MIWC aqueous pore, residues 72 and 188 were mutated individually to the larger amino acid tryptophan. P_f in oocytes expressing mutants G72W or A188W (1.3-1.4 x 10^{-3} cm/s) was not greater than that in water-injected oocytes even though these proteins were expressed at the oocyte plasma membrane as shown by quantitative immunofluorescence. Coinjection of cRNAs encoding WT MIWC and G72W or A188W indicated a dominant negative effect: P_f (in cm/s x 10^{-3}) was: 22 (0.25 ng WT), 10 (0.25 ng WT + 0.25 ng G72W) and 12 (0.25 ng WT + 0.25 ng A188W). These results suggest the MIWC is mercurial insensitive because of absence of a cysteine residue near the NPA motifs, and that residues 70-73 and 189 are located at or near the MIWC aqueous pore. The finding of a dominant negative phenotype for mutants G72W and A188W indicates that MIWC monomers interact at a functional level.

W-Pos213

VOLTAGE-ACTIVATED PROTON CURRENTS IN HUMAN THP-1 MONOCYTES

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Depolarization-activated H^+-selective currents were studied by whole-cell and excised-patch voltage clamp in human monocytic leukemia THP-1 cells. H^+ currents generally resembled those in other mammalian cells. Increased pH_o or decreased pH_i shifted the H^+ conductance-voltage (g_H-V) relationship to more negative potentials, turning on the g_H. The g_H activated slowly and sigmoidally during depolarizing pulses. Fitted by a single exponential following a delay, the activation time constant, τ_{act}, was ~5-10 s at threshold potentials and decreased at more positive potentials (pH_i 5.5). Tail currents upon repolarization decayed mono-exponentially at all potentials. The time constant, τ_{tail}, was voltage dependent, decreasing from 2-3 s at 0 mV to ~200 ms at -100 mV. Surprisingly, although τ_{act} depended strongly on pH_o, τ_{tail} was independent of pH_o. This may result from a gating mechanism in which the first step in deactivation is a deprotonation reaction (Cherny *et al*, 1995. *J. Gen. Physiol.* 105:861-896). In inside-out membrane patches, H^+ currents were larger and activated more rapidly at lower bath pH (pH_i). This result is consistent with H^+_i setting the rate of g_H activation, and both H^+_i and H^+_o determining the position of the g_H-V curve (*cf.* DeCoursey & Cherny, 1995. *J. Physiol. in press*).

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W-Pos214

DETECTION OF ALTERNATIVELY SPLICED VARIANTS OF THE PLASMA MEMBRANE CALCIUM PUMP IN RAT TISSUES USING POLYCLONAL ANTIBODIES

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Ethidium bromide staining and Southern blots of mRNAs prepared from animal and human tissues showing the tissue-specific distribution of the different isoforms of the plasma membrane calcium pump and its splices provide valuable insight in the physiological importance of the pump in the cells. Physiologically, the occurrence of an alternate splice at the carboxyl-terminus, resulting in the spliced a and unspliced b form, is particularly important since the regulatory properties of the calcium pump reside in this region. This report shows the distribution, at the protein level, of the a and b form of the different isozymes of the plasma membrane calcium pump in different rat tissues using isoform-specific antibodies. Polyclonal antibodies were raised against specific regions of the different isoforms and were screened for their specificity using the appropriate isoforms which were expressed in COS-1 cells. The antibodies were shown to have high specificity by Western blot and competition studies. Supported by NIH grants GM 28835 and DK 44902).

W-Pos215

INFLUENCE OF SODIUM ION CONCENTRATION ON MEMBRANE CAPACITANCE INCREMENTS DUE TO ELECTROGENIC TRANSPORT IN Na,K-ATPase.

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Electrogenic transport by Na,K ATPase was studied. Transient current was induced by photo release of ATP from an inactive precursor, caged ATP. Small changes of the net system capacitance, C, can be determined in such a system from alternating currents under application of alternating voltages with various frequencies (Sokolov et al, 1992, *Biol.Membr.* 6, 1263; Sokolov et al, 1994, in The Sodium Pump, eds Bamberg & Schoner, Springer, New York, 529). Increments of C decreased with increasing frequency, f, of applied voltage. The dependence C vs f has a sigmoidal shape. The characteristic frequency f_0 of the steepest slope of the curve significantly decreased in solutions with high ionic strength, in which Na^+ transport is decelerated (Wuddel & Apell, 1995, *Biophys.J.* 69:909). The value of C correlated with the total charge, Q, transported across the membrane. C decreased when the $[Na^+]$ was reduced. At <2 mM [Na+] the increments became negative. The increase of C can be attributed to charge relaxing process inside the protein as discovered by (Nakao & Gadsby, 1986, *Nature* 323:628). The characteristic frequency f_0 depended on the time constants of charge redistribution processes. The amplitude of the C increment depended on (1) the amount of charge moved through the membrane dielectric and (2) membrane potential bias. Nonlinear dependencies of C vs. Q were explained by a voltage bias across the membrane fragments due to pumping. The potentials corresponding to maximum C change were similar to midpoint potentials of the equilibrium charge distribution (Rakowski, 1993, *J.Gen.Physiol.* 101:117) and depended on $[Na^+]$.

W-Pos216

CATIONIC LIPIDS INDUCE CHANGES IN THE PHYSICAL PROPERTIES OF THE DIPALMITOYLPHOSPHATIDYLCHOLINE BILAYERS. ((R.B. Campbell[2], S.V. Balasubramanian[1], and R.M. Straubinger[1,2],)) [1]Department of Pharmaceutics, University at Buffalo/State University of New York, Amherst, NY 14260 and [2]Department of Biophysics, Roswell Park Cancer Institute, Buffalo, NY 14263

Vesicles composed of dialkyl cationic lipids combined with phospholipids have been shown to deliver oligonucleotides and plasmids into cells in culture. The specific mechanism by which cationic lipids aid gene delivery are not well understood; membrane fusion or destabilization may be an essential step. We have investigated the effect of two cationic lipids, DOTMA (N[1-(2,3-dioleyloxy) propyl] N,N,N trimethyl-ammonium)) and DOTAP (1,2-bis (oleoyloxy)-3-(trimethylamino)propane, on the physical, domain, and non-bilayer properties of DPPC (dipalmitoylphosphatidylcholine) vesicles, using differential scanning calorimetry (DSC), circular dichroism (CD) and fluorescence spectroscopy. The presence of cationic lipids perturbs the packing of DPPC bilayers, and induces changes in such physical properties as the lipid order parameter and the phase transition temperature. Laurdan (6-dodecanoyl-2-dimethylaminonaphthalene) is under investigation to determine the lamellar domain structures and the non-bilayer properties of DPPC membranes containing DOTAP. The studies to date suggest a possible role of cationic lipids in inducing structural and dynamic changes in the membrane bilayer that may underlie phenomena enabling gene delivery.

W-Pos217

PERMEABILITY OF LECITHIN/PHOSPHATIDYLETHANOLAMINE LIPOSOMAL MEMBRANES WITH MIXTURES OF ETHANOL-INDUCED INTERDIGITATED AND NORMAL BILAYER STRUCTURES. ((H. Komatsu and S. Okada)) National Institute of Health Sciences, Osaka 540, JAPAN.

It has been suggested that the interdigitated structure plays an important role in regulating many functions of biological membranes. In our previous study (*Biochim. Biophys. Acta*, **1237**, 169(1995)), control of permeability was focused on as one of the biomembrane functions, and the effects of ethanol on the permeability of large unilamellar vesicles composed of dipalmitoyl phosphatidylcholine (DPPC) were studied by monitoring the leakage of the fluorescent dye, calcein, entrapped in the inner aqueous phase of the vesicles. Large permeabilities were observed in the range of 0.6M to 1.3 M ethanol, where the normal bilayer and interdigitated structures coexist, being due to the instability of the boundary regions, the interdigitated membrane being characterized by a thinner structure and more rigid hydrocarbon regions in the layer than its non-interdigitated counterpart. In the present study, mixed membranes, composed of DPPC and dilauroyl phosphatidylethanolamine (DLPE), were examined. Permeabilities were more prominent in phase separations with interdigitated and normal gel phases than with interdigitated and normal liquid-crystalline phases. It has been already demonstrated that in the absence of ethanol, DPPC and DLPE are miscible with each other in the gel or liquid-crystalline phase but the presence of ethanol leads to phase separation. These results thus suggest that ethanol can disturb the normal control of biological membrane permeability and that ethanol-induced interdigitated-membrane formation may be concerned with alcohol-caused diseases, relating to biomembrane permeability.

W-Pos218

STRUCTURAL PROPERTIES OF PHOSPHATIDYLETHANOL
((Olga Bondar and Elizabeth S. Rowe.)) Department of Biochemistry and Molecular Biology, University of Kansas Medical School, and VA Medical Center, Kansas City, MO 64128.

The structural organization of the negatively charged phospholipids, dimyristoylphosphatidylethanol (DMPEth) and dipalmitoylphosphatidylethanol (DPPEth), have been studied using differential scanning calorimetry, fluorescence spectroscopy and ^{31}P NMR. It was found that the phase behavior of these phospholipids below and during the main chain melting transition is different in 50 mM Tris buffer compared to salt solutions or water. The temperature of the main phase transition shifted to 50.47 °C for DPPEth in water compared to 40.4 °C in Tris buffer. A less significant shift was observed for DMPEth. Fluorescence studies using Prodan suggest that DMPEth can form an interdigitated structure in 50 mM Tris buffer without additives, such as ethanol. Prodan fluorescence studies of DPPEth suggest full interdigitation in the presence of Tris buffer and 2M ethanol. These results are discussed in terms of the effect of cations on the structure of anionic phospholipids and also in terms of influence of these structural properties on the ethanol adaptation of membranes.(Supported by the Department of Veterans Affairs.)

W-Pos219

A THEORETICAL MODEL FOR HELIX ASSOCIATION IN LIPID BILAYERS. MONTE CARLO SIMULATION STUDIES.
((Maria Maddalena **Sperotto**)) Department of Physical Chemistry, Umeå University, S-901 87 Umeå, Sweden.

A theoretical model is proposed for the trans-bilayer helix association in lipid bilayers. The model is built on the 10-state Pink model for the pure lipid bilayer, which accounts accurately for the most important conformational states of the lipids and their mutual interactions and statistics. According to the Pink model the bilayer is formed by two independent monolayers, each represented by a triangular lattice, on which sites the lipid chains are arrayed. Each trans-bilayer helix occupies laterally a seven-site hexagon. It is then assumed that each helix possesses hydrophilic residues in its hydrophobic sequence, so to interact with the sorrounding lipid matrix via selective anisotropic forces. The helices would therefore assembly in order to shield their hydrophilic residues from the hydrophobic sorrounding. The model describes the self-assembly of helices in lipid bilayer via lateral and rotational diffusion, anisotropic lipid-helix interactions, and non-specific helix-helix interactions.

The aim behind these model studies is to understand whether the assembly of trans-bilayer polypeptides into stable structures—or their dispersion in the lipid matrix—is lipid-mediated. Monte Carlo simulations are used to analyse the physical conditions necessary for helix assembly.

W-Pos220

INTERACTION BETWEEN CEU DERIVATIVES AND LIPID BILAYERS: A FTIR AND ^2H SOLID-STATE NMR SPECTROSCOPIC STUDY. ((Nadine Boudreau*, René C. Gaudreault%, Patrick Poyet% and Michèle Auger*)) *Département de Chimie, CERSIM, Université Laval, Québec, Canada, G1K 7P4, %Centre de recherche de l'Hôpital St-François d'Assise, Québec, Canada, G1L 3L5.

1-aryl-3-(2-chloroethyl) ureas (CEUs) are a new class of antineoplastic agents. CEU are hybrid molecules designed from the unnitrosated pharmacophoric moiety of nitrosoureas and the prosthetic group of aromatic nitrogen mustards and their general structure is $RNHCONHCH_2CH_2Cl$. These molecules are active both *in vitro* and *in vivo* but their mechanisms of action are not defined yet. The interactions between CEU derivatives and model membranes of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) have been studied using Fourier transform infrared spectroscopy (FTIR) and deuterium solid-state nuclear magnetic resonance spectroscopy (^2H NMR). Model membranes containing cholesterol have also been investigated. The results indicate that the incorporation of CEUs derivatives decreases the phase transition temperature of the pure lipid and increases the conformational disorder of the acyl chains, both in the absence and presence of cholesterol. The effects are strongly dependent on the R substituent and are related to the cytotoxicity of the drugs.

W-Pos221

APPLICATION OF A PICONEWTON MAGNETIC FORCE TRANSDUCER FOR MEASURING THE BENDING STIFFNESS OF A PHOSPHOLIPID BILAYER. ((V. Heinrich, J. Butler and R.E. Waugh)) Department of Biophysics, University of Rochester School of Medicine and Dentistry, Rochester, NY 14642.

Phospholipid bilayers exhibit a small but finite elastic resistance to bending. In this report we present recent refinements in an approach to measure the bending stiffness by a direct mechanical method, in which a thin tubular strand of membrane bilayer (tether) is formed from the surface of a giant phospholipid vesicle. The forces required to form the tether can be directly related to the membrane bending stiffness by a simple force balance. In this most recent refinement, the force on the tether was generated by the action of an electromagnet on a paramagnetic bead attached to the vesicle surface. Appropriate calibrations in which the velocity of the bead in a still fluid was measured as a function of the current applied to the magnet enabled us to calculate the force on the bead as a function of the measured current. This force is expected to be proportional to the square root of the membrane tension, which can be applied and controlled via the aspiration pressure in a micropipette used to hold the vesicle. The constant of proportionality contains the membrane bending stiffness. Measurements were performed on 12 different vesicles composed of stearoyl oleoyl phosphatidylcholine (SOPC) plus 5% (by mole) biotinylated phosphatidylethanolamine (biotin-X-DHPE) to mediate adhesion to streptavidin-coated beads. From each vesicle, tethers of a certain length were formed repeatedly at increasing and decreasing values of the membrane tension. Between 40 and 50 measurements were performed on each vesicle. The expected relationship between membrane tension and tether force was observed, and the mean value of the bending stiffness obtained from the twelve vesicles was 1.09×10^{-19} J, with a standard deviation of 0.08×10^{-19} J. The precision of these data indicate the reliability of this experimental approach, which avoids uncertainties of interpretation and measurement inaccuracies that may be associated with other methods for determining the bending stiffness of bilayer membranes. (Supported under NIH grant no. HL18208.)

W-Pos222

Effect of Cholesterol and Anionic Phospholipids on Pore Formation in Bilayers by the Amphipathic pH Sensitive GALA Peptide , F. Nicol, S. Nir+, F. C. Szoka, Jr., +The Hebrew University of Jerusalem and School of Pharmacy, University of California, San Francisco, 94143-0446

The peptide GALA, binds to large neutral liposomes at low pH and aggregates irreversibly in the membrane to form a pore consisting of 10 + /-2 peptides. Introducing an anionic phospholipid(POPC/POPG liposomes) had no effect on the leakage. However, a dramatic decrease in the efficiency of GALA to induce leakage was observed when cholesterol content was increased in the liposomes (20 to 40 mole %). An analysis of the results revealed a different leakage pattern with liposomes containing larger fractions of cholesterol. One explanation of the cholesterol effect is that in the presence of cholesterol the surface aggregation of the peptide is reversible, which implies that only a fraction of the bound peptide forms sufficiently large aggregates to form a pore. Another possibility is that the binding is significantly reduced so that it is the only cause of the decrease of the leakage efficiency. The second possibility is not supported by the preliminary analysis which assumed a range of partition coefficients of the peptide in cholesterol containing membranes. Fluorescence measurements indicate a blue shift in the wavelength of the maximum intensity of the tryptophan when GALA binds to POPC and POPC/POPG liposomes. The magnitude of the shift is gradually reduced with an increase of cholesterol content in the membranes suggesting that the peptide is more exposed to water in the presence of cholesterol in the bilayer. Supported by NIH GM30163/DK46052.

W-Pos223

EFFECTS OF POLYMYXIN B AND ITS NONAPEPTIDE ON DIMYRISTOYLPHOSPHATIDYLGLYCEROL; AN INFRARED SPECTROSCOPIC INVESTIGATION. ((D. Carrier, L. Khoury, A. Kielar and N. Chartrand)) Department of Biochemistry, University of Ottawa, Ottawa, Canada K1H 8M5.

Polymyxin B (PMB) is produced by *Bacillus Polymyxa* and is a very potent antibiotic used to treat infections caused by Gram-negative bacteria. PMB consists on an oligocationic decapeptide linked to a hydrocarbon chain. Under physiological conditions, the peptidic portion bears five positive charges and can thus interact with negatively charged amphiphiles, like dimyristoylphosphatidylglycerol (DMPG). Our infrared spectroscopic investigation revealed that PMB induces an unusually complex thermotropic behavior of DMPG bilayers, very different from that reported for dipalmitoylphosphatidylglycerol. In addition, an important hysteresis was observed in the presence of PMB. Our spectra suggest that the multiple phase transitions seen with PMB are not caused by lipid lateral phase separation, nor by a macroscopic phase separation. The nonapeptidic portion alone (PMBN) simply induced a single, shifted transition and no hysteresis.

W-Pos224

ON THE DYNAMICS OF THE ELECTRIC FIELD INDUCED BREAKDOWN IN LIPID MEMBRANES ((M. Winterhalter)) Biophys. Chem., Biozentrum, Klingelbergstr. 70, CH-4056 Basel, Switzerland

Electric field methods are powerful tools for cell characterization and manipulation. Important biotechnological applications, e.g. electrofusion of cells or electroinjection of macromolecules into living cells, rely on membrane breakdown. Despite the widespread use of these techniques, the process of membrane breakdown is poorly understood: a better understanding can be expected to lead to higher efficiencies. The present study used planar lipid bilayers in order to limit the number of experimental parameters. Membrane rupture was induced by careful application of short electric field pulses: measurement of the subsequent increase in membrane conductivity with time permitted the underlying mechanism to be characterised. The initial process of pore formation starts a few µsec after the onset of the pulse, and the ensuing breakdown of the entire lipid membrane occurs within about a millisecond (pore widening velocity of a few cm/s). The kinetics of the pore-opening process could be influenced qualitatively by adding surfactants and lipid-attached macromolecules respectively. Changing the effective mass per unit area of membrane, which could be brought about by using known percentages of lipids with covalently-bound, hydrophilic polymers, allowed a model for the pore widening process to be quantitatively tested.

W-Pos225

X-RAY STRUCTURE DETERMINATION OF FULLY HYDRATED L_α PHASE DPPC BILAYERS ((John F. Nagle[1,2], Ruitian Zhang[1], Stephanie Tristram-Nagle[2], Wenjun Sun[1], Horia Petrache[1], and Robert M. Suter[1])) [1]*Department of Physics and* [2]*Department of Biological Sciences, Carnegie Mellon University , Pittsburgh, PA 15213*

Bilayer form factors obtained from X-ray scattering data taken with high instrumental resolution are reported for multilamellar vesicles of L_α phase lipid bilayers of DPPC at 50°C under varying osmotic pressure. Artifacts in the magnitudes of the form factors due to liquid crystalline fluctuations have been eliminated by using modified Caillé theory. The Caillé fluctuation parameter η_1 increases systematically with increasing D spacing and this explains why some higher order peaks are unobservable for the larger D spacings. The corrected form factors fall on one smooth continuous transform F(q); this shows that the bilayer does not change shape as D decreases from 67.2 Å to 53.9 Å. The distance between headgroup peaks is obtained from Fourier reconstruction of samples with four orders of diffraction and also from electron density models that use 38 independent form factors. By combining these results with previous gel phase results, area A^F per lipid molecule and other structural quantities are obtained for the fluid L_α phase. Comparison with results that we derive from previous neutron diffraction data is excellent, and we conclude that $A^F = 62.9 \pm 1.3 Å^2$ from diffraction studies, in excellent agreement with a previous estimate from NMR data. (Supported by NIH GM44976)

W-Pos226

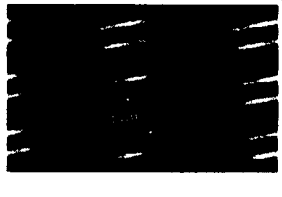
GEL ($L_{\beta'}$) PHASE STRUCTURES OF SATURATED LECITHIN BILAYERS AS A FUNCTION OF TEMPERATURE AND CHAIN LENGTH ((W.-J. Sun*, S. Tristram-Nagle†, R. M. Suter*, and J. F. Nagle*†)) *Department of Physics* and Department of Biological Sciences†, Carnegie Mellon University, Pittsburgh, PA 15213*

Systematic low-angle and wide-angle x-ray scattering studies have been performed on fully hydrated unoriented multilamellar vesicles of saturated lecithins with even chain lengths N_C from 16 to 24 as a function of T in the regular gel ($L_{\beta'}$) phase. For all N_C's as T increases, the area per chain A_C increases linearly with an average slope $dA_c/dT = 0.027 Å^2/°C$, and the lamellar D-spacings also increase linearly with an average slope $dD/dT = 0.039 Å/°C$. At the same T, longer chain lengths have more densely packed chains, i.e. smaller A_C's, than shorter chain lengths. The chain packings of longer chain lengths are found to be more distorted from the hexagonal packing than that of smaller N_C's, and the distortions of all N_C's approach the same value as the pretransitions are reached. The volume expansions of these lipids are found to be mainly due to the expansions in the hydrocarbon chain regions. Electron density profiles are reconstructed using four orders of low angle lamellar peaks. $D - X_{HH}$, the interfacial region thickness, is nearly constant (~20Å) for all N_C's and T. (Supported by NIH GM44976)

W-Pos227

STRUCTURE OF THE RIPPLE PHASE IN LECITHIN BILAYERS ((W.-J. Sun*, S. Tristram-Nagle†, R. M. Suter* and J. F. Nagle*†)) *Departments of Physics* and Biological Sciences†, Carnegie Mellon University, Pittsburgh, PA 15213*

The phases of the form factors are derived for the ripple ($P_{\beta'}$) phase in the lecithin bilayer system. By combining these phases with the experimental form factor amplitude data (D. C. Wack and W. W. Webb, Phys. Rev. A 40:2712-2730 (1989)), the electron density map of the ripple phase of DMPC is reconstructed and shown in the enclosed figure. These phases are obtained by fitting the above mentioned data to a 2D electron density model, which is constructed by convoluting an asymmetric triangular ripple profile with a trans-bilayer electron density profile. Accurate ripple structures, which include the ripple amplitude, the slopes of both the major and the minor sides of the ripple, the degree of ripple asymmetry, the bilayer head-head spacings and the water spacings are also achieved from the fitting. The advantage of this model over the "pattern recognition" method, which is done by finding the correct phase combination out of all possible combinations of 20 or so peaks, is quite obvious. Furthermore, this model provides a more direct, quantitative way of obtaining the degree of ripple asymmetry, compared to the qualitative connection between unit cell obliquity and ripple asymmetry made by other groups. (Supported by NIH GM44976)



W-Pos228

INFLUENCE OF LIPID HYDRATION ON HYDROCARBON CHAIN ORDER - A ^{2}H NMR STUDY. ((L.L.Holte and K.Gawrisch)) NIAAA, NIH, Bethesda, MD 20892-2088

Deuterium NMR order parameters are sensitive to perturbations in the area per lipid molecule as small as ±0.2 Å^2 which may result from changes in temperature or hydration. We determined chain order parameters for saturated DMPC-d54, monounsaturated SOPC-d35, and (six-fold) polyunsaturated SDPC-d35, as a function of hydration and temperature. At a lipid to water molar ratio of 1:6, saturated and monounsaturated PC's experience a collective increase in acyl chain order over the entire chain when compared with fully hydrated lipids at the same reduced temperature. When dehydrated, the docosahexaenoic acid-containing SDPC-d35 showed increased order mostly for methylene groups near the glycerol backbone but a much smaller increase for methylene groups near the center of the bilayer. The data may help to improve precision of values for hydrophobic thickness, area per molecule, and the thickness of the water layer between lipid bilayers obtained by diffraction methods.

W-Pos229

MEMBRANE STRUCTURE AT THE SOLID/WATER INTERFACE STUDIED WITH NEUTRON REFLECTIVITY AND ATOMIC FORCE MICROSCOPY

((B. W. Koenig[+], K. Gawrisch[+], S. Krueger[$], W. Orts[$], C. F. Majkrzak[$], N. Berk[$], and J. V. Silverton[#]))
[+] NIAAA, [#] NHLBI, NIH, Bethesda, MD 20892; [$]NIST, Reactor Radiation Division, Gaithersburg, MD 20899

Specular reflection of neutrons (SRN) and atomic force microscopy (AFM) were combined to study the structure of single lipid bilayers in water adsorbed to planar single crystalline silicon wafers. SRN provides the mean neutron scattering length density profile perpendicular to the solid/water interface. AFM yields a surface profile of the lipid coated interface, thus allowing insight into the lateral organization of the lipid film. AFM was used to optimize the experimental conditions for lipid adsorption at the interface of the wafer. The lipid forms a continuous layer with a height of approximately 50 Å which is interrupted by irregularly shaped uncovered areas which are 100 to 500 Å in size. A maximum surface coverage of 90 % could be achieved. SRN measurements were performed on several single phosphatidylcholine lipid bilayers below and above the main phase transition temperature of the lipid. Partial deuteration of the lipid as well as contrast variation of the water were applied. The measurements show a SiO_2 layer, a water layer between SiO_2 and lipid, and a single lipid bilayer. The thickness values obtained are in agreement with trends found in bulk samples by diffraction techniques.

W-Pos230

WATER PERMEATION ACROSS UNSATURATED BILAYERS MEASURED BY ^{17}O NMR.

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Equilibrium water permeation rates were measured across PC and PC/PE bilayers as a function of unsaturation (one to twelve double bonds per lipid), ethanol, and temperature. Using the ^{17}O NMR signal of water allowed measurement of permeation rates that are two orders of magnitude shorter than possible with the ^{1}H signal of water. Large, unilamellar liposomes of 87 nm diameter (as measured by dynamic light scattering) containing 50 mM $MnCl_2$ were prepared by extrusion, followed by dialysis. The final phospholipid concentration was determined by ^{31}P NMR and the entrapped water volume by ^{23}Na NMR. ^{2}H NMR on chain deuterated POPC did not indicate a perturbation of chain packing due to the presence of Mn^{2+}. The permeation coefficients increased dramatically with increasing unsaturation. Mixtures of DOPC and DOPE (1:1) have lower permeation rates than pure PC membranes. Addition of ethanol reduced water permeation. All permeation rates increased with temperature. Thermal activation energies of water permeation were identical for all lipid preparations except for the DOPC/DOPE mixture which gave values that were 30% higher.

W-Pos231

THE REDUCTION OF ACYL CHAIN PACKING FREE VOLUME BY CHOLESTEROL DEPENDS ON ACYL CHAIN UNSATURATION

((Drake C. Mitchell, and Burton J. Litman)) NIAAA, NIH, Rockville, MD 20892

In this study the fluorescent membrane probe diphenylhexatriene (DPH) was used to characterize L-C phase lipid bilayers containing a wide range of acyl chain polyunsaturation, with and without 30 mol% cholesterol. DPH anisotropy decays were analyzed with a model based on the P2-P4 formalism which characterizes acyl chain packing free volume. In pure phospholipid bilayers at 10 °C packing free volume increased monotonicaly with acyl chain unsaturation. However, at 40 °C 16:0, 18:1 PC and 16:0, 22:6 PC had equal packing free volumes. This indicates that unsaturation reduces the ability of the bilayer to convert increased thermal energy into increased packing free volume, which is consistent with the limited conformational degrees of freedom of polyunsaturated acyl chains. Packing free volume was reduced by ~50% by 30 mol% cholesterol in a bilayer consisting of 16:0, 18:1 PC, and by only ~20% in a bilayer consisting of di 22:6 PC. Changes in DPH fluorescent lifetime were interpreted as corresponding to changes in water penetration into the bilayer. Acyl chain unsaturation had a large effect on water penetration, with DPH in di 22:6 PC having an intensity-weighted average lifetime 2 ns. shorter than DPH in 16:0, 18:1 PC. The incremental change in water penetration into the bilayer due to cholesterol was the same regardless of acyl chain unsaturation. This suggests that the ability of cholesterol to reduce water penetration, presumably by reducing packing defects in the interfacial region, is not strictly coupled to its ability to reduce acyl chain packing free volume in the bilayer hydrophobic core.

W-Pos232

PHASE SEPARATION IN BILAYERS CONTAINING DISATURATED AND DIPOLYUNSATURATED ACYL CHAINS.

((C.D. Niebylski and B.J. Litman)) Section of Fluorescence Studies, LMBB, DICBR, NIAAA, NIH, Rockville MD, 20892.

In order to explain the packing properties of mixed chain polyunsaturated phospholipids (i.e. 18:0,22:6n3-PC), the mixing behavior of disaturated PC (di16:0 and di18:0) with the dipolyunsaturated PC, di22:6n3-PC, was studied with calorimetry and fluorescence microscopy. DSC of the mixed system revealed that the T_M of the low melting component (di22:6n3-PC) was unchanged, but T_M decreased and transition width increased for the higher melting component (disaturated PC). Low concentrations (7mol%) of di22:6n3-PC decreased the onset of the di18:0 melt to below 40°C, while >35mol% di22:6 further reduced the onset of the di18:0 melt to near 20°C. Enthalpy of the di18:0 melt did not significantly vary with increasing di22:6. These results are consistent with lateral phase separation of di18:0PC from the di22:6PC and demonstrates that the presence of a liquid-crystalline matrix of di22:6PC reduces the cooperativity of the melting of the di18:0PC gel phase. Phase separation in these mixed systems is supported by microscopic imaging. The de-mixing properties of disaturates from dipolyunsaturates suggest that preferential chain interactions govern the lateral packing properties of mixed chain polyunsaturated species (i.e. 18:0,22:6n3-PC). Studies have indeed supported the presence of preferential interactions between saturated acyl chains in mixed chain polyunsaturates (***Biochemistry*** 30:313-319,1991). Our findings highlight the degree to which saturated acyl chains self-associate when present in mixtures containing 22:6n3 acyl chains and thereby have the potential to promote the formation of lateral packing domains .

W-Pos233

THE EFFECT OF ELECTRIC FIELD ON THE DEUTERIUM AND PHOSPHORUS NMR SPECTRA OF LIPID DISPERSIONS.

((P.D. Osman, B.A. Cornell and F. Separovic)) CSIRO Division of Applied Physics, Lindfield NSW 2070, CSIRO DFST, PO Box 52, North Ryde, NSW 2113, Australia.

Solid-state NMR has been used to study the effect of electric fields on dimyristoylphosphatidylcholine (DMPC) deuterated at the choline methyls and the headgroup α and β methylenes. Hydrated DMPC dispersions and mixtures of DMPC with egg yolk phosphatidylcholine (EYPC) were placed between gold coated glass coverslips and subjected to fields ranging between 1 MVm^{-1} and 16 MVm^{-1}, using biphasic pulses which were generated either continuously or synchronously with the NMR pulse program[1]. Continuous frequencies varied from 100 Hz to 10 kHz, and the synchronous pulse lengths were between 1.0 ms and 100 ms. The effects observed included an irreversible collapse of the deuterium quadrupolar splitting and the appearance of a reversible isotropic line. P-31 NMR was used to monitor the lipid phase. Sample hydration and power dissipation were assessed using impedance spectroscopy.

1. Osman, P. and Cornell, B. (1994) *Biochim. Biophys. Acta* **1195**, 197-204.

W-Pos234

EFFECTS OF TEMPERATURE AND DIPALMITOYLGLYCEROL ON PHOSPHOLIPASE A_2 ACTIVITY. ((J.D. Bell, M. Burnside, J.A. Owen, M.L. Royall, and M.L. Baker)) Dept. of Zoology, Brigham Young University, Provo, Utah 84602.

Phospholipase A_2 catalyzes the hydrolysis of phospholipid to yield fatty acid and lysophospholipid. Bilayers composed of phosphatidylcholine initially resist catalysis upon addition of the enzyme. However, they become susceptible after a latency period as sufficient reaction products accumulate in the bilayer. Temperature near the thermotropic phase transition of the bilayer and the presence of saturated long-chain diacylglycerol modulate the effectiveness of the reaction products. Experiments with various fluorescent probes (dansyl-labelled phospholipid, 6-propionyl-2-(dimethylamino)naphthalene, 6-dodecanoyl-2-(dimethylamino)naphthalene, and pyrene-labelled fatty acid and lysolecithin) produced the following results regarding mechanisms by which temperature and diacylglycerol modulate the reaction. First, the length of the latency period as a function of temperature correlated with the apparent degree of binding of the enzyme to the bilayer surface at the end of the latency. Second, the reaction products disrupted the surface of the bilayer for both gel-phase and liquid crystalline phospholipid. This disruption correlated with the enhancement of phospholipase activity by the products. Depending on the aqueous calcium concentration, the disruption affected deeper regions of the bilayer in the liquid crystalline phase. Third, as previously established for fatty acid, the increase in phospholipase A_2 activity caused by reaction products coincided with lateral segregation of lysolecithin in the bilayer. Fourth, diacylglycerol promoted the lateral segregation of reaction products. This effect correlated with the tendency of diacylglycerol to augment the effect of reaction products below the phase transition temperature.

W-Pos235

THE LOCATION OF ETHANOL IN A LIPID BILAYER INVESTIGATED WITH 2-D NMR AND MAGIC ANGLE SAMPLE SPINNING. **((L.L.Holte and K. Gawrisch)) NIAAA, NIH, Bethesda, Maryland 20892-2088**

We have used the 2-dimensional NOESY experiment to investigate cross-relaxation between proton resonances of the monounsaturated phospholipid stearoyloleoyl-phosphatidylcholine (SOPC) and ethanol in multilamellar dispersions using Magic Angle Spinning (MAS). Cross-relaxation is strongest between resonance signals of the lipid headgroup, glycerol backbone, and upper parts of the acyl chain close to the glycerol. These measurements provide solid support for the preferential interaction of ethanol with the lipid-water interface. The same lipid resonances show cross peaks with water, indicating that ethanol and water compete for identical binding sites on the lipid.

W-Pos236

PLANAR LIPID BILAYERS: A TOOL TO STUDY GAMETE INTERACTIONS. ((A. Liévano, W. J. Lennarz*, K. Ohlendieck*, and A. Darszon)). Dept. Genética y Fisiología Celular, Instituto de Biotecnología-UNAM, Cuernavaca, México and *Dept. of Biochemistry and Cell Biology, SUNY at Stony Brook.

Fertilization involves intergamete plasma membrane fusion, and there is a need for model systems to study cell fusion. Membrane vesicles fusion with lipid bilayers provides a novel model for cell-cell interaction. We have previously shown that it is possible to transfer ion channels directly from mouse and sea urchin sperm to lipid bilayers. To fertilize, sperm must undergo the acrosome reaction (AR), which involves dramatic morphophysiological and permeability changes making sperm more fusogenic. We found that unreacted *S. purpuratus* sea urchin sperm had an ion channel transfer probability to lipid bilayers of 0.15. Experimental conditions that increase this probability are: AR (0.4), nystatin (0.9, a plasma membrane fusion monitor, *Biophys J.* **58**:833-839), and hypoosmotic media. Recently the egg-sperm receptor from *S. purpuratus* was purified and cloned (*Science* **259**:1421-1425). It binds to bindin, a sperm protein exposed during AR, and has a single putative transmembrane domain. Acrosome reacted sperm ion channel transfer increased (1.0) when this purified *S. purpuratus* egg-sperm receptor was previously added to the lipid bilayer.

Supported by DGAPA-UNAM, NIH and CONACyT. A. D. is an international Scholar of the Howard Hughes Medical Institute.

W-Pos237

DIPALMITIN- AND UNSATURATED FATTY ACID-INDUCED LIPID BILAYER PERTURBATIONS CORRELATE WITH SYNERGISTIC ACTIVATION OF PROTEIN KINASE C. ((E.M. Goldberg,* D.S.Lester,‡ D.B. Borchardt,§ and R. Zidovetzki*)) Departments of *Biology and §Chemistry, University of California, Riverside, CA 92521, and ‡DRT/CDER Food and Drug Administration, Laurel, Maryland 20708

Our previous studies have demonstrated that the saturated diacylglycerol (DAG) dipalmitin (DP) induces lateral phase separation of phosphatidylcholine/phosphatidylserine (4:1 mol/mol) bilayers into gel-like and liquid crystalline domains at 37°C. Under these conditions DP does not activate protein kinase C (PKC). We now show that addition of unsaturated arachidonic acid (AA) or docosahexaenoic acid (DHA) decreases the relative amount of the DP-induced gel phase, induces an isotropic phase and results in activation of PKC. Only a two-fold increase of PKC activity was observed with 25 mol% AA or DHA without DP, whereas addition of 25 mol% DP caused a ~20-fold increase in PKC activity with AA and ~50-fold with DHA. Palmitic acid did not significantly affect bilayer structure or PKC activity in the presence or absense of DP. The results demonstrate that the addition of unsaturated free fatty acids (FFAs) may enable DP to function as an activating DAG through effects on lipid bilayer structure. Further, the results may be relevant in understanding the synergism between unsaturated FFAs and DAGs in PKC activation.

W-Pos238

CERAMIDE INDUCES STRUCTURAL DEFECTS INTO PHOSPHATIDYLCHOLINE BILAYERS AND ACTIVATES PHOSPHOLIPASE A_2. ((R. Zidovetzki, E.M. Goldberg, and H.-W. Huang)) Department of Biology, University of California, Riverside, CA 92521.

We studied effects of bovine brain ceramide on the structure of dipalmitoylphosphatidylcholine (DPPC) bilayers and cobra venom phospholipase A_2 (PL-A_2) activity using ^2H NMR and specific enzymatic assays. Addition of ceramide to DPPC at 45°C induces lateral phase separation of the bilayers into regions of gel and liquid crystalline phases. The order parameters of the DPPC acyl chains in the liquid crystalline phase are only slightly affected by the presence of ceramide, indicating that the latter is largely partitioned in the gel phase of DPPC. At 60°C, above T_m, the presence of ceramide induced a large increase of the order parameters of DPPC side chains. No significant perturbation of DPPC structure was observed upon addition of 25 mol% sphingomyelin. The observed structural effects of ceramide correlated with ceramide-induced activation of cobra venom PL-A_2. Ceramide activated PL-A_2 in a concentration-dependent manner, with the significant effect observed at 5 mol% ceramide, which caused ~3-fold increase in PL-A_2 activity. The results showing activation of PL-A_2 by ceramide may provide an additional aspect of the biological effects of this second messenger and suggest a possibility of cross-talk between the sphingomyelinase and PL-A_2 signal transduction pathways.

W-Pos239

DIRECT IMAGING OF MOLECULE/ION TRANSPORT THROUGH BILAYER LIPID MEMBRANES USING SCANNING ULTRAMICRO-ELECTRODES. ((N.Ikeda, Y.Noritomi, and H.Nakanishi)) Advanced Research Lab., Research and Development Center, Toshiba Corp., Kawasaki 210, Japan

We have been studying the effect of light irradiation on electrical properties of the bilayer lipid membranes (BLMs) which include azobenzene (AZ) chromophores as a model of visual light processing[1]. In this study, we tried to measure the direct molecule/ion transport profile from the planar BLMs using scanning ultramicroelectrodes. In order to measure molecules or ions transported from the membranes, two kinds of electrodes were used; ion selective microelectrodes (50μmφ) and platinum microdisk electrodes (10μmφ). The molecule/ion transport was caused by the concentration gradient formed between the BLM consisted of monoolein and AZ amphiphilic derivatives. Various kinds of molecule/ion flow were investigated, such as K^+, Cl^-, p-benzoquinone, N,N,N',N'-tetramethyl-p-phenylenediamine, 1,4-diazabicyclo[2,2,2]octane, etc.

As a result, we succeeded to obtain spacial pattern of molecule/ion concentration at the distance between 50 μm – 500 μm from the BLM. It was also found that, in the case of K^+, the diffusion profile was changed by the 355 nm light irradiation which corresponded to the *trans–cis* photoisomerization of AZ in the BLM.

1)H.Nakanishi, Prog. Surf. Sci., 49, 197(1995).

W-Pos240

DIFFUSION THROUGH PORES AND CHANNELS. ((YE Korchev, GM Alder, GN Hill, C McGiffert, CA Pasternak, TK Rostovtseva & CL Bashford)) Division of Biochemistry, St. George's Hospital Medical School, Cranmer Terrace, London SW17 0RE, UK.

The rates at which ions ($^{86}Rb^+$, [^{3}H]-choline, $^{36}Cl^-$, ^3H_2O and nonelectrolytes ([^{14}C]-urea, [^{14}C]-glycerol, and [^{14}C]-sugars) equilibrate across pores in track-etched polyethyleneterephthalate (PETP) membranes depends on: 1) pore size: all tracers equilibrate faster the longer the membranes are etched; 2) ionic strength; and 3) pH; (2) and (3) affect ions but not neutral solutes or water. Diffusion of cations decreases: (a) as ionic strength increases, (b) as pH decreases and (c) after addition of Zn^{2+} or Ca^{2+}; under these conditions $^{36}Cl^-$ diffusion increases. The decreased cation diffusion found under these conditions contributes to decreased ionic conductance. In general small pores exhibit greatest ion selectivity. However, some low-conducting states (low pH, high ionic strength) of individual pores have less ion selectivity than high-conducting states. Similar results are found in toxin-induced pores in planar lipid bilayers. We conclude that charged surfaces differentially modulate flow of ions and nonelectrolytes and may contribute to the special features of ion conductance seen in biological pores and channels.

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W-Pos241

COMPARISON OF THE INTERACTIONS OF CONJUGATED AND UNCONJUGATED BILE SALTS WITH MODEL MEMBRANES. ((K.A. Parthum[1], K.M. Phillips[1], S.A. Tomellini[1] and I.W. Levin[2])) [1]Chemistry Dept., University of New Hampshire, Durham, NH 03824, [2]Laboratory of Chemical Physics, NIDDK, NIH, Bethesda, MD 20892. (Spon. by S.A. Tomellini)

Infrared spectroscopic studies were performed to evaluate the effects of various bile salt species on a model membrane. Specifically, the interactions of the unconjugated, taurine-conjugated, and glycine-conjugated forms of the two bile salt epimers, sodium ursodeoxycholate and sodium chenodeoxycholate, with multilamellar vesicles of dipalmitoylphosphatidylcholine (DPPC) were examined. The DPPC bilayers were hydrated using solutions with initial pH's of 5, 7, and 8. Experiments were also performed using hydrating solutions containing ethanol concentrations within the range known to induce interdigitation of the DPPC acyl chains. Significant differences in bilayer perturbations occur depending upon bile salt species, solution pH, and bilayer architecture. For example, the effect of the bile salts on the cooperativity of the phase transition for the DPPC bilayer are reduced in the presence of ethanol.

W-Pos242

THERMAL PHASE BEHAVIOR AND PACKING CHARACTERISTICS OF FULLY HYDRATED BILAYERS COMPOSED OF DHPC AND DPPE.
((M. M. Batenjany, J. T. Mason, I. W. Levin* and T. J. O'Leary)) Department Of Cellular Pathology, AFIP, Washington, D. C. 20306 and *Laboratory of Chemical Physics, NIDDK, NIH, Bethesda, MD 20892.

The thermal behavior and packing characteristics of multilamellar vesicles (MLVs) composed of DHPC and DPPE in excess water were studied using Differential Scanning Calorimetry (DSC) and Raman spectroscopy. DSC heating thermograms indicate 0-6 mol% DPPE causes a downward shift in the pretransition temperature of DHPC bilayers ($L_{\beta i} \rightarrow L_\beta$) from 33.5 °C to 27 °C and results in a complete loss of a DHPC pretransition above 8 mol% DPPE. In addition, an increase in DPPE (0-100 mol%) shows a linear rise in the main phase transition temperature of this binary mixture from 43.5 °C to 63.5 °C. At each binary mixture, a single main transition was observed. Moreover, the corrected phase diagram (Mabrey, S. & Sturtevant, J. (1976) *Proc. Natl. Acad. Sci.* 73:3862-3866) is consistent with an ideal phase relationship, which suggests fully miscible mixtures of these phospholipids from 0-100 mol%. Raman spectroscopy, using d-62-DPPE was utilized to delineate differences in the hydrocarbon packing characteristics of DPPE and DHPC within these mixtures, as well as, to identify the presence of any lateral phase separations of these lipids. Based upon the DSC and Raman spectroscopic data a phase diagram for DHPC/DPPE MLVs is constructed and the packing characteristics of binary mixtures of these phospholipids in excess water are discussed.

W-Pos243

MOLECULAR DYNAMICS SIMULATION OF A PLPC LIPID BILAYER: EFFECTS OF TWO DOUBLE BONDS
((Marja Hyvönen[1,2]*, Mika Ala-Korpela[1,2,3], Juha Vaara[1], Tapio.T. Rantala[1] and Jukka Jokisaari[1])) [1]Department of Physical Sciences, University of Oulu, Oulu, Finland; [2]A.I. Virtanen Institute for Molecular Sciences, University of Kuopio, Kuopio, Finland; [3]Hammersmith Hospital, NMR Unit, London, U.K..
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Structural variations of lipid molecules, such as length and unsaturation state of the fatty acid chains, are known to alter metabolic attributes of membranes. In this study, we have assessed the effects of two double bonds on the hydrocarbon interior using biologically relevant PLPL molecules (1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphatidylcholine, 16:0/18:2).

A PLPC lipid monolayer (consisting of 36 PLPC molecules plus 1368 water molecules) was simulated using the CHARMm molecular modeling package. Use of periodic boundary conditions and rotation-reflection make the simulation to model an infinite bilayer covered with water on both sides.

The orientational behavior of the fatty acid chains in the PLPC bilayer was found to be seemingly different from that in monounsaturated or in saturated phospholipid bilayers [Hyvönen *et. al.*, Chem. Phys. Letters, in press]. Particularly, the double bond regions of the sn-2 chains seem to induce structural changes into the parts of the saturated sn-1 chains which are at the same depth in the layer. Thus, our results point out that marked changes in the membrane structure can arise from slight changes in the unsaturation state, which elucidates the crucial role of the double bonds in membrane functions.

W-Pos244

FRACTIONAL STEROL CONCENTRATION DEPENDENCE OF ACRYLAMIDE QUENCHING OF DEHYDROERGOSTEROL FLUORESCENCE IN LIQUID-CRYSTALLINE PHOSPHOLIPID BILAYERS. ((Mei-Mei Wang and Parkson Lee-Gau Chong)) Dept. of Biochemistry, Temple University School of Medicine, Philadelphia, PA 19140.

In previous studies, we used fluorescence intensity dips (1) and fluorescence polarization peaks (2) as evidence that dehydroergosterol (DHE) and cholesterol can be regularly distributed into hexagonal superlattices in lipid bilayers. We hypothesized that the ratio of regularly distributed areas to irregularly distributed areas reaches a local maximum at critical sterol mole fractions and that the vertical position of sterols in the regular region is different than in the irregular region. In the present study we have examined the fractional sterol concentration dependence of acrylamide quenching of DHE fluorescence in the liquid-crystalline state of DHE/DMPC, DHE/cholesterol/DMPC and DHE/ergosterol/DMPC mixtures. It was found that the quenching rate constant reaches a local maximum at the critical sterol concentrations, at which DHE fluorescence dips are observed. This result supports our hypothesis that in the regular region, sterol molecules are embedded less deep into the bilayer and experience a higher dielectric constant (1). This result not only explains why fluorescence intensity drops at critical mole fractions but also provides additional supporting evidence for sterol regular distribution in both two-component and three-component lipid membranes (supported by AHA).

(1) Chong (1994) *Proc.Natl.Acad.Sci.USA* 91:10069-10073.
(2) Chong (1994) the Proceedings of the 23rd Steenbock Symposium.

W-Pos245

REGULAR DISTRIBUTION OF CHOLESTEROL AND ERGOSTEROL IN THREE-COMPONENT LIPID MEMBRANES. ((Fang Liu*, Istvan P. Sugar# and Parkson Lee-Gau Chong*)) *Dept. of Biochemistry, Temple University School of Medicine, Philadelphia, PA 19140 and #Dept. of Biomathematical Science, Mt. Sinai Med. Center, New York, NY 10029.

We have extended our previous work (1,2) of sterol regular distribution from two-component systems to three-component systems. We have examined the fractional sterol concentration dependence of dehydroergosterol (DHE) fluorescence in the liquid-crystalline state of DHE/cholesterol/DMPC and DHE/ergosterol/DMPC mixtures. Both fluorescence intensity and fluorescence lifetime exhibit local minima at the sterol concentrations previously reported for lipid regular distribution into hexagonal superlattices (1). In addition to the fluorescence dips for hexagonal superlattices, we have observed intermediate fluorescence dips at concentrations predicted by the square superlattice model (3). However, the dips for square superlattices in DHE/ergosterol/DMPC mixtures become diminished after long incubation (~14 days), whereas the dips for square superlattices in DHE/cholesterol/DMPC mixtures remain discernible after long incubation at room temperature. This result suggests that subtle differences in sterol conformation cause changes in sterol lateral organization in membranes. Apparently, in the case of DHE/ergosterol/DMPC, the square superlattices are metastable relative to the hexagonal superlattices (supported by AHA).

(1) Chong (1994) *Proc.Natl.Acad.Sci.USA* 91:10069-10073.
(2) Chong (1994) the Proceedings of the 23rd Steenbock Symposium.
(3) Virtanen et al. (1995) *Biochemistry* 34:11568-11581.

W-Pos246

PHYSICOCHEMICAL CHARACTERIZATION BY ^2H AND ^1H NMR OF AQUEOUS LIPID DISPERSIONS RESEMBLING BILE. ((P. W. Westerman*†, R. Jacquet*, B. Quinn*, P. Rinaldi§ and Y. Sun§)) *Northeastern Ohio Universities College of Medicine, Rootstown, Ohio 44272; †Kent State University, Kent, Ohio 44242 and §University of Akron, Akron, Ohio 44325.

The phase properties of aqueous dispersions of model bile mixtures containing cholesterol, lecithin and bile salts have been characterized by ^2H NMR. We have chemically incorporated a deuteriomethyl (CD_3) group into one lipid component, and utilized the differences in magnitude of the motionally-averaged quadrupole splitting ($\delta\nu$) to determine by spectral integration, the distribution of that lipid between solid, multilamellar and micellar phases. By ^2H-labelling both cholesterol and lecithin in a system of a given overall composition we have used the different CD_3 chemical shifts and $\delta\nu$ values, to determine directly the chemical composition of the micellar and multilamellar phases. ^2H relaxation times of C^2H_2 sites in the lecithin component have been measured for the micellar phases in these same mixtures. Differences between T_1 and T_2 values were employed to estimate particle size, assuming either a spherical or ellipsoidal shape. The structure of the micellar particles in model bile systems were investigated by ^1H NMR. 600 MHz 1D and 2D ^1H NMR spectra of cholesterol/lecithin/taurocholate (NaTC) dispersions in D_2O were recorded at 37°C. On the basis of cross-peaks in 2D homonuclear NOESY experiments a "golf-ball" like spherical structure for the mixed micelles is proposed.

LIPID-PROTEIN INTERACTIONS

W-Pos247

STUDY OF THE INTERACTION BETWEEN ACTIN AND LIPID BILAYERS BY FTIR AND ^{19}F NMR ((Mario Bouchard*, Chantal Paré*, Jean-Pierre Dutasta[%], Jean-Paul Chauvet[%], Claude Gicquaud[#], and Michèle Auger*)) *Département de Chimie, CERSIM, Université Laval, Québec, Canada, G1K 7P4, [%]École Normale Supérieure de Lyon, Lyon, France, [#]Département de Chimie-Biologie, UQTR, Trois-Rivières, Québec, Canada, G9A 5H7.

Actin is a ubiquitous cytosqueletal protein which is involved in cell motility and morphogenesis. Actin can exist as a monomer, G-actin, or as a polymer, F-actin. The polymerization of G-actin into F-actin can be induced by millimolar concentrations of salts. We have investigated by attenuated total reflectance (ATR) infrared spectroscopy the effect of F-actin on the acyl chains of several lipids with different chain lengths and polar head groups. The results indicate that the conformational order of the lipid acyl chains is not affected by the presence of F-actin. On the other hand, we have selectively attached a fluorinated probe, 3-bromo-1,1,1-trifluoropropanone, on the cysteines 10, 284 and 374 of the protein. By ^{19}F NMR spectroscopy, we have observed a conformational change of these cysteines in the presence of some charged lipids. More specifically, the results obtained indicate a conformational change of G-actin in the presence of DMPG, a negatively charged lipid, and in the presence of positively charged liposomes made of DMPC and stearylamine.

W-Pos248

MODEL OF INTERACTION BETWEEN A CARDIOTOXIN AND DIMYRISTOYLPHOSPHATIDIC ACID BILAYERS DETERMINED BY SOLID-STATE ^{31}P NMR SPECTROSCOPY. ((Frédéric Picard*, Michel Pézolet*, Pierre E. Bougis[%] and Michèle Auger*))*Département de Chimie, CERSIM, Université Laval, Québec, Québec, Canada, G1K 7P4, [%]Laboratoire de Biochimie, URA 1455 du CNRS, Institut Fédératif de Recherche Jean Roche, Université de la Méditerranée, Faculté de Médecine Secteur Nord, Bd. P. Dramard, 13326 Marseille Cedex 20, France.

The interaction of cardiotoxin IIa, a small basic protein extracted from *Naja mossambica mossambica* venom, with dimyristoylphosphatidic acid (DMPA) membranes has been investigated by solid-state ^{31}P nuclear magnetic resonance spectroscopy. Both the spectral lineshapes and transverse relaxation time values have been measured as a function of temperature for different lipid-to-protein molar ratios. The results indicate that the interaction of cardiototoxin with DMPA gives rise to the complete disappearance of the bilayer structure at a lipid-to-protein molar ratio of 5:1. However, a coexistence of the lamellar and isotropic phases is observed at higher lipid contents. In addition, the number of phospholipids interacting with cardiotoxin increases from about 5 at room temperature to approximately 15 at temperatures above the phase transition of the pure lipid. The isotropic structure appears to be an inverted micellar phase that can be extracted by a hydrophobic solvent.

W-Pos249

INTERACTIONS OF THE NBF-1 DOMAIN OF CFTR WITH PHOSPHOLIPID MEMBRANES.
((Shoshana BarNoy, Peter McPhie, Y. Wang, Eric Sorscher, George Lee, Ofer Eidelman, and Harvey B. Pollard)) LCBG & LBP, NIDDK, NIH, Bethesda, MD, and Departments of Physiology and Medicine, University of Alabama at Birmingham, Birmingham, AL.

The nucleotide binding fold domain (NBF-1) of CFTR is the locus of the main ΔF508 mutation in cystic fibrosis. Even though NBF-1 has been assumed to be principally cytosolic, the 20 kDa rNBF-1 was shown to form ion-conducting pathways in planar lipid bilayers. Recently, rNBF-1 has been shown to gain access to the extracellular side of the membrane. To further understand the molecular basis of lipid interactions with NBF-1 we studied the reciprocal interactions of both components. rNBF-1 induced the permeabilization of PS membranes to calcein, a large, negatively charged dye molecule, in a dose dependent manner. This process was dependent on ionic strength and on lipid composition. Permeabilization caused by rNBF-1(ΔF508) was characteristically different. rNBF-1 also drove the aggregation of PS liposomes, in a sub-milimolar Ca-dependent manner. On the other hand, addition of PS liposomes induced conformational changes in the polypeptide as evidenced by tryptophan emission, accessibility to aqueous quenchers and CD.

W-Pos250

MODELING MEMBRANE-PROTEIN INTERACTIONS: A *de novo* DESIGN FOR A PEPTIDE THAT INSERTS INTO LIPID BILAYERS. ((L. A. Chung and T. E. Thompson)) University of Virginia, Biochemistry Dept., Charlottesville, VA 22908.

The biogenesis of all membrane proteins requires the partitioing of hydrophobic sequences into a non-polar environment. This is true for *sec*-independent proteins, which do not need a fully functional translocation complex; spontaneously inserting proteins, which incorporate into membranes post-translationally; and membrane proteins that must be released from the translocon complex into the surrounding lipid. To study the partitioning of hydrophobic sequences from polar to non-polar environments, we have designed a peptide sequence, H_2N-Ala_2-Leu_3-Ala_{22}-Tyr-Lys_6-$CONH_2$, that is highly soluble in buffer and also incorporates into lipid bilayers from aqueous solutions. The solution and membrane-bound conformations of the peptide were characterized with infrared (IR) spectroscopy and confirmed with circular dichroic (CD) spectroscopy. Using Fourier Transform IR (FTIR) we analyzed both the solution and membrane-bound structures of the peptide while Attenuated Total Reflectance IR (ATR-IR) yielded an orientation for the helical peptide in a planar lipid bilayer. Recently, new information obtained from curvefitting analysis of the solution spectra gave three conformations for the peptide in solution: helix, β-sheet, and extended structures. Curvefitting analysis also gave evidence for the lipid-bound peptide in helical, unordered, and β-structures with the helical conformation oriented perpendicular to the plane of the bilayer. Here we report both the results of our curvefitting analysis and a revised model for the binding and insertion of this peptide into lipid bilayers. (This work is funded by NIH grant GM-14628.)