W-Pos297

EXTENDED DIPOLAR CHAIN MODEL FOR ION CHANNELS: ELECTROSTRICTION EFFECTS AND THE TRANSLOCATION ENERGY BARRIER. ((Miguel Sancho*, Michael B. Partenskii and Peter C. Jordan)) Department of Chemistry, Brandeis University, Waltham, MA 02254; *Departamento de Fisica Aplicada III, Universidad Complutense de Madrid, E-28040 Madrid, Spain.

The dipolar chain model introduced to describe the aqueous pore of single-file transmembrane ion channels (TMIC) indicates that the susceptibility of water in unoccupied channels is high while the presence of a monovalent ion reduces water's orientational susceptibility to nearly zero (Partenskii, Cai & Jordan, Chem. Phys., 153, 125 [1991]). The associated energy barrier for translocation is high, calling into question the electrostatic rationalization of channel function. Static ion-pore interaction is insufficient to lower the energy barrier to physiologically reasonable values (Partenskii and Jordan, Quart. Rev. Biophys., in press). Here, we modify the analysis to account for non-electrostatic ion-water and water-water Lennard-Jones interactions. We focus on a model system with gramicidinlike dimensions and find that electrostriction has only a limited influence on the total energy barrier for permeation. However, there is a clear ion-dependent modulating effect on translocation that corresponds to Eisenman selectivity sequence I (low site field strength), in complete accord with experiment. The computed ratios of translocation rates are in reasonable agreement with those found experimentally.

W-Pos298

INFLUENCE OF A CHANNEL FORMING PEPTIDE ON ION ENERGY BARRIERS VIEWED IN A CONTINUOUS THREE-DIELECTRIC MODEL FOR ION CHANNELS. ((Michael B. Partenskii and Peter C. Jordan)) Department of Chemistry, Brandeis University, Waltham, MA 02254.

A three dielectric model of transmembrane ion channels of finite length is presented, with ϵ_1 the permittivity of interchannel water ($r < R_1$), ϵ_2 that of the highly polarizable region of the channel forming peptide ($R_1 < r < R_2$) and ϵ_3 that of the remainder of the system, the membrane and the nonpolar regions of the peptide ($r > R_2$). Setting $\epsilon_w = \infty$ for bulk water and using the method of images the electrostatic problem is exactly soluble. Comparison with numerical solutions to the Poisson-Boltzmann equation with $\epsilon_w = 80$ demonstrates that the model is accurate, especially for ionic strengths ≥ 0.5M. The analysis is applied to a system with gramicidinlike geometry. Choosing $\epsilon_1 \sim 4$-5, to account for the strongly nonlinear dielectric behavior of water in the ionic field (Partenskii and Jordan, J. Phys. Chem., 96, 3906 [1992]), we varied ϵ_2 to assess the possible influence of peptide. It appears difficult to reproduce the results of our previous semimicroscopic study of the influence of the carbonyls on the ionic energy barrier within a continuum approach for reasonable values of ϵ_2. The discrepancies arise because ϵ_2 is actually nonlocal and because the model's fixed geometry can not account for significant local reorientation of polar groups near the ion.

SARCOPLASMIC RETICULUM I

W-Pos299

BLOCK OF THE RYANODINE-MODIFIED SR Ca^{2+}-RELEASE CHANNEL. ((A. R. G. Lindsay, A. Tinker and A. J. Williams.)) National Heart and Lung Institute, University of London, LONDON, SW3 6LY, UK.

We have previously reported that the large, impermeant, cations tetrabutyl ammonium (TBA), tetrapentyl ammonium and the local anaesthetic QX314 interact with the cytosolic vestibule of the conduction pathway of the purified sheep cardiac sarcoplasmic reticulum (SR) ryanodine receptor-channel. The interaction of each of these cations results in the production of a unique reduced conductance state, the occurrence of which is both voltage- and concentration-dependent (Tinker et al., 1992, Biophys. J., 61: 1122-1132). Following modification with ryanodine, which causes the receptor-channel to enter a reduced conductance state with long open dwell time, these cations block the receptor-channel to a level which is indistinguishable from the closed state. The voltage-dependence of TBA's interaction both before and following modification was determined by Boltzmann analysis and by determining k_{on} and k_{off}. In control, the two methods yield an equivalent gating charge of 1.66 and 1.81 respectively. Following modification these values are 1.74 and 1.61. TBA concentration dependence has been examined in control and after modification; K_ds are 45 and 165 μM respectively. We suggest that the interaction of ryanodine with the receptor-channel involves an alteration to the cytosolic vestibule which is reflected in the differing affinity of the TBA site. We have also examined the interaction of several polycations which are known to inhibit Ca^{2+}-release from isolated SR vesicles. Gentamycin, neomycin, streptomycin, spermine and spermidine block the ryanodine-modified receptor-channel. Unlike the large tetraalky ammonium cations, these polycations block to a reduced conductance state.

This work was supported by the British Heart Foundation and the MRC.

W-Pos300

CHARGED LOCAL ANAESTHETICS BLOCK THE SHEEP CARDIAC RYANODINE RECEPTOR-CHANNEL. ((A.Tinker and A.J. Williams.)) National Heart & Lung Institute, University of London, London SW3 6LY, UK.

We have examined the effect of QX314, QX222 and Procaine on K^+ conduction in the ryanodine receptor-channel purified from sheep cardiac sarcoplasmic reticulum. QX222 and Procaine act as classical voltage-dependent blockers from the cytoplasmic face of the channel. At $\leq$ 500 Hz filtering this is manifest as a smooth reduction in single-channel current amplitude at positive holding potentials. Quantitative analysis gives an effective valence of approximately 0.9 for both ions and $K_d(0)$s of 9.2 and 15.8 mM for QX222 and Procaine respectively. Analysis of the concentration-dependence suggests that QX222 is binding to a single site with a K_m of 491 μM at 60 mV holding potential. Investigation of the excess open channel current noise at higher frequencies of filtering by amplitude distribution analysis reveals that both the voltage-dependence and concentration-dependence occur due to changes in K_{on}. The addition of QX314 to the cytosolic face of the channel is different and leads to the production of a substate with an amplitude ½ of control. Quantitative analysis reveals that the effect is highly voltage-dependent with a valence of approximately 1.5 accounted for by nearly equal changes in K_{on} and K_{off}. Analysis of concentration-dependence reveals positive co-operativity due to changes in K_{on} with at least two QX314s binding to the conduction pathway. A paradoxical increase in K_{off} at high positive holding potentials and with increasing concentration at 80 mV suggests the existence of a further QX314-dependent reaction. The substate block is interpreted as a form of partial occlusion in the vestibule of the conduction pathway reducing current by electrostatic means.

Supported by the MRC and BHF.

W-Pos301

HYDROGEN PEROXIDE MODIFIES THE GATING OF THE SHEEP CARDIAC SARCOPLASMIC RETICULUM CALCIUM-RELEASE CHANNEL ((A. Boraso & A. J. Williams)) National Heart & Lung Institute, University of London, LONDON SW3 6LY, UK

Some pathophysiologic conditions cause an overproduction of reactive oxygen species (ROS) to such an extent that intracellular defence mechanisms may be unable to limit the damage. The ROS singlet oxygen has been applied to the cardiac sarcoplasmic reticulum (SR) Ca^{2+}-release channel and shown to cause an initial increase in open probability (Po), followed by irreversible loss of channel function (Holmberg et al. Cardioscience (1991), 2: 19-25). The effect of the ROS hydrogen peroxide has been investigated on the channel in this study. Vesicles of heavy SR from sheep cardiac muscle were incorporated into planar phospholipid bilayers and Ca^{2+} used as the permeant ion. Free $[Ca^{2+}]$ on the cytosolic side of the channel was initially 10μM and was 40-60mM on the luminal side. At 10μM cytosolic $[Ca^{2+}]$ hydrogen peroxide (1-10mM) increased the open probability of the sheep cardiac SR Ca^{2+}-release channel, but no loss of channel function was observed. In a second series of experiments, the cytosolic $[Ca^{2+}]$ was lowered from 10μM to 0.5nM. The Po of the Ca^{2+}-release channel dropped to zero and, under these conditions, hydrogen peroxide (3-5mM) produced openings within 3-5mins. After washing out hydrogen peroxide, raising the $[Ca^{2+}]$ to 10μM or lowering it to nM or pM levels no longer modified the Po of the channel. No change in channel amplitude at 0mV was seen. The ryanodine binding site appeared to be unaffected by hydrogen peroxide (3mM) since ryanodine (1-2μM) still characteristically locked the channel into a subconductance open state. These results suggest that hydrogen peroxide induces Ca^{2+}-independent openings of the sheep cardiac SR Ca^{2+}-release channel.

W-Pos302

ACTIVATION OF THE SHEEP CARDIAC CA^{2+}-RELEASE CHANNEL BY CARDIAC GLYCOSIDES : STUDIES ON THE STRUCTURAL REQUIREMENTS FOR ACTIVITY. ((S.J.McGarry and A.J. Williams)), National Heart and Lung Institute, University of London, LONDON, SW3 6LY, UK.

The effects of cardiac glycosides on sheep cardiac and skeletal sarcoplasmic reticulum (SR) Ca^{2+}-release channels have been studied using rapid $^{45}Ca^{2+}$ efflux from SR vesicles and at the single-channel level. Therapeutic concentrations of digoxin (1nM) increased rapid $^{45}Ca^{2+}$ efflux at 0.1μM extravesicular Ca^{2+} from 144 $\pm$ 20 to 258 $\pm$ 24 nmoles $^{45}Ca^{2+}$/mg protein/sec. At an activating $[Ca^{2+}]$ of 0.1μM, 1nM digoxin increased single-channel Po from 0.012 $\pm$ 0.003 to 0.110 $\pm$ 0.029 when added to the cytosolic face of the channel; at 10μM Ca^{2+}, 1nM digoxin increased Po from 0.048 $\pm$ 0.012 to 0.281 $\pm$ 0.065. The increase in Po was achieved mainly by increasing the frequency of channel opening. However, higher concentrations of digoxin increased Po by increasing the duration of open events as well, giving an Ec50 of approximately 0.91nM for digoxin. Ouabain was less potent than digoxin. At 10μM Ca^{2+} 10nM ouabain increased Po from 0.040 $\pm$ 0.011 to 0.232 $\pm$ 0.061. There was no effect of cardiac glycosides on skeletal muscle Ca^{2+}-release channels. Prednisolone, β-estradiol, chlormadinone and spironolactone failed to activate the cardiac SR Ca^{2+}-release channel or affect the digoxin-activation of the channel. Similarly, digoxigenin and ouabagenin, and digitoxose and rhamnose, at up to 100μM failed to increase single-channel Po. The genins also failed to affect the digoxin or ouabain-induced increases in Po. In conclusion, both sugar moieties and a genin or steroid nucleus are required for binding to the glycoside site on the cardiac channel and the composition, and possibly the number, of the sugar residues are determinants of activity.

Supported by The British Heart Foundation.

W-Pos303

REGULATION OF THE SHEEP CARDIAC SARCOPLASMIC RETICULUM Ca^{2+}-RELEASE CHANNEL BY LUMINAL Ca^{2+}.

((R. Sitsapesan & A.J. Williams)) National Heart and Lung Institute, University of London, LONDON SW3 6LY, UK.

We have demonstrated that when the sheep cardiac sarcoplasmic reticulum (SR) Ca^{2+}-release channel is activated solely by cytosolic Ca^{2+}, changes in luminal $[Ca^{2+}]$ have no effect on steady-state open probability (Po) (Sitsapesan & Williams, 1992, J. Physiol. 446, 552P). We now report on the effects of changes in luminal $[Ca^{2+}]$ when the channel is activated by the phosphodiesterase inhibitor, sulmazole, at sub-activating cytosolic $[Ca^{2+}]$. Current fluctuations were recorded in symmetrical 250mM Cs^+ PIPES, pH 7.2, at $\pm$40mV after incorporation of isolated SR vesicles into planar lipid bilayers. With 100pM free $[Ca^{2+}]$ on both sides of the channel, Po was zero and was unchanged by addition of sulmazole (1-10mM). At 10μM luminal $[Ca^{2+}]$, occasional brief openings (mean open time < 1ms) were observed at +40mV and longer openings (>2ms) were observed at -40mV. At 1-4mM luminal $[Ca^{2+}]$, large increases in Po and mean open time (>3ms) were observed at both +40mV and -40mV. The effects of luminal Ca^{2+} were completely reversible and could not be reproduced with other cations (Ba^{2+},1-10mM; Cs^+, 1.1M; K^+, 1.2M). Sulmazole had no effect on single channel conductance. Similar results were obtained with the purified ryanodine receptor-channel in symmetrical 210mM KCl. The results indicate that luminal $[Ca^{2+}]$ may be an important regulator of the cardiac SR Ca^{2+}-release channel.

This work was funded by the British Heart Foundation.

W-Pos304

SKELETAL MUSCLE CALCIUM RELEASE CHANNEL (RYANODINE RECEPTOR) AND ITS MODULATION BY FATTY ACIDS IN MALIGNANT HYPERTHERMIA.

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Human anesthesia-induced malignant hyperthermia (MH) is associated with a loss of Ca^{2+} regulation in skeletal muscle. Swine with porcine stress syndrome (PSS) exhibit signs similar to those observed in MH humans when exposed to triggering anesthetic agents. A defect in the ryanodine receptor (*ryr*$_1$) has been identified as the cause of PSS (Science 253 448, 1991). The present study examines whether a defect in *ryr*$_1$ alone can trigger the MH syndrome, or even abnormal Ca^{2+} release, in the absence of fatty acids (FAs). Swine confirmed as homozygous for the PSS mutation, while found to be MH susceptible, were unable to trigger into an MH syndrome at all times. Under physiological conditions anesthetic (halothane)-induced Ca^{2+} release was normal in a heavy sarcoplasmic reticulum fraction from porcine and human MH muscle. FAs, which are elevated in MH muscle, greatly reduced the threshold of halothane-induced Ca^{2+} release from the terminal cisternae in a temperature-dependent manner (antagonized only at 37°C by ruthenium red), in agreement with the known temperature dependence of halothane-induced contractures in MH muscle. While human MH exhibits heterogeneity, a mutation in *ryr*$_1$ is necessary, but not sufficient, for PSS. Elevated FA production appears to be required for the altered structure of the *ryr*$_1$ to be manifest as an abnormality in halothane-induced Ca^{2+} release.

W-Pos305

MODULATION BY CAFFEINE OF THE RYANODINE RECEPTOR CA2+ CHANNEL PURIFIED FROM NORMAL AND MALIGNANT HYPERTHERMIA SUSCEPTIBLE PIGS.

((N.H. Shomer, J.M. Mickelson, and C.F. Louis.)) University of Minnesota, Dept. of Vet. Biology St. Paul, MN 55108

We have previously demonstrated that a mutation in the skeletal muscle sarcoplasmic reticulum Ca^{2+} release channel (CRC) results in a defect in the Ca^{2+} regulation of the CRC derived from pigs with the inherited skeletal muscle disorder Malignant Hyperthermia (MH). We have now tested the hypothesis that the altered caffeine sensitivity of intact MH muscle could be attributed to a defect in the single channel properties of the purified, detergent-solubilized CRC isolated from MH pigs. All channel recordings were made in 200 mM CsCl, 20 mM MOPS, pH=7.4. Under these conditions, addition of up to 10 mM caffeine to the cis (cytoplasmic) side of the bilayer had no effect on the single channel conductance of either MH or normal channels. In the presence of 0.125μM Ca^{2+}, which is well below the Ca^{2+} required for maximal channel opening (7μM), caffeine had little effect on channel open time and gating kinetics; there were no differences between MH and normal channels with respect to these properties. In the presence of optimal activating calcium (7μM), caffeine applied to the cis side of the bilayer significantly increased the open probability of both MH and normal channels. The addition of 10 mM caffeine resulted in both a 2-4 fold increase in percent open time compared to baseline and a profound change in gating kinetics; CRCs derived from MH pigs did not differ significantly from normal CRCs in their sensitivity to caffeine. We conclude that the altered caffeine sensitivity of intact MH pig muscle does not appear to be reflected in an altered open channel probability of the purified MH CRC. Supported by NIH grant GM 31382.

W-Pos306

CALMODULIN INTERACTION WITH THE SR CALCIUM CHANNEL PROTEIN IN NORMAL AND MALIGNANT HYPERTHERMIA SUSCEPTIBLE MUSCLE.

((H.-C. Yang, M.M. Reedy, J.R. Mickelson, C.F. Louis, and G.M. Strasburg)) Michigan State Univ. and University of Minnesota

The skeletal muscle disorder malignant hyperthermia (MH) derives from a mutation in the sarcoplasmic reticulum (SR) Ca channel protein which, in turn, alters SR Ca channel function. We examined the binding of calmodulin (CaM), a partial inhibitor of SR Ca release, to the channel protein in SR from normal and MH susceptible swine to determine whether an abnormality in CaM regulation of the channel protein is associated with the altered Ca release in MH. Wheat germ CaM was labeled with rhodamine-maleimide, and its binding to the channel protein in SR was determined by fluorescence anisotropy. Binding of CaM to the high affinity class of sites in the channel protein in normal and MH SR was not significantly different in the presence of 0.1 mM $CaCl_2$ (B_{max1} = 46 pmol/mg, K_{d1} = 11 nM). Based on ryanodine binding of 10 pmol/mg, this corresponds to approximately 1 CaM/channel protein subunit. Addition of 1 mM $MgCl_2$ with 0.1 mM $CaCl_2$ resulted in a shift of B_{max1} to 20 pmol/mg (K_{d1} = 2 nM) for MH SR and to 10 pmol/mg (K_{d} = 2 nM) for normal SR, consistent with 2 CaMs and 1 CaM, respectively, per channel protein tetramer. The altered CaM binding in MH SR suggests that the Arg^{615} to Cys^{615} mutation in the MH channel protein results in altered cooperative subunit interactions indicative of altered Ca channel activity in MH.

W-Pos307

REDUCED OXYGEN SPECIES STIMULATE CALCIUM RELEASE FROM SKELETAL MUSCLE SARCOPLASMIC RETICULUM.

((Favero, T.G., Henry, R., and Abramson, J.J.)) Department of Physics, Portland State University, Portland, OR 97207.

Several reduced oxygen species (ROS) were evaluated for their ability to induce Ca^{2+} release from SR vesicles and to modify [^{3}H]-ryanodine binding. Hypochlorous acid (HOCl) at micromolar concentrations induced Ca^{2+} release from actively loaded SR vesicles and stimulated high affinity [^{3}H]-ryanodine binding (2-3 fold). HOCl reduced the EC_{50} for Ca^{2+} activation by 50%, and decreased the k_d for ryanodine binding by 3-fold. These effects of HOCl were inhibited by ruthenium red (RR). Hydroxyl radicals (HR) produced via the Fenton reaction also stimulated Ca^{2+} release from SR vesicles. Release was inhibited by RR, deferoxamine (Fe chelator) and mannitol (HR quencher). Superoxide had no effect on Ca^{2+} release or high affinity ryanodine binding. These results support the hypothesis that (ROS) directly interact with the Ca^{2+} release mechanism from skeletal muscle SR. Supported by MRF of Oregon, and NIH (GM44337-01 to J.J.A. and HL08388 to T.G.F.)

W-Pos308

HYDROGEN PEROXIDE STIMULATES CALCIUM RELEASE FROM SKELETAL MUSCLE SARCOPLASMIC RETICULUM.

((Favero, T.G., Henry, R., and Abramson, J.J.)) Department of Physics, Portland State University, Portland, OR 97207.

Hydrogen peroxide (H_2O_2) at millimolar concentrations induces Ca^{2+} release from actively loaded SR vesicles. High affinity [^{3}H]-ryanodine binding is enhanced at concentrations from 100 uM to 10 mM (2-3 fold). At H_2O_2 concentrations greater than 10 mM binding is inhibited. H_2O_2 decreases the k_d for [^{3}H]-ryanodine binding, increases its association rate, but has no effect on the rate of dissociation of [^{3}H]-ryanodine from its receptor. H_2O_2 also reduces the EC_{50} for Ca^{2+} activation from 900 nM to 400 nM in the presence of 1 mM H_2O_2. These effects are completely abolished in the presence of catalase, ruthenium red, and Mg^{2+}(mM). H_2O_2 stimulated [^{3}H]-ryanodine binding is not further enhanced by either doxorubicin or caffeine. These data indicate that H_2O_2 directly interacts with the Ca^{2+} release mechanism from skeletal muscle SR. Supported by MRF of Oregon, and NIH (GM44337-01 to J.J.A. and HL08388 to T.G.F.)

W-Pos309

SEPARATING THE DUAL ACTIONS OF RYANODINE ON THE SARCOPLASMIC RETICULAR CALCIUM RELEASE CHANNEL.
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Ryanodine, an alkaloid purified from *Ryania speciosa* Vahl, exhibits two opposing actions on the sarcoplasmic reticular Ca^{2+} release channel (SR CRC). Lower (nM) concentrations of ryanodine activate (open) the SR CRC, whereas higher (μM) concentrations deactivate (close) the channel. The biphasic nature of ryanodine's effects may result from its interaction with more than one receptor site on the SR CRC. However, direct binding studies have failed to establish unequivocally the number of binding sites on the SR CRC. Attempts to separate the activator and deactivator actions of ryanodine may help to better define the functional relationship, if any, among ryanodine binding site(s). Accordingly, we have examined the ability of adenine nucleotides (allosteric effectors of the SR CRC) to modulate ryanodine's actions on the SR CRC. Ca^{2+} efflux from SR vesicles was measured as an index of the activator, and separately, the deactivator effects of ryanodine. AMP-PCP, a non-hydrolyzable ATP analogue, acted synergistically with ryanodine to decrease the EC_{50} for activation by approximately 67-fold. It also decreased the EC_{50} for deactivation, but the shift in this case was only 2.7-fold. 8-alanyl-ryanodine is a O_{10eq} derivative of ryanodine previously shown to be our highest potency activator of the SR CRC. In the presence of AMP-PCP, the EC_{50} for activation by 8-alanyl-ryanodine was shifted leftward by the same degree as seen with ryanodine. In summary, AMP-PCP separated the EC_{50}'s for activation and deactivation by ryanodine. AMP-PCP also induced identical shifts in the EC_{50} for activation by both ryanoids. Finally, 8-alanyl-ryanodine exhibited only activator activity both in the absence and presence of AMP-PCP. These results suggest that independent binding sites on the SR CRC are responsible for the biphasic effects of ryanodine.

W-Pos310

POTENTIAL ROLE OF PALMITOYL CARNITINE MODULATION OF THE AVIAN Ca^{2+} RELEASE CHANNEL IN MUSCULAR NONSHIVERING THERMOGENESIS.
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In ducklings, prolonged cold exposure induces the development of a nonshivering thermogenesis (NST) of muscular origin. ^{45}Ca uptake and [^{3}H]ryanodine binding measurements with muscle homogenates and sarcoplasmic reticulum (SR) vesicles showed that cold acclimation (5 wk) induced an increase in the content of both the Ca^{2+}-ATPase and the Ca^{2+} release channel (RyR), suggesting that NST could result from an increase in ATP-dependent cycling of Ca^{2+} between the SR and the cytosol. We investigated the effects of free fatty acids and their metabolites on the RyR (EL-HAYEK et al., 1991, Biophys. J.61(2): A25). Palmitic acid was found ineffective in stimulating [^{3}H]ryanodine binding and Ca^{2+} release. Palmitoyl carnitine (PC) stimulated [^{3}H]ryanodine binding in a dose dependent manner in avian skeletal SR, but not in dog cardiac SR, suggesting a specific action on the skeletal isoform. PC also induced a rapid Ca^{2+} release from passively loaded SR vesicles, at concentrations thought to be compatible with *in vivo* levels. Further investigations are currently in progress to understand the nature of PC activation of the RyR and its potential role in modulating SR permeability in avian skeletal muscle and NST.

W-Pos311

THE FASTEST CONTRACTING SKELETAL MUSCLES OF NON-MAMMALIAN VERTEBRATES EXPRESS ONLY ONE ISOFORM OF THE RYANODINE RECEPTOR.
((John O'Brien and Barbara A. Block)) Department of Organismal Biology and Anatomy, University of Chicago, IL 60637.

The SR Ca^{2+} release channel (ryanodine receptor RyR) has been extensively characterized in mammalian muscles. Two isoforms of the RyR exist in mammalian muscles, skeletal (slow and fast-twitch muscles) and cardiac, which have distinct amino acid sequences and physiological properties. While mammalian skeletal muscles express a single isoform of the RyR, avian, amphibian, reptilian and fish skeletal muscles have been shown to express two. Immunological studies have demonstrated similarity between the two non-mammalian isoforms, co-expressed within a single muscle fiber, and the mammalian skeletal and cardiac RyRs. The functional significance of the expression of both isoforms in non-mammalian skeletal muscle fibers is unknown yet important considering that models for EC coupling are based on anatomical, physiological and biochemical results from these species. We examined the RyR isoform expression in fish skeletal muscles using immunological techniques. The fastest twitch muscles in vertebrates are the extraocular muscles. Immunoblot analysis with a monoclonal antibody that recognizes both isoforms of the RyR in fish and a polyclonal antibody specific to the skeletal RyR (provided by G. Meissner) show that only the skeletal isoform is expressed in the extraocular muscles of fish. Both isoforms are expressed in the fish epaxial muscles. SDS-PAGE gels also reveal one high molecular weight polypeptide in extraocular muscle while two polypeptides are seen in epaxial muscle. This pattern was confirmed in avian extraocular muscle, which expresses only the skeletal isoform, while two forms are observed in neck muscles. To investigate the functional correlation with contraction speed we probed the swimbladder muscle of toadfish, a fast-twitch, 400 Hz sound-producing muscle. Contrary to previous reports, we found that toadfish swimbladder muscle expresses only the skeletal isoform as in extraocular muscles, while the epaxial muscles expressed two isoforms. The appearance of only one isoform in the fastest twitch fibers in vertebrates provides functional evidence that the skeletal isoform of the RYR has been evolutionarily selected for speed.

W-Pos312

NEGATIVELY CHARGED VESTIBULE AT THE LUMENAL MOUTH OF THE SARCOPLASMIC RETICULUM CALCIUM RELEASE CHANNEL.
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The cardiac sarcoplasmic reticulum (SR) Ca^{2+} release channel exhibits directional conduction asymmetry (Biophys.J. 61: A433, 1992) suggesting that surface charge may effect ion transport. To address this possibility, we measured Ba^{2+} currents (lumen→myoplasm) at different ionic strengths in neutral bilayers (8:2 ratio; PE:PC). Unidirectional Ba^{2+} current was assured by adding Ba^{2+} only into the trans chamber. At low ionic strength (10 mM HEPES, PH 7.4, 20 μM free Ca^{2+}), Ba^{2+} current (0 mV) could be measured at very low [Ba^{2+}] (< 2 mM). At high ionic strength (symmetrical 400 mM Cs^+ or $Tris^+$), Ba^{2+} current was significantly reduced. Furthermore, in symmetrical current carrier (50 mM $CsCH_3SO_3$, 10 mM HEPES, 20 μM free Ca^{2+}), 1 mM carbodiamide (EDC; 20 mM CH_3NH_2 trans only) treatment neutralized negatively charged carboxyl groups and reduced the Cs^+ conductance (lumen→myoplasm) from 460 ± 45 pS (n=10) to 327 ± 105 pS (n=5). These two lines of evidences strongly suggest that the SR Ca^{2+} release channel has a negatively charged vestibule at its lumenal mouth (supported by NIH AR41197).

W-Pos313

PORPHYRINS ACTIVATE THE CALCIUM RELEASE MECHANISM OF SKELETAL MUSCLE SR VIA AN OXIDATION REACTION.
((Buck, E., Milne, S.T., *Pessah, I.N., and Abramson, J.J.)) Depart. of Physics, Portland State Univ., Portland, OR 97207, and *Depart. of Pharm. & Toxic., Univ. of California, Davis, CA 95616.

Micromolar concentrations of the porphyrin meso-Tetra(4-N-methylpyridyl)porphine tetraiodide (TMPyP) induces rapid Ca^{2+} release from skeletal muscle SR vesicles. Ca^{2+} release is stimulated by ATP (Kd_{ATP} = 100 μM) and Ca^{2+} (Kd_{Ca} = 1 μM), and is inhibited by Mg^{2+} (K_I = 220 μM) and ruthenium red (K_I = 7 nM). TMPyP stimulates high affinity [^{3}H]-ryanodine binding (2-3 fold), and sensitizes the receptor to activation by Ca^{2+}. Reconstituted Ca^{2+} channel activity is also stimulated by porphyrins, and is sensitized to activation by Ca^{2+}. These observations show that porphyrin induced Ca^{2+} release is caused by a direct interaction with the Ca^{2+} release protein from sarcoplasmic reticulum. Reduction of TMPyP by $Na_2S_2O_4$ inhibits its ability to stimulate Ca^{2+} release, suggesting that release occurs by an oxidation reaction. Supported by MRF of Oregon and NIH (GM44337-01 to J.J.A. and ES05002 to I.N.P.).

W-Pos314

CALCIUM RELEASE FROM SARCOPLASMIC RETICULUM (SR) OF PRESSURE-OVERLOAD INDUCED HYPERTROPHIC HEART.
((F.O. Mkparu, C.-R. Kim and D.H. Kim)) Univ. of Connecticut Health Center, Farmington, CT 06030-1305

We previously showed that doxorubicin and caffeine sensitivity of rat SR Ca^{2+} release channel (CRC) increased with mild left ventricular (LV) hypertrophy (Carroll et al., Biophys. J. 61:A431, 1992). Present study further characterized the Ca^{2+} release mechanism in the hypertrophied LV using both whole homogenates and SR vesicles. Whole homogenates of severely hypertrophied LV (76%) had lower Ca^{2+}-activated ryanodine binding ($\bar{x}$ ± SE) (Vmax: 57.3 ± 8.9, n=5 vs. 89.3 ± 7.9 pmol/g, n=4, $p < 0.03$) without significant changes in Ca^{2+} affinity (K_{Ca}: 0.055 ± 0.005, n=5 vs. 0.055 ± 0.03 uM, n=4) and cooperativity (nH=3). However, % increase in ryanodine binding to LV homogenates by 5 uM doxorubicin or 0.5 mM caffeine was higher in hypertrophied animals than sham. This finding supports the previous finding that CRC in hypertrophied LV was more sensitive to the Ca^{2+} release drugs. Total cholesterol amounts of the SR in mildly hypertrophied LV were 20% higher than those in sham ($\bar{x}$ ± SE, 74 ± 6, n=4 vs. 62 ± 2 ug cholesterol/mg SR, n=4). Our results suggest that pressure-overload induced LV hypertrophy is associated with both qualitative and quantitative changes in SR CRC. Supported by NIH (HL-33026 and HL-07420) and AHA-CT. D.H.K. is an Established Investigator of AHA.

W-Pos315

CA^{2+} OCCLUSION IN THE CRATP-ATPASE COMPLEX. ((Carol Coan, Ji-Ying Ji, and Jose Adalberto Amaral)) University of the Pacific, San Francisco, CA 94115.

When CrATP is bound to the SR ATPase, 2 Ca^{2+} are held in an occluded state. We use two different approaches to study these sites; we add varying amounts of EGTA or $^{40}Ca^{2+}$ to the CrATP-$E^{45}Ca_2$ complex and observe release or exchange, respectively, and we form the CrATP-E complex first and observe $^{45}Ca^{2+}$ binding under different conditions. In the latter experiments we can obtain separate binding curves for the two Ca^{2+} (Kd(1) = .2 mM, Kd(2) = 2mM). Ca^{2+} is released from these sites at very different rates (k(1) = .4 min^{-1}, k(2) = .02 min^{-1}, 37°C). We can show that the fast release is from the higher affinity Ca^{2+} site. However, we also find that only the Ca^{2+} on the site with the lower affinity can exchange with Ca^{2+} in the medium. There is a significant ionophore dependency on the rate of exchange, indicating that the main access to the site is through the interior of the vesicle. Thus, it appears that the access to the lower affinity site is through the bottom of the Ca^{2+} channel.

W-Pos316

CORRELATION OF Ca-ATPase PHOSPHORYLATION KINETICS WITH PROTEIN CONFORMATIONAL CHANGES DETECTED BY TIME-RESOLVED EPR. ((James E. Mahaney, Jeffrey P. Froehlich, and David D. Thomas)) Department of Biochemistry, University of Minnesota Medical School, Minneapolis, MN 55455, and National Institute on Aging, NIH, Baltimore, MD 21224.

Rapid acid quenching and time-resolved EPR spectroscopy were used to study the relationship between ATP-induced phosphorylation and conformational transitions in the sarcoplasmic reticulum Ca-ATPase at 2°C. Phosphorylation of the enzyme with 100 μM ATP was biphasic with a 50:50 distribution of fast (65 s^{-1}) and slow (3 s^{-1}) components. Addition of 900 μM caged ATP reduced the rate and amount of pre-steady state phosphorylation in both phases. Dephosphorylation with ADP revealed that the ADP-insensitive phosphoenzyme (E_2P) accumulates rapidly. For the EPR experiments, an iodoacetamide spin label was used, but NEM pre-treatment was required to achieve selective attachment of the label to the enzyme. NEM and spin labeling reduced the rate and amount of pre-steady state phosphorylation in both phases, but steady state phosphorylation levels were also reduced (~35 %). The EPR spectrum of spin-labeled Ca-ATPase consisted of two resolved components, the mole fractions of which depended on ligand environment, and therefore, ATPase conformation. Immediately upon laser flash-photolysis of caged ATP, which produced 100 μM ATP in the EPR sample, the EPR signal increased to a level indicating decreased probe mobility, with kinetics similar to the slow phase of phosphorylation. We conclude that (1) caged ATP binds to and inhibits the Ca-ATPase, (2) reduced temperature does not inhibit the conversion of E_1P to E_2P, and (3) the EPR signal likely reflects changes in quarternary protein structure as well as secondary and tertiary structure.

W-Pos317

THAPSIGARGIN INDUCED CALCIUM RELEASE FROM SR AND ASOLECTIN VESICLES ((Favero, T,G., Pessah, I.N.[#], and Abramson, J,J.)) Dept. of Physics, Portland State University, Portland OR, 97207. [#]Depart. of Pharmacology and Toxicology, University of California, Davis CA, 95616. (Spon: P. Smejtek)

Thapsigargin has been used in many cell preparations to induce an increase in cytosolic Ca^{2+} concentration purportedly by inhibition of Ca^{2+}-ATPase activity. Thapsigargin induces Ca^{2+} release from actively loaded SR vesicles at concentrations higher than required to inhibit Ca^{2+}-ATPase activity. The rate and amount of thapsigargin-induced Ca^{2+} release is inhibited by increasing Mg^{2+}(mM) but not ruthenium red. Thapsigargin also induces a similar concentration-dependent release in Ca^{2+}-loaded asolectin liposomes devoid of any protein. This effect was highly lipid dependent as increasing the lipid concentration decreased both the rate and amount of Ca^{2+} release induced by thapsigargin. These data suggest that Ca^{2+} release induced by micromolar concentrations of thapsigargin may be due to an ionophoric effect on the lipid membrane. Supported by MRF of Oregon and NIH (GM44337-01 to J.J.A., HL08388 to T.G.F., and ES05002 to I.N.P.)

W-Pos318

The Effect of Senescence on the Conformational Stability of Skeletal Muscle Sarcoplasmic Reticulum Ca-ATPase. Deborah Ferrington, Terry Jones, Thomas Squier, and Diana Bigelow; Dept. of Biochemistry, University of Kansas, Lawrence, KS 66045.

Sarcoplasmic reticulum (SR) membranes purified from the skeletal muscle of young (4-6 months) and old (26-28 months) Fischer strain 344 rats, exhibit no age-related differences in abundance of the Ca-ATPase, Ca-ATPase activity, cholesterol or phospholipid content. Conformational stability of the Ca-ATPase, assessed by monitoring enzyme activity during incubation at 37°C, is decreased in SR membranes isolated from old animals relative to those from young animals. On the other hand, rates of inactivation of Triton- or $C_{12}E_9$-solubilized preparations exhibit no age-related differences; both young and old preparations showed rapid inactivation compared to vesicular preparations. Thus altered lipid-protein or protein-protein interactions upon aging may result in decreased stability of the Ca-ATPase. Therefore we have probed the dynamics of the Ca-ATPase and surrounding lipids in the SR membrane using spin-label EPR. Using a stearic acid spin probe, we find no age-dependent or time-dependent differences in membrane lipid motion upon heating at 37°C. Overall rotational mobility of maleimide spin-labeled Ca-ATPase is the same in SR membranes from young and old animals. However, upon heating at 37°C, Ca-ATPase mobility decreased more rapidly in vesicles from old animals compared to that from young animals, suggesting a greater tendency to aggregate.

W-Pos319

Affinity Column Purification of the Ca-ATPase of Cardiac and Skeletal Sarcoplasmic Reticulum. **Qing Yao and Diana J. Bigelow, Department of Biochemistry, University of Kansas, Lawrence, Kansas 66045.**

Isolation of skeletal sarcoplasmic reticulum (SR) membranes by differential centrifugation results in a vesicular preparation in which the Ca-ATPase is the predominant, but not the only protein. The Ca-ATPase is much less abundant in cardiac SR membranes. Therefore, we have utilized detergent solubilization followed by a Reactive Red-120 agarose affinity column for experiments that require highly purified Ca-ATPase protein. Such chromatography has been remarkably successful in isolating the Ca-ATPase from skeletal SR as a single band on SDS polyacrylamide gels while maintaining high specific activity of the enzyme. We find that as little as 1 mg SR protein can be purified in this manner, at the same time, removing contaminating ATPases. On the other hand, the cardiac Ca-ATPase shows very different binding properties to this column. An integral part of this isolation procedure includes optimal solubilization of the SR membrane with detergent before application to the column. We report here our comparison of several detergents (C12E8, C12E9, deoxycholate, Triton X-100, CHAPS) for solubilization of several fractions of skeletal and cardiac SR membranes for use with this column.

W-Pos320

THAPSIGARGIN INHIBITION OF CALCIUM- AND MAGNESIUM-DEPENDENT NTPase ACTIVITY OF CANINE CARDIAC SARCOPLASMIC RETICULUM. **((C. Tate, B. Elkenburg, M. Lewallen, P. McBride, G. Taffet, and M. Entman))Univ. of Houston, Baylor College of Medicine, Houston, TX 77030**

ATPase activity of isolated canine cardiac SR is 90% Ca-sensitive, while GTPase activity is only 0-20% Ca-sensitive depending on the [Mg]. GTP and ATP apparently bind to different sites on the CaATPase protein (JBC, 276:16165, 1991). The specific inhibitor of the SR CaATPase protein, thapsigargin (TH), was used to explore nucleotide binding (photoaffinity labeling) and NTP hydrolysis (including phosphoenzyme content [EP]) using two concentrations of TH: "low" (750 pmol/mg SR) and "high" (250 nmol/mg SR). At "low" TH Ca-dependent ATPase activity was inhibited 100% (I_{50}=300-350 pmol/mg SR) resulting from a 100% decrease in Ca-dependent EP content and a 80-90% decrease in ATP binding. In contrast, when Ca-dependent GTPase activity and Ca-dependent EP were observed (at low [Mg]), they were inhibited by "low" TH like CaATPase activity; however, no significant change in GTP binding or Mg-dependent, Ca-insensitive GTPase activity was noted. At "high" TH, though, GTP binding was decreased by 60%, and Mg-dependent GTPase activity and Mg-EP from GTP were lowered 50-60%. These results provide additional evidence that ATP and GTP enter the hydrolysis cycle(s) through different sites and that the two nucleotide binding sites and hydrolysis cycle(s) have differing sensitivities to modification by TH. Support: NIH HL13870, AG06221, AG00428.

W-Pos321

EFFECTS OF ANESTHETICS ON THE OLIGOMERIC STATE AND FUNCTION OF THE CALCIUM ATPASE OF SARCOPLASMIC RETICULUM. ((H. Kutchai, J.E. Mahaney, L.M. Geddis and D.D. Thomas)) Dept. of Physiol., U. Virginia, Charlottesville, VA 22908 and Dept. of Biochem., U. Minnesota, Minneapolis, MN 55455.

Ca-ATPase of sarcoplasmic reticulum (SR) of rabbit fast skeletal muscle was labeled with erythrosin isothiocyanate (ERITC). Time-resolved phosphorescence anisotropy was used to estimate the mole fractions and the rotational correlation times of putative monomers, dimers, and tetramers of Ca-ATPase. Treatment of ERITC-labeled SR with butanol, hexanol, and octanol--general anesthetics--resulted in decreased rotational correlation times and shifted the state of aggregation of the Ca-ATPase toward smaller oligomers and monomers. In the same ranges of alcohol concentration, the alcohols enhanced the Ca-ATPase activity of unlabeled SR. By contrast, treatment of ERITC-labeled SR with the local anesthetic lidocaine resulted in the formation of aggregates of Ca-ATPase that are larger than tetramers. Lidocaine in this concentration range inhibits the Ca-ATPase activity of unlabeled SR. EPR studies with fatty acid and phospholipid spin labels suggest that treatment of SR with butanol, hexanol, or octanol results in a decreased population of SR phospholipids that are motionally-restricted by the Ca-ATPase and in decreased order and increased fluidity of both the restricted and the unrestricted phospholipids. These results are consistent with a single physical model -- inhibition by lateral aggregation -- that explains the effects of both general and local anesthetics on the Ca-ATPase. (Supported by grant GM 27906 from NIH and a grant-in-aid from AHA, Virginia Affiliate.)

W-Pos322

EFFECT OF HALOTHANE ON THE CALCIUM ATPASE IN SARCOPLASMIC RETICULUM. ((Brad S. Karon and David D. Thomas)) Dept. of Biochemistry, University of Minnesota Medical School, Minneapolis, MN 55455.

We have studied the effect of the volatile anesthetic halothane on the molecular dynamics and function of the Ca-ATPase in sarcoplasmic reticulum (SR). Using time-resolved phosphorescence anisotropy (TPA), we estimated the rotational correlation times and mole fractions of putative monomers, dimers, and tetramers of the Ca-ATPase. Lipid fluidity was measured as a function of halothane concentration, using EPR of spin labeled lipids. In agreement with previous studies, we found that halothane activated the Ca-ATPase and fluidized the SR membrane. Halothane increased the rate of rotational motion of all Ca-ATPase aggregates, and promoted the formation of Ca-ATPase monomers and dimers from larger aggregates. Comparison of the effects of halothane with temperature and other fluidizing perturbations indicates that the activation of the Ca-ATPase by halothane cannot be explained by an increase in lipid fluidity alone. Therefore, we propose that the primary mechanism of halothane activation of the Ca-ATPase involves a change in the aggregation state of the enzyme.

W-Pos323

THAPSIGARGIN AND CYCLOPIAZONIC ACID CAUSE AGGREGATION OF CALCIUM ATPASE OF SARCOPLASMIC RETICULUM. ((Joseph V. Mersol, Howard Kutchai†, James E. Mahaney, and David D. Thomas)) Department of Biochemistry, The University of Minnesota, Minneapolis, MN and † Department of Physiology, The University of Virginia, Charlottesville, VA

The Ca-ATPase of longitudinal sarcoplasmic reticulum (SR) of rabbit skeletal muscle was labeled with erythrosin isothiocyanate (ERITC-SR). The effects of the specific inhibitors thapsigargin (TG) and cyclopiazonic acid (CPA) on the time-resolved phosphorescence anisotropy of ERITC-SR was studied. In the absence of inhibitors, ERITC-SR shows a decreasing anisotropy which is best fit as the sum of three exponential decays plus a constant term. The addition of amounts of TG or CPA stoichiometric to the Ca-ATPase caused a monotonic increase in the phosphorescence anisotropy. This increase is attributed to the aggregation of Ca-ATPase by each inhibitor. Thapsigargin is thought to inhibit the Ca-ATPase by locking it into the E2 conformation, while Ca^{2+} stabilizes the E1 conformation. Preincubation of the ERITC-SR in 100 μM or 1 mM Ca^{2+} before the addition of of each inhibitor was found to slow down the changes in phosphorescence anisotropy. These results are consistent with other data from this laboratory which suggest that formation of higher oligomers of the Ca-ATPase is associated with inhibition of its activity.
This research was supported by NIH Grant GM27906. JVM was supported by NIH Grant 5T32HL07284.

ERYTHROCYTE MEMBRANE

W-Pos324

IDENTIFICATION OF A SORBITOL PERMEASE IN HUMAN ERYTHROCYTES. ((G.R. Kracke, G.G. Preston and T.H. Stanley*)) Departments of Anesthesiology, University of Missouri School of Medicine, Columbia, Missouri 65212 and University of Utah School of Medicine*, Salt Lake City, Utah 84132.

Sorbitol, a polyol derived from glucose by aldose reductase, is a common organic solute in many cells. It plays an important role in osmotic regulation in some cells and in others its accumulation is responsible for the sugar cataracts and peripheral neuropathy of uncontrolled diabetes. In order to characterize sorbitol transport, we measured D-^{14}C-sorbitol influx in human erythrocytes. Sorbitol influx at 37°C was time-dependent and a linear function of sorbitol concentration over the range of 0.05-100 mM. Influx over the temperature range of 10-50 °C had an activation energy of 10.0 kcal/mol ($Q_{10} \approx 1.8$), higher than predicted for diffusion through an aqueous pore. Sorbitol influx was not sensitive to changes in cell volume. Glucose transport inhibitors either had no effect or minimally inhibited sorbitol influx. Influx was stimulated two-fold by 0.5 mM PCMB, a sulfhydryl reagent. Molar excesses of other sugars did not decrease influx. We conclude that there is a specific sorbitol transport pathway in human erythrocytes, similar to the sorbitol permease recently described for renal epithelial cells. (Supported by the American Heart Association- Missouri Affiliate and the Stanley Research Foundation.)

W-Pos325

TRANSPORT OF NATURAL LONG CHAIN FATTY ACIDS ACROSS RED CELL GHOSTS IS PROTEIN-MEDIATED. ((A. M. Kleinfeld and P. Chu)) Medical Biology Institute La Jolla, CA 92037

The mechanism by which long chain fatty acids cross membranes has been thought to involve rapid movement across the lipid phase of the membrane. Contrasting this is evidence for protein-mediated transmembrane transport. Most studies of long chain natural fatty acid "transport" have in fact been measurements of cell association of fatty acid. At least in the case of red cells, no direct measure has been made of fatty acid transport in which the fatty acid moves from one aqueous phase to the other. Since fatty acids readily bind to the lipid phase of biological membranes, it is difficult to distinguish binding from transport if only binding is measured. What is required is a way to rapidly detect and quantify the intra-cellular aqueous phase concentration of long chain fatty acid following rapid addition of fatty acid to the outer aqueous phase. We have recently developed a fluorescent method of measuring the concentration of aqueous phase FFA using intestinal fatty acid binding protein from rat intestine modified at Lys 27 with acrylodan (ADIFAB) (G.V. Richieri, R.T. Ogata and A.M. Kleinfeld, J. Biol. Chem., 1992, in press). The interaction of long chain fatty acids with ADIFAB is rapid, specific for fatty acids, and results in substantial increase in the ratio of 505 to 432 nm fluorescence. In the present study we have trapped ADIFAB in resealed human red cell ghosts and monitored ADIFAB fluorescence after rapid addition of sodium oleate at 37°C. We find that in these untreated ghosts transport from outside to inside is extremely rapid, equilibrium is reached within times less than 200 ms. Treating these sealed ghosts with DIDS, H_2DIDS, or proteases, eliminates this rapid transport. Specifically, we observed a dose dependent elimination of transport with H_2DIDS, complete inhibition occurs with concentrations of [H_2DIDS] ~ 1μM. These results strongly suggest that transport of long chain fatty acid is mediated by a membrane protein. This work is supported by NIH grant RO1,GM44171.

W-Pos326

OBSERVATION OF FROZEN-HYDRATED ERYTHROCYTE MEMBRANE SKELETONS BY CRYO-ELECTRON MICROSCOPY. ((Li Li and Robert Josephs)) Laboratory for Electron Microscopy and Image Processing, The University of Chicago, Chicago, IL 60637

We have examined unstained, unfixed hydrated membrane skeletons of human red blood cells utilizing cryo-electron microscopy. Isolated membrane skeletons dialyzed in low ionic strength buffer were embedded in a layer of vitreous ice supported by a fenestrated carbon film. We have obtained clear images of single layers of skeletons over holes in the supporting film. Each junctional actin complex contains 5 to 8 attached spectrin molecules. Usually there was one spectrin molecule bridging adjacent actins but about 20% of the adjacent actin oligomers were connected by two spectrin molecules. The average distance between actin protofilaments was 151.2 nm. The average contour length of spectrins was 174.9 nm and the correlation length was 255.8 nm (measurements were made in 0.2 mM sodium phosphate buffer, pH 7.4). These data are consistant with the spectrin molecules being largely extended linear structures. (Supported by NIH Grant HL22654)

W-Pos327

MULTIPLE, SPECTRIN-RELATED BUT INDEPENDENT MECHANICAL RELAXATION COMPONENTS IN ERYTHROCYTE GHOSTS UNDERGOING FUSION. ((A.E. Sowers, Y.-K. Wu, G.K. Lewis, and R.A.Sjodin)) Depts. Biophys. and Microbiology & Immunology, Univ. Maryland, Sch. Med, Baltimore, MD. 21201.

Our ability to *control* the induction of a fusion zone between two erythrocyte ghosts and to quantitatively follow the post-fusion swelling or rounding-up of a two ghost fusion product, as a model for cell fusion, may allow the measurement, dissection, and discovery of new membrane/spectrin biomechanical properties and cytoskeletal influences as could come into play in, for example, virus-induced syncytium formation. A biophysical characterization of the rounding-up process (see other abstract by Wu et al. and BJ 60: 1026-1037 [1991]) revealed that the rate of rounding-up (the diameter of the fusion zone) could have up to three (I-III) highly *distinct*, but *time-invariant*, kinetic phases, depending on conditions (including a heat-treatment known to affect specifically the spectrin network [Brandts et al.,1977]). The presence of glycerol does not affect the fastest phase (Phase I - 1.0-1.2 sec), suggesting that permebility, suspension aqueous viscosity, and headgroup hydration may not be limiting. Diamide treatment affected only the first 0.5 sec interval of the Phase I, while 2,3-diphosphoglycerate had an influence only in the Phase III interval (> 4.0 sec). N-ethylmaleimide (NEM) had an effect only if there was a heat treatment. The effect of NEM was that it appeared to lower the temperature threshold for the heat-treatment. Supported by ONR, NSF, and NIH.

W-Pos328

DNDS (4,4'-DINITRO-STILBENE-2,2'-DISULFONATE) DOES NOT ACT AS A PURELY COMPETITIVE INHIBITOR OF RED BLOOD CELL BAND 3-MEDIATED ANION EXCHANGE. ((P.A. Knauf, E.A. Ries, L.A. Romanow, S. Bahar, and E.S. Szekeres)) Dept. of Biophysics, Univ. of Rochester Med. Ctr., Rochester, NY 14642.

With equal [Cl^-] inside and outside the cell ($Cl_i = Cl_o$), the apparent dissociation constant for DNDS binding to band 3 increases linearly with [Cl^-], as expected if DNDS is a competitive inhibitor of Cl^- exchange (Frohlich, *J. Memb. Biol.* *65*:111, 1982). If DNDS is a nonpenetrating external competitive inhibitor, it should bind *only* to E_o, the form of band 3 with the transport site facing outward and unloaded with Cl^-. To test this, we have used a Dixon plot intersection technique to measure the dissociation constant for binding of DNDS at 0°C to E_i, the form of band 3 with the transport site facing inward. Surprisingly, we find a value of 1.01 ± 0.05 μM, 12 times larger than the dissociation constant for E_o reported by Frohlich (0.084 ± 0.004 μM), but not infinite as predicted for a competitive inhibitor. No Dixon plot curvature was seen. The simplest interpretation is that DNDS binds to a site other than the transport site, with different affinities for E_i and E_o, although the possibility that DNDS can bind to the transport site of E_o and also to a noncompetitive site, but not to both at the same time, cannot be ruled out at present. If DNDS binds only to E_i and E_o, the predictions of either model fit perfectly with the effects of changes in $Cl_i = Cl_o$ on DNDS affinity reported by Frohlich. (Supported by NIH Grant DK27495.)

W-Pos329

CALCIUM PUMP MEASURED IN SINGLE CELLS BY MICRO-PHOTOLYSIS AND LASER SCANNING MICROSCOPY. ((L.Pratsch*, U.Kubitscheck*, H.Passow# and R.Peters*)) *Institut für Medizinische Physik und Biophysik der Universität, Münster, and #Max-Planck-Institut für Biophysik, Frankfurt, Germany

In the past, the plasma membrane calcium pump playing a pivotal role in the homeostasis and modulation of cell calcium has been extensively studied by macroscopic flux measurements. Here, we report the first measurement of calcium pump activity in single human erythrocyte ghosts. Ghosts were loaded with calcium, a fluorescent calcium indicator and caged ATP. In a suitably modified laser scanning microscope (Leica) ATP was released by a short UV pulse and the calcium ion concentration measured inside single ghosts at physiologically relevant concentration (μM) and time resolution (s). Results were consistent with expectation based on previous macroscopic measurements and revealed, for the first time, the sample population distribution of pump parameters. Optical single-cell and single-channel flux measurements open new possibilities for characterizing membrane transport proteins (c.f. EMBO J. 9 (1990) 2447-2451) and have the potential to be employed for screening cells transfected with mutant membrane proteins. (Supported by Deutsche Forschungsgemeinschaft, Pe138/12)

W-Pos330

Mg^{2+} TRANSPORT IN ERYTHROCYTES; REGULATION BY INTRACELLULAR pH OR cAMP. ((J.Hwa, A.Romani, C.Marfella and A.Scarpa)) Dept. of Physiology and Biophysics, Case Western Reserve University, Cleveland OH 44106. (Spon. by E.Carafoli)

In erythrocytes, Mg^{2+} movement has recently been shown to occur through two different pathways, Na^+-dependent and Na^+-independent. In the absence of extracellular Na^+, Cl^- is a potent stimulator of Mg^{2+} efflux (Gunther T. & Vormann J. FEBS.LETT 271:149-151 1990). We induced Mg^{2+} efflux by adding increasing concentrations of choline chloride (0,5,20,100mM) to an isoosmotic sucrose-hepes solution (pH 7.4). After 6 min of incubation, with increasing Cl^- concentrations net Mg^{2+} efflux increased from 0.1mM to 1.3mM/mg prot. Using met-haemoglobin as an intrinsic intracellular pH indicator and dual wavelength spectrophotometry, we were able to observe a dose dependent decrease in intracellular pH with increasing extracellular Cl^- concentrations. Inhibition of the HCO_3^-/Cl^- exchanger with 4,4'-dinitrostilbene-2,2-disulfonic acid (DNDS 50uM) inhibited both acidification and Mg^{2+} efflux. Intracellular pH was also changed by the addition of nigericin (5uM) and increasing concentrations of extracellular K-Hepes (0,5,100mM). Even under these conditions a relationship between increasing rates of Mg^{2+} efflux and intracellular acidification was observed. As with other cell types, in erythrocytes, the addition of dibutyrylcAMP (100uM) also induced an efflux (0.6mM at 6 minutes), however, its addition did not produce a change in intracellular pH and the efflux required extracellular Na^+. Thus, Na^+-dependent Mg^{2+} efflux can be regulated by intracellular cAMP and Na^+-independent efflux by intracellular pH.

W-Pos331

FACTORS DETERMINING THE CONFORMATION AND QUATERNARY STRUCTURE OF HUMAN ERYTHROCYTE BAND 3 IN DETERGENT SOLUTION. ((Lawrence M Schopfer and James M. Salhany)) VA and Univ. of Nebraska Medical Centers Omaha, NE 68105.

Fluorescence spectroscopy was used to follow the kinetics of covalent binding of DIDS (4,4'-diisothiocyanato-2,2'-stilbene disulfonate) to isolated band 3 in the detergent $C_{12}E_8$. We have discovered a dilution-induced loss in the ability of band 3 monomer to form a covalent adduct with DIDS. The loss in DIDS reactivity with dilution followed a 50:50 biphasic time course despite the use of a homogeneous preparation of band 3 oligomers. The loss in reactivity generally correlated with the association of band 3 dimers and tetramers to oligomers which were larger than the tetramer. The final oligomeric product was capable of binding BADS (4-benzamido-4'-amino-2,2'-stilbenedisulfonate) reversibly, but with an affinity nearly 30-fold lower than the starting material. Removal of the cytoplasmic domain of band 3 slowed the conformational interconversion of the integral domain by about 5-fold and inhibited the aggregation process. The conformational interconversion was also slowed by the presence of 150 mM chloride but not 90 mM sulfate. Covalent binding of DIDS inhibited the dilution-induced aggregation process. Addition of 250 uM lipid inhibited both the loss of DIDS reactivity and the protein aggregation process. The apparent half-effect value for lipid protection of band 3 conformational change was about 20 uM for phosphatidylethanolamine. While several types of lipid offer protection, phosphatidic acid accelerated the decay process by eliminating the biphasicity. We conclude that the conformation of the integral domain of band 3 can be modulated allosterically, by the addition of ligands, including various lipids. The results offer direct evidence for cooperative interactions between band 3 subunits during loss of activity, and they show that the cytoplasmic domain participates in the control of this transition.

W-Pos332

IMMUNOASSAYS OF ERYTHROCYTE ANKYRIN(2.3) PRIOR TO AND DURING ACUTE LITHIUM THERAPY.

((Yatian Zhang, M.D., Ph.D.)) Dept. of Biology, New York University, New York, New York 10003

SDS-PAGE gels and Western blotting were used for quantitative studies of erythrocyte membranes from 28 female inpatients with bipolar illness and 12 controls at the University of Pisa Female Psychiatric Service. Samples from patients were obtained prior to beginning lithium therapy and then again after one week of lithium therapy. Densitometry done was on the electrophoretic bands in conjunction with the Western blotting using anti-ankyrin monoclonal antibody ZA23 which gave an independent estimate of the actual ankyrin content. In addition, I had also investigated the possibility of utilizing ZA23 for direct measurement of ankyrins by an ELISA procedure without membrane separation on SDS-PAGE. There was no significant difference in the ratio of the densitometric readings of ankyrin(2.3) to band 4.1 prior to and during acute lithium therapy whereas ankyrin(2.3) in membranes from bipolar subjects was significantly higher than the control population and the mean values for ratios of ankyrin(2.3) to band 4.1 were more than 50% greater than the controls. This study suggests the genetic or post-translational Ankyrin(2.3).

W-Pos333

KINETICS OF THE PROTEIN 4.1-ERYTHROCYTE MEMBRANE INTERACTION STUDIED BY TIR-FRAP.

((A.L. Stout[1], R.C. MacDonald[3], R.I. MacDonald[3] and D. Axelrod[1,2])) [1]Biophysics Research Division, [2]Dept. of Physics, U. of Michigan, Ann Arbor, MI 48109, and [3]Dept. of Biochem. Mol. Biol. and Cell Biol., Northwestern U., Evanston, IL 60208.

Protein 4.1 is a major component of the membrane skeleton, mediating the spectrin-actin interaction as well as the attachment of the spectrin network to the red cell membrane. Using the technique of total internal reflection fluorescence recovery after photobleaching (TIR-FRAP) microscopy, we have studied the kinetics of the interaction of carboxyfluorescein-labeled 4.1 with the inside surfaces of individual human red cell membranes adhered and hemolyzed on polylysinated glass. The specificity of the observed CF-4.1 binding was evaluated by comparison of fluorescence emitted from the membranes under various conditions. Red cells stripped of spectrin, actin, ankyrin, and 4.1 could bind CF-4.1 at their exposed cytoplasmic surfaces but not at their outside surfaces. Removing only spectrin and actin from the membranes resulted in a 62% reduction in fluorescence as compared to fully stripped membranes; mild trypsin digestion of fully-stripped membranes led to a 87% reduction under identical conditions. Analysis of the TIR-FRAP data reveals that there are at least three classes of CF-4.1-membrane interaction. One population, representing approximately 37% of the total membrane-bound CF-4.1, is described by a k_{off} of (2.1 ± .1) sec^{-1}. A second class, about 18% of the total, is characterized by a k_{off} of (.069 ± .004) sec^{-1}. The remaining 45% of the CF-4.1 is irreversibly bound on the time scales spanned by these experiments. These kinetic rates are as fast or faster than rates of membrane skeleton rearrangements deduced by others from morphological or physiological measurements. Supported by NSF DMB 8805296 (to D.A.).

W-Pos334

HEMOGLOBIN-MEMBRANE INTERACTIONS AS PROBED BY NITROBLUE TETRAZOLIUM.

((O. Abugo and J.M. Rifkind)) NIH/NIA Gerontology Research Center, Baltimore, MD 21224

Hemoglobin is known to bind to the membrane band 3 with deoxyhemoglobin having a higher affinity than oxyhemoglobin.[1] Nitroblue-tetrazolium (NBT) was shown to be reduced both by superoxide formed during autoxidation of hemoglobin and directly by deoxygenated hemoglobins chains bound to the membrane. By comparing lysates with ghosts, it is shown that membrane interactions produce an order of magnitude increase in the NBT reduction. By studying the stoichiometry of this enhancement and the competition with glyceraldehyde-3-phosphate dehydrogenase it was shown that the membrane-associated reduction of NBT results from binding of hemoglobin to band 3.

This method was used to indicate significant membrane interactions which occur throughout the oxygenation curve with a particular enhancement already observed when the first oxygen is removed from hemoglobin at 90% oxygenation. The reduction of NBT was also used to indicate hemoglobin - membrane interaction in intact erythrocytes.

[1] J.A. Walder, R. Chatterjee, T.L. Steck, P.S. Low, G.F. Musso, F.T. Kaiser, P.H. Rogers, and A. Arnone, *J. Biol. Chem.* 259, 10238 (1984).

W-Pos335

OXYRADICAL DAMAGE TO ERYTHROCYTES ASSOCIATED WITH HEMOGLOBIN-MEMBRANE INTERACTIONS.

((J.M. Rifkind and O. Abugo)) NIH/NIA Gerontology Research Center, Baltimore, MD 21224

Enhanced autoxidation of hemoglobin at intermediate oxygen pressures produces superoxide. Within the red cell potential damage by the production of these oxyradicals should be inhibited by the cytoplasmic enzymes superoxide dismutase, catalase, and glutathione peroxidase. From studies of the reduction of nitroblue tetrazolium it was shown that hemoglobin binds to the membrane even at these intermediate oxygen pressures. Oxyradicals originating from the fraction of hemoglobin bound to the membrane may be relatively inaccessible to the cytoplasmic enzymes resulting in erythrocyte damage. By incubation of erythrocytes at various partial pressures of oxygen we have been able to delineate the membrane damage associated with the production of oxyradicals near the membrane. This damage includes membrane protein crosslinking, swelling of the cells, a decrease in deformability, and eventually lysis. These oxyradicals can also escape the erythrocyte providing a potential source for damage to other cells and tissues.

W-Pos336

SPIN-LABELED-H_2-DADS-MALEIMIDE, A NEW PROBE FOR THE STUDY OF ERYTHROCYTE MEMBRANE PROTEIN DYNAMICS USING EPR SPECTROSCOPY

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A new nitroxide spin-label derivative of the compound 4,4'-diamino-dihydro-stilbene-2,2'-disulfonate (H_2-DADS) which forms a covalent attachment with the anion-exchange (band 3) protein of the human erythrocyte membrane has been synthesized. This new EPR spin-label probe contains: a pyrroline-nitroxide group, which provides the requisite paramagnetic center required for EPR spectroscopic studies; a reactive maleimide group for covalent attachment of the probe to the protein; and a stilbenedisulfonate backbone to provide specificity for the exofacial domain of the anion-exchanger. V_1 EPR spectra of labeled intact human erythrocytes and ghost membrane preparations show a discrete signal indicative of a probe bound to a single motional environment which is immobilized on the linear EPR time-scale. V'_2 Saturation-Transfer EPR spectra of intact erythrocytes reveal very slow rotational motions of spin-labeled band 3. ST-EPR spectra of labeled ghost membrane preparations show only small increases in protein mobility relative to intact cells. Protein mobility was more sensitive to temperature in ghosts versus intact cells. Band 3 protein mobility in ghost membranes which were treated with trypsin to cleave the 43K cytoplasmic domain was studied and found to be increased slightly. Chymotrypsin cleavage of the extracellular loop which splits the integral membrane domain into 58K and 38K segments showed no increase in protein mobility, suggesting that the membrane-protein complex was not disrupted by this proteolytic cleavage. (Supported by NIH HL34737 and RR04075.)

W-Pos337

EFFECTS OF INHALATION ANESTHETICS ON ROTATIONAL MOBILITY OF HUMAN ERYTHROCYTE BAND 3.

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One mode of action of inhalation anesthetics is believed to be the result of cell membrane fluidity changes following partitioning of a lipophilic anesthetic into the membrane lipid. Such changes in membrane lipid ordering can alter the mobility and/or interactions of integral membrane proteins and their functional characteristics. We have taken advantage of this fluidizing effect of the anesthetics diethyl ether, chloroform, and halothane to study the effect of fluidization on the rotational mobility of the erythrocyte anion exchange protein (band 3). We have used the magnetic resonance technique of saturation-transfer EPR spectroscopy (ST-EPR) with intact cells and ghost membranes whose band 3 was affinity labeled with the spin probe 4-^{15}N,$^{2}H_{15}$-PROXYL-4'-maleimido-dihydrostilbenedisulfonate. We have also used the complimentary optical technique of transient phosphorescence emission anisotropy (TPA) with ghost membranes whose band 3 was affinity labeled with the probe eosin-5-maleimide. Results from both techniques indicate that all three anesthetics tested caused an increase in the rotational mobility (decreased rotational correlation time) of band 3. At the levels of each anesthetic employed results from ST- EPR and TPA correlated exactly, with ether causing the smallest effect, halothane an intermediate effect, and chloroform the largest effect. Freeze-fracture electron microscopy on control and anesthetic-treated membranes indicated that the observed changes in rotational dynamics were not related to changes in intramembraneous particle distribution. Studies are in progress to determine the effects of these anesthetics on kinetic and thermodynamic properties of anion exchange. Supported by NIH grants HL34737 & RR04075 (A.H.B.) and the L.P. Markey Foundation (J.M.B).

W-Pos338
FLUORESCENCE EMISSION SPECTRA OF LAURDAN IN HEMOGLOBIN FREE ERYTHROCYTE MEMBRANES IN CHILDREN WITH TRISOMY 21.
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The fluorescent probe Laurdan (2-dimethylamino (6-lauroyl)naphtalene) has been reported to be incorporated at the hydrophilic-hydrophobic interface of the membrane with the lauric acid tail anchored in the hydrophobic region of the bilayer. Moreover, it has been demonstrated that Laurdan displays spectral sensitivity to the polarity of its surrounding, showing a blue-shift of the emission in apolar solvents, with respect to polar solvents. We have investigated fluorescence emission spectra of Laurdan incorporated in hemoglobin free erythrocyte membranes obtained from children with trisomy 21 and healthy controls. Our results show a blue shift of the emission spectra of Laurdan in erythrocyte membranes from subjects with trisomy 21. This decrease in polarity could be in part attributed to the previously reported (Biol Cell 1992;75,135-138) increase of membrane phospholipid packing, which may cause a reduction of water penetration.

W-Pos339
EFFECT OF NX06999 ON THE ELECTROPHORETIC MOBILITY OF NORMAL AND SICKLE BLOOD, RED CELLS AND PLASMA.
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Blood from normal persons and sickle-cell patients was studied by continuous-flow electrophoresis. Sickling was induced by sodium metabisulfite, and the effect of an antisickling agent, NX06999, extracted from Fagara xanthoxyloides root, was investigated. The mobility of sickle cells did not differ significantly from that of normal red blood cells, 1.08×10^{-4} $cm^2 V^{-1} sec^{-1}$. Sickle whole blood showed a 25% decrease in mobility over normal whole blood. Plasma from sickle-cell patients showed a 43% mobility increase over normal plasma, with a high deviation in the gamma-globulin region. Incubation with sodium metabisulfite decreased the mobility of sickle whole blood to 0.73×10^{-4} $cm^2 V^{-1} sec^{-1}$, but subsequent treatment with NX06999 restored it to that of normal blood, 1.08×10^{-4} $cm^2 V^{-1} sec^{-1}$.
Supported by DRR/NIH RCMI and NASA.

W-Pos340
ELECTROPHORESIS AND PARTITIONING OF ERYTHROCYTES (RBC) FROM ALZHEIMER DISEASE PATIENTS (AD) AND HEMATOLOGICALLY NORMALS (HN).
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Some manifestations of AD are reflected in changes in the membrane properties of many cell types including RBC. To determine whether these chemical alterations in AD RBC are apparent at the cell surface, RBC from AD and HN subjects were collected in citrate, washed and partitioned in both charge-sensitive and non charge-sensitive dextran (Dx)-poly(ethylene glycol) (PEG) aqueous phase systems and subjected to electrophoresis. The partition ratio, P, of AD RBC was within the normal range, as was the electrophoretic mobility (EPM) in saline of -1.07 to -1.10 $\mu m\ sec^{-1}\ v^{-1}$ cm. The viscosity corrected EPM of AD RBC and HN RBC in top, PEG-rich, phase were identical (-1.80 $\pm$ 0.02 and -1.81 $\pm$ 0.02, respectively). In the bottom, Dx-rich phase, the viscosity corrected EPM differed (-4.87 $\pm$ 0.08 and -4.53 $\pm$ 0.07, respectively). The difference in EPM was eliminated by first treating the cells with neuraminidase or trypsin but not with chymotrypsin. Thus the differential polymer adsorption between AD and HN RBC can be eliminated by appropriate enzymatic modification of the cell surfaces.

W-Pos341
RELATIONSHIPS AMONG IRREVERSIBLE DEFORMATION, CELL DEFORMABILITY AND CELL DENSITY IN SICKLE CELLS (SS CELLS).
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It is well known that blood from patients with sickle cell disease (SCD) contains irreversibly deformed cells and dense cells which may play an important role in the clinical course. However, the mechanism for the formation of such cells is not well understood, especially in circulation *in vivo*. Previously, we demonstrated that the degree of irreversible deformation, which was determined by an image analysis system, directly correlated with cell density and inversely correlated with Hb F (fetal hemoglobin) levels. Hb F is known as a potent inhibitor of polymerization of hemoglobin S (HbS). These data suggest that cell density is directly related to the degree of sickling in circulation *in vivo* as well as the degree of irreversible deformation. In this study, we assessed the effect of mean cell hemoglobin concentration (MCHC) and Hb F levels on the degree of irreversible deformation and cell deformability, determined by an ektacytometer, in relation to dense cell formation *in vivo*. We followed two sickle cell patients with different α gene numbers (2 and 5) on hydroxyurea (HU) treatment to increase Hb F levels. It is well known that MCHC in SS cells from patients with α gene deletion is lower than that from patients with normal or greater number of α genes. Hb F levels in the two patients increased from less than 5% to about 20% during HU treatment. Our data demonstrated that Hb F levels affected dense cell formation differently in the two patients while both the degree of irreversible deformation and cell deformability directly correlated with dense cell formation. This result suggests that sickling plays an important role in the formation of dense cell *in vivo*. Additional data on the relationships among irreversible deformation, cell deformability, cell density, and Hb F level are currently being collected from patients with SCD and different α gene numbers who are not on HU treatment.

W-Pos342
CORNEAL ENDOTHELIAL CELLS EXPRESS CHIP28-TYPE WATER CHANNELS.
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We have previously reported that bovine corneal endothelial cells (CBCEC) apparently express water channels, since the sulfhydryl reagent pCMBS 1 mM reduced CBCEC osmotic permeability P_f by 75%, such inhibition could be reverted by 5 mM dithiothreitol, and the activation energy for P_f was 4.7 $\pm$ 0.7 Kcal/mole. To identify the putative channels, we prepared poly-A^+ RNA from CBCEC and injected (50 ng) into isolated *Xenopus laevis* oocytes. We measured oocyte P_f four days after injection from the rate of volume increase upon exposure to 92% hypoosmotic medium. Oocyte cross-sectional area was determined by capturing its microscopic image with a computer-driven frame grabber every 20 seconds. The P_f of control oocytes (injected with 50 nl of water) was 13.4 $\pm$ 0.3 $\mu m/s$ (n=63). Injection of CBCEC-poly-A^+ RNA resulted in a P_f of 40.9 $\pm$ 1.6 $\mu m/s$ (n=72). This increase in P_f due to expression was inhibited by 78% with 0.3 mM $HgCl_2$; such inhibition was in turn reversed by incubating the oocytes in 5mM β-mercaptoethanol. The coinjection of CBCEC-poly-A^+ RNA with an excess of an antisense oligonucleotide for CHIP28 resulted in nearly complete abolition of the increase in oocyte P_f due to expression. We conclude that a major part of the osmotic water flow across the plasma membrane of CBCEC is accounted for by water traversing CHIP28-type water channels.
Supported by NIH Grants EY06178, HL33991, and the Intl. Soc. Nephrology.

W-Pos343

H^+ BUFFER SYSTEMS: DIFFERENTIAL EFFECTS ON CARDIAC $I_{K\text{-}ATP}$ SINGLE CHANNEL CONDUCTANCE AND OPEN PROBABILITY.

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The ATP-sensitive K^+ current ($I_{K\text{-}ATP}$) is implicated in the pathogenesis of ischemia, thus the effect of intracellular acidosis on $I_{K\text{-}ATP}$ is important. Using inside-out patches from rabbit ventricular cells, we have previously shown that intracellular protons (H^+_i) decreased unitary conductance (γ) and also antagonized ATP inhibition thereby increasing open probability (P_o) after 'rundown' was retarded by trypsin treatment. To further study the mechanism of H^+_i effects, we investigated $I_{K\text{-}ATP}$ for 3 buffer systems with 0.5 mM ATP present (Table). At pH 7.4, γ and P_o were constant whether the buffer was HEPES (5 mM, pK_a 7.5) or PIPES (5 mM, pK_a 6.8). When pH was lowered to 6.3, the H^+_i effect on γ was independent of the buffer used, but the H^+_i effect on P_o was less with HEPES than with PIPES. Histidine (5 mM, pK_a 6.5), a non-sulfonate buffer, behaved similarly to PIPES. These results suggest that H^+ acts at two distinct sites: one to decrease γ and one to increase P_o, and that the site for P_o is more sensitive to the solution buffering power.

	γ(pS) pH7.4	γ pH6.3	P_o pH7.4	P_o pH6.3
HEPES	72.0±1.4	60.0±0.6	.03±.02	.20±.07
PIPES	74.0	60.0±1.8	.02±.02	.67±.03

W-Pos344

GLYBURIDE-SENSITIVE K^+ CHANNELS REGULATE MEMBRANE POTENTIAL OF DOG CORONARY ARTERY SMOOTH MUSCLE CELLS.

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Recent studies have suggested that resting membrane potential (V_m) and tone of coronary vascular smooth muscle cells (VSMC) are controlled by a glyburide-sensitive K^+ channel (K_{ATP}). Therefore, we studied freshly isolated dog coronary artery VSMC using microelectrode, whole cell, and inside-out patch methods to assess the existence of K_{ATP} and their role in determining V_m. Whole cell voltage clamp (Tyrode's in bath; 1mM ATP+140 mM KCl in pipette) showed a large, time-dependent, outward rectifying K^+ current which activated at -30 mV and reached 1.4 ± 0.2 nA at 100 msec into a step from -60 to +60 mV. Single channel experiments related this current to a Ca^{++}-activated K^+ channel with a unitary conductance of 190 pS in symmetrical 140 mM KCl solutions. However, around V_m (-80 to -40 mV) no time-dependent current was identified. Instead, a small, linear, time-independent current was observed between -90 and -40 mV that reversed at -53 ± 4 mV with a slope conductance of 265 ± 40 pS. Glyburide (Gly, 1 μM) caused the current to reverse at -38 ± 5 mV and decreased the conductance to 149 ± 23 pS. Cromakalim (Crm,10 μM) caused the current to reverse at -82 ± 1 mV and increased the conductance to 1946 ± 321 pS. In current clamp Gly depolarized cells from -60 ± 5 mV to -41 ± 2 mV, while Crm hyperpolarized cells from -53 ± 3 mV to -81 ± 1 mV. Gly reversed or prevented the effects of Crm. In inside-out patches we identified an infrequently appearing 45 pS channel that was sensitive to Gly. These data indicate that the activity of K_{ATP}, in part, regulate V_m in dog coronary VSMC. (NIH HL32469 and Upjohn Labs)

W-Pos345

A SPECIFIC ACTIVATOR OF THE ATP INHIBITED K^+ CHANNELS IN GUINEA PIG VENTRICULAR CELLS.

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The discovery of potent activators (PCOs) and blockers (glyburide) of the ATP inhibited K^+ channel (K_{ATP}) has helped in the identification of K_{ATP} in various tissues. However, many were found to affect other ion channels as well. We examined the specificity of a new compound, P-1075, on cardiac K_{ATP} using whole cell and single channel recordings. At 10 μM, the compound shortened ventricular cell action potential duration by about 60%. The effect was completely reversible by 1 μM glyburide. Whole cell current experiments showed that the compound elicited a time independent outward current which had a linear current-voltage relationship between -70 to 0 mV. This current was sensitive to glyburide. Elevation of intracellular ATP from 1 mM to 5 mM strongly reduced the effect of P-1075 on the current, from 25.5 ± 1.8 pA/pF to 3.4 ± 0.6 pA/pF at $5*10^{-3}$M, for example. Removal of internal ATP, however, markedly diminished the drug effect, suggesting phosphorylation of the channel may be a pre-requisite for drug action. Curve fit to the dose response curves yielded a K_D of 20 μM and a Hill coefficient of 3.8 for the 1 mM ATP experiment and a corresponding 37 μM and 2.2 for the 5 mM ATP. Single channel experiments suggest that P-1075 promotes K_{ATP} channel activity by increasing channel open probability. The compound, at doses up to 25 μM, did not affect the inward and outward rectifying K^+ currents nor the L-type Ca^{2+} current. We conclude that P-1075 is a specific and effective activator of phosphorylated K_{ATP} channels.

W-Pos346

TRYPSIN AND α-CHYMOTRYPSIN DIGESTION ABOLISHES GLIBENCLAMIDE SENSITIVITY OF K_{ATP} CHANNELS IN RAT VENTRICULAR MYOCYTES.

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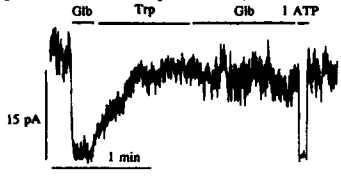
Cytoplasmic trypsin-treatment of voltage-sensitive potassium channels has been shown to cleave domains of the channel responsible for inactivation of the channel. Trypsin has also been reported to remove slow, irreversible inactivation, or run-down in ATP-sensitive potassium (K_{ATP}) channels (Furukawa et al., 1992, Biophys. J. 61, A249). Our attempts to reproduce this effect were unsuccessful and cytoplasmic treatment of rat ventricular K_{ATP} channels with either crude, or pure, trypsin failed to prevent a slow run-down of channel activity. However, 1-2 mg/ml trypsin (porcine pancreatic type IX, crude type II (Fig. 1), or bovine pancreatic α-chymotrypsin, all Sigma Chem. Co.) rapidly (within 1 minute) and irreversibly abolished glibenclamide-sensitivity (10 μM) in inside-out, or outside-out, patches, without affecting channel conductance or removing sensitivity to 1 mM ATP. Glibenclamide-binding (to a 140 kDa receptor) and channel inhibitory affinities are very closely correlated. It is possible, and a widely held view, that the receptor is an integral part of the channel.



The results suggest that glibenclamide must bind to either a separate protein, or to a domain on the K_{ATP} channel that is separate from the conducting pore in order to inhibit the channel, and that this domain is disconnected from the channel by trypsin-treatment.

W-Pos347

COMPARISON OF THE ELECTROPHYSIOLOGICAL EFFECTS OF P1075 AND CROMAKALIM IN CANINE AND GUINEA PIG CARDIAC MUSCLE.

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Previous electrophysiological studies suggest that pinacidil is 2-5 fold less potent than cromakalim (CR) in shortening action potential duration (APD) and increasing outward K^+ current (IKo). This study examined the effects of the pinacidil analog N"-Cyano-N'-(1,1-dimethylpropyl)-N-(3-pyridinyl)-guanidine (P1075) on APD and IKo in canine and guinea pig (GP) cardiac tissues. APD was studied in canine Purkinje fibers (PF) and papillary muscles (PM) and in GP PM using standard microelectrode techniques. EC_{50}'s for APD shortening in canine PF and PM were ~100 nM (n=6) and 80 nM (n=2), respectively. EC_{50} for APD shortening in GP PM was 1 μM (n=3). The EC_{50} for CR in canine PF was 700 nM (n=6). The APD induced shortening by P1075 and CR in PF and PM was reversed by addition of 10-100 nM glyburide. Whole cell patch-clamp techniques were used to study IKo in isolated ventricular myocytes (VM). IKo was elicited using a slow voltage ramp (6 mV/sec, from -130 to +50 mV). In VM, 10 μM CR was the lowest concentration that increase IKo, measured at +20 mV, in both canine (0.36 ± 0.07 nA to 2.04 ± 0.26 nA, n=10, p<0.001) and GP (0.9 ± 0.1 to 1.1 ± 0.1 nA, n=5, p<0.05) VM. P1075 was 100 fold more potent: 100 nM was the threshold concentration which significantly increased IKo in both canine (0.39 ± 0.01 nA to 0.81 ± 0.14 nA, n=7, p<0.05) and GP (1.18 ± 0.23 nA to 1.40 ± 0.16 nA, n=5, p<0.01) VM. Since CR has previously been shown to open ATP-sensitive K^+ channels in cardiac cells, these results suggest that P1075 is a more potent activator of these channels.

W-Pos348

MUSCARINIC AGONISTS SUPPRESS ATP-SENSITIVE K^+ CHANNEL CURRENTS IN SMOOTH MUSCLE CELLS FROM GUINEA-PIG URINARY BLADDER

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Potassium channels (K_{ATP}) closed by intracellular ATP and inhibited by glibenclamide have been identified in cardiac muscle, pancreatic beta cells, skeletal muscle, neurons and arterial smooth muscle. These channels are thought to link changes in cellular metabolism (e.g. ATP changes) to the cell's membrane potential and appear to be targets of a wide variety of synthetic (e.g. lemakalim) and endogenous vasodilators (e.g. calcitonin gene-related peptide). We used the patch clamp technique to measure whole cell and single channel potassium currents in smooth muscle cells isolated from the detrusor muscle of the guinea pig urinary bladder. Lemakalim (10 μM) increased whole cell K^+ currents by about 50 pA at -80 mV with 60 mM external K^+ when the cells were dialyzed with 0.1 mM ATP and 140 mM K^+. Glibenclamide (10 μM) inhibited the entire lemakalim-stimulated current as well as 19 pA of steady-state K^+ current. Increasing intracellular ATP from 0.1 mM to 3.0 mM reduced the glibenclamide-sensitive K^+ current both in the presence and absence of lemakalim by about 4-fold. External barium (100 μM), which blocks K_{ATP} channels in skeletal muscle, reduced K_{ATP} channel currents in bladder smooth muscle by 50% at -80 mV. Lemakalim increased the open state probability (NP_O) of single potassium channels in outside-out patches (with 0.1 mM internal ATP) by 6-fold. The single channel conductance was about 7 pS at 0 mV with a physiological K^+ gradient and is in accord with estimates made from the noise of the lemakalim-induced whole cell current. Muscarinic agonist carbachol (CCh) (1-10 μM) reduced the whole-cell glibenclamide sensitive currents by 56%. Atropine (10 μM) prevented the suppression of K_{ATP} channel current by CCh. CCh had no effect on the membrane current in the presence of glibenclamide. We conclude that urinary bladder smooth muscle has K_{ATP} channels and that they contribute to the membrane potential. We propose that cholinergic stimulation may depolarize bladder smooth muscle in part through inhibition of K_{ATP} channels.

W-Pos349

ATP-SENSITIVE POTASSIUM CHANNELS FROM CORONARY SMOOTH MUSCLE RECONSTITUTED IN LIPID BILAYERS. ((M. Ottolia, & L. Toro)) Molecular Physiology & Biophysics. Baylor College of Medicine, Houston, TX 77030.

K_{ATP} channels from pig coronary smooth muscle were incorporated into lipid bilayers. K_{ATP} channels were recorded after incorporation of membrane vesicles banding at the 20:25% interface of a discontinuous sucrose gradient. Experiments were performed using a 250/5 KCl gradient in the presence of 5 μM free Ca^{2+}. Under these conditions, K_{ATP} channels had at least four subconductance levels with values ranging between 20 and 50 pS (n=4). In most cases at 0 mV, channel activity spontaneously run-down. Initially, their open probability was low (Po ≈ 0.01), their openings were brief separated by long closings (mean open times < 5 ms; mean closed times > 200 ms) and they gradually disappeared (within 2-3 min) (n=8). In some occasions K_{ATP} channels had a higher Po (≈0.7) and longer open times (≈50 ms) (n=3). K_{ATP} channels could be stimulated upon addition of 2.5 to 5 mM UDP-TRIS in the presence of 0.5 to 1 mM $MgCl_2$ (n=3) and by 10-50 μM pinacidil a "K channel opener". Pinacidil promoted long-lived openings (≈100 ms) of an intermediate subconductance level (26 pS) (n=4). Spontaneously active K_{ATP} channels and channels previously stimulated by UDP or pinacidil, could be inhibited by ATP-Na_2 (50-200 μM) (n=6). In conclusion, K_{ATP} channels from coronary smooth muscle have several subconductances and similar pharmacology than those present in cardiac and skeletal muscles. Reconstitution of K_{ATP} channels in lipid bilayers will be useful to study the mechanisms of drug action. Supported by AHA-National Center (900963) and NIH (HL47382).

W-Pos350

SINGLE CHANNEL PROPERTIES OF AN ATP- AND Ca^{++}- SENSITIVE K^+ CHANNEL IN THE RAT SUBSTANTIA NIGRA. C. Jiang, F. J. Sigworth & G. G. Haddad, Depts. Pediatrics (Section of Respiratory Medicine) and Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, CT 06510

ATP-sensitive K^+ channels are believed to play an important role in the neuronal response to metabolic stress. However, the detailed regulation at the single channel level has not been defined in central neurons. We examined single channel kinetics of ATP-sensitive K^+ channels in dissociated neurons from the substantia nigra (SN), a region known to be rich in glibenclamide-binding sites. A large conductance channel (~200pS) was recorded in about 1/3 of SN neurons (n=200), which was selectively permeable to K^+ but not to Na^+, Ca^{++} and Cl^-. The open time histograms of this channel could be fitted by 2 exponentials, and closed times by 3 exponentials. Changes of Vm from -60mV to 60mV revealed an outward rectification in the open probability (P_o) with an almost linear unitary conductance. ATP markedly suppressed P_o by increasing the long closed times with no effect on unitary conductance. A similar, but less potent, result was observed with ADP and AMP-PNP, a non-hydrolyzable ATP analogue. P_o was enhanced by increasing cytoplasmic Ca^{++} and was inhibited by Ca^{++} chelation with EGTA. External charybdotoxin (50nM) and apamin (5nM) did not have any effect on channel activity, but glibenclamide (10-20μM) inhibited it significantly. ATP did not inhibit channel activity via Ca^{++}-chelation since MgATP plus Mg^{++} (2.5mM) decreased P_o, and ATP (1mM) inhibited the channel in a high Ca^{++} background (5mM). These results suggest that 1) SN neurons have a K^+ channel that is independently sensitive to ATP and Ca^{++}; and 2) the action of ATP on this channel is not mediated by protein phosphorylation.

W-Pos351

RECONSTITUTED K^+ FLUX VIA THE PARTIALLY PURIFIED K^+/ATP CHANNEL FROM CARDIAC SARCOLEMMAL. ((P. Paucek and K. D. Garlid)). Department of Pharmacology, Medical College of Ohio, Toledo, OH 43699

K^+ transport was monitored in sarcolemmal vesicles and liposomes loaded with the fluorescence probe, PBFI. Sarcolemmal proteins were extracted using Triton X-100 and fractionated on DEAE celluose column. Electrophoretic K^+ flux was observed in only one fraction. K^+ flux was inhibited by ATP (K_i = 639 μM) and glibenclamide (K_i = 60 nM). The sarcolemmal and mitochondrial K+/ATP channels were readily distinguished by the fact that ATP inhibition of the sarcolemmal channel did not require Mg^{2+}, whereas ATP inhibition of the mitochondrial channel has an absolute requirement for Mg^{2+} (Paucek et al., J.B.C. in the press, 1993). Supported by NIH Grant HL 36573.

W-Pos352

EFFECTS OF ATP AND Ca^{2+} ON K^+ CHANNELS OF THE MDCK CELL. ((Shyue-Fang Hsu and Ronald R. Burnette)) University of Wisconsin, School of Pharmacy, Madison, WI 53706.

Inside-out patches were formed from MDCK cells. The patches were shown to contain K^+ channels by the successful suppression of channel activity by the addition of 10 mM TEA into the bathing solution. The action of TEA was reversible upon washout. One class of K^+ channels was observed to be reversibly activated when ATP (maximal response occurred at ~ 2 mM ATP) was added to the bathing solution. The channel had a conductance of 34 pS (between -80 mV to -40 mV) when bathed with a symmetrical K^+ solution (160 mM). In addition, the symmetrical K^+ solution contained: 2 mM Cl^-, less than $1X10^{-7}$ M Ca^{2+}, 1 mM Mg^{2+}, 10 mM glucose and no Na^+. In contrast, another class of K^+ channels was reversibly activated when Ca^{2+} (2 mM) was present in the bathing solution and reversibly deactivated when ATP (2 mM) was present in the bathing solution. The channel conductance was 37.7 pS (between -100 mV to -60 mV) when the channel was bathed with 160 mM K^+ in the pipette solution and 5 mM K^+ in the bathing solution. Both the bathing and pipette solutions had 2 mM Cl^-, 1 mM Mg^{2+} and 10 mM glucose.

W-Pos353

ATP-SENSITIVE K^+ CHANNELS IN PANCREATIC ALPHA CELLS ((A.S.Rajan, L.Aguilar-Bryan, D.A.Nelson, C.G.Nichols*, S. W.Wechsler, J.Lechago and J.Bryan)) *Depts. of Medicine, Cell Biology, and Pathology, Baylor College of Medicine, Houston TX 77030 and *Dept. of Cell Biology & Physiology, Washington Univ. School of Medicine, St. Louis, MO 63110*

Glucagon secretion from pancreatic islet alpha cells is regulated by changes in blood glucose, but the molecular mechanism(s) by which this occurs remains elusive. In islet beta cells, ATP sensitive K^+ (K_{ATP}) channels play a major role in coupling glucose metabolism to the stimulation of insulin release. Using clonal alpha cells (αTC-6), we tested the hypothesis that K_{ATP} channels are present in alpha cells. Ionic flux assays using ^{86}Rb revealed that an outward K^+ flux in ATP-depleted cells was attenuated by the specific K_{ATP} channel blocker, glyburide (5 nM). Diazoxide (400 μM) an activator of K_{ATP} channels, reversed the inhibition of $^{86}Rb^+$ efflux induced by glyburide. Likewise in whole cell patch clamp experiments, an outward K^+ conductance that developed upon cell break-in and ATP dialysis, was attenuated by glyburide. The K_{ATP} conductance inwardly rectified at positive potentials. Single channel recordings in inside-out patches showed attenuation of the K_{ATP} current when the bathing medium on the cytoplasmic surface was switched rapidly from 0 to 1 mM ATP. The K_{ATP} current was restored when the bathing solution was changed from 1 to 0 mM ATP. Using a ^{125}I-labeled glyburide analog, we also identified two specific high affinity sulfonylurea receptors in the αTC-6 cell membranes. These results demonstrate the presence of K_{ATP} channels in alpha cells which may play a role in their metabolic regulation. The concurrent identification of high affinity sulfonylurea receptors adds to the growing body of evidence that these receptors are integral components of K_{ATP} channels. *(Sponsored by L.C.Smith)*

W-Pos354

MODE-SPECIFIC REGULATION OF ATP-DEPENDENT GATING BY NUCLEOTIDE DIPHOSPHATES IN THE ATP-SENSITIVE K CHANNEL. ((A. Terzic, I. Findlay, Y. Hosoya and Y. Kurachi)) Mayo Clinic, Rochester, MN 55905.

The ATP-sensitive K channel (K_{ATP}) is regulated by the ATP_i-dependent gating and activated by depletion of ATP_i. Nucleotide diphosphates (NDPs), such as ADP and GDP, antagonize the inhibition of K_{ATP} by ATP_i, which may play an essential role in regulating K_{ATP} during cardiac ischemia and glucose-dependent insulin secretion. The molecular mechanism underlying the interaction of NDPs and K_{ATP} has been poorly understood. Here we report that the effect of NDPs on the ATP_i-dependent gating of K_{ATP} in cardiocytes critically depends on the mode of the channel with reference to its "phosphorylation". Upon formation of an I-O patch, K_{ATP} appears in the ATP-free solution, but spontaneously becomes inoperative (rundown), presumably due to its dephosphorylation. After complete rundown, mM concentrations of NDPs made "dephosphorylated" K_{ATP} operative. Hence, K_{ATP} has two distinct operative modes: (1) spontaneous "phosphorylated" operative mode, and (2) NDP_i-induced operative mode of the "dephosphorylated" K_{ATP}. In Mode 1, ATP_i inhibited K_{ATP} with an IC_{50} of ~20 μM. In the presence of 5 mM UDP 5 mM, IC_{50} became ~500 μM. This indicates that UDP_i decreases the sensitivity of K_{ATP} to ATP_i, which suggests a direct interaction of UDP with the ATP_i-dependent gating mechanism of Mode 1 K_{ATP}. By contrast, in Mode 2, the UDP-induced openings of K_{ATP} after rundown were inhibited by ATP_i with an IC_{50} of 20 μM, suggesting that UDP does not interact with the ATP_i-dependent gate of Mode 2. In conclusion, the mode of the channel with reference to rundown determines the molecular interaction of NDPs and the ATP-binding gating of K_{ATP}. Supported by AHA Grant in Aid 91013540.

W-Pos355

REGULATION OF NEURONAL POTASSIUM CURRENT BY INTRACELLULAR NUCLEOTIDE TRIPHOSPHATES. ((M. A. Simmons and C. R. Schneider)) Neuropharmacology Lab., Dept. Pharmacology, Marshall Univ., Huntington, WV 25755.

The M current (I_M) is a voltage- and time-dependent K current present in sympathetic neurons, brain, spinal cord and smooth muscle. We have examined the role of intracellular ATP and GTP in the sustenance of I_M.

Whole cell recordings were made from single neurons enzymatically dissociated from bullfrog sympathetic ganglia. The rate of rundown of I_M was observed. Electrodes were filled with solutions containing different [ATP]. The rate of rundown of I_M was slowed as $[ATP]_i$ was increased from 0.1 to 1 mM. Rundown became more rapid as $[ATP]_i$ was further increased from 1 to 10 mM. AMP-PNP inhibited the ability of low concentrations of ATP to maintain I_M. Addition of pyruvate or glucose to the extracellular medium was equivalent to including ATP in the pipette. The effect of ATP was blocked by adding UDP (10 mM) to the pipette. GTP (1-8 mM) in the pipette solution also slowed rundown of I_M. The effect of GTP was not altered by UDP.

These results show that I_M is regulated by intracellular ATP and GTP. Furthermore, the cellular level of a nucleotide triphosphate depends not only on its pipette concentration, but also on the contents of the extracellular medium and on the metabolic state of the cell. The results also suggest that the ability of ATP to maintain I_M results from its transphosphorylation to GTP. Thus, the level of GTP, rather than the level of ATP, may be more important in determining the magnitude of I_M.

W-Pos356

BLOCKADE OF THE DELAYED RECTIFIER BY CALCIUM IONS. RELATIONSHIP TO THE EFFECTS OF DIVALENT CATIONS ON GATING. John R. Clay, NINDS, NIH, Bethesda, MD 20892

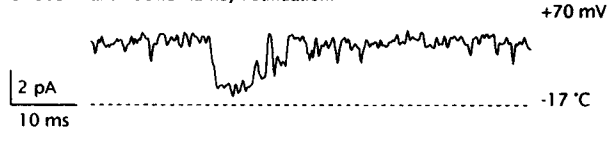
A significant, voltage-independent blockade of I_K in squid giant axons was observed with Ca_o^{+2}, using a relatively negative holding potential (HP = -90 mV). The K_D (50% block) was 105± 55 mM (n=6, ± s.d.). Similar results were obtained with Mg_o^{+2}, but not Mg_i^{+2} (100 mM). The I_K inactivation curve was shifted along the voltage axis by increasing Ca_o^{+2}, thereby removing inactivation with HP = -60 mV. The I_K increase due to this removal of inactivation is approximately offset by Ca_o^{+2} blockade, which may explain why block of I_K has not been previously reported. Changes in Ca_o^{+2} produce a well known voltage shift of the I_K activation curve. However, I_K kinetics are differentially effected (Armstrong and Matteson, 1986, J. Gen. Physiol. 87:817). In this study a shift of the activation curve was observed with Mg_i^{+2} with effects on kinetics which are the mirror image of the effects of external divalents. These results are difficult to reconcile with surface charge theory. Alternatively, they can be modeled in a Hodgkin & Huxley context in which the activation curve is given by $(\alpha/(\alpha+\beta))^4$, the kinetics are described by $(\alpha+\beta)^{-1}$, and α and β are specifically modified by extra- and intracellular divalents, respectively, similar to the original proposal of Gilly and Armstrong (1982, J. Gen. Physiol. 79:965).

W-Pos357

GATING AT SUBZERO TEMPERATURES. ((J. Miodownik and W. Nonner)) Department of Physiology and Biophysics, University of Miami, Miami, FL 33101.

Open-shut transitions of ion channels usually appear instantaneous at the time resolution of the patch clamp technique. On the expectation that low-temperature experiments would reveal details of the transition *per se*, we have developed a technique for recording from excised membrane patches cooled down to -35°C.

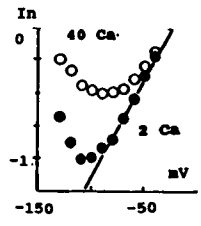
Ca-activated large K channels of cultured rat myotubes were examined at temperatures between +20°C and -35°C in the presence of up to 40 vol% of the anti-freeze compound, ethyleneglycol. Channel openings were observed throughout. Cooling reduced unitary conductance with increasing Q_{10} (1.56 at 10°C, 2.05 at -15°C median temperature), whereas open probability passed through a minimum near 0°C (P_o at $[Ca]_i$= 30 μM and +30 mV was 0.58 for -8°C, and 0.92 for -17°C). In the cold, a transition phase was resolved between well-defined sojourns at open or shut current levels. This phase was characterized by oscillatory and/or graded changes in current. Oscillations occurred between open, shut, and intermediate levels, or outlined a gradually shifting level (see Figure). Thus, cold channels remain capable of rapid modulations of their current, but become less capable of rapidly stabilizing open or shut states. Supported by NIH GM30377 and Lucille Markey Foundation.



W-Pos358

EXTERNAL CALCIUM BLOCKS SHAKER CHANNELS. ((F. Gómez-Lagunas and Clay M. Armstrong)) Dept of Physiology, Univ Pennsylvania, Philadelphia. (Spon. by A. Obaid)

Shaker H4 channels were expressed in Sf9 cells by infection with a recombinant baculovirus, provided by Dr. C. Miller. Macroscopic currents were recorded 2 days after infection. External Ca^{++} reversibly blocks inward tail currents, producing a region of negative slope in the instantaneous IV curve. Ca^{++} block is apparent even at low concentrations (1- 2 mM). Above 2 mM, Ca^{++} block follows a Langmuir isotherm. With 40 mM external K^+, the Ki is 4.4 mM, at -120 mV. Ca^{++} block is voltage dependent and, in 40 mM Ca^{++}, becomes apparent at about -50 mV. Fitting the Boltzmann function to the fraction blocked, gave an electrical distance of 0.5 (minimum estimation). External Ca^{++} also blocks the slow, inward, tails seen during recovery from inactivation, but it does not changes the rate of recovery.



W-Pos359

MODIFYING THE ORIENTATIONAL CONFIGURATIONS OF A PROTEIN-PROTEIN INTERACTION THROUGH SITE SPECIFIC MUTAGENESIS. ((P. Hidalgo Jimenez and R. MacKinnon)) University of Chile, Santiago Chile and Harvard Medical School, Boston, MA 02115 .

When amino acid Asp 431 on a Shaker K^+ channel is mutated to Asn (D431N), scorpion toxin sensitivity is lost if the mutation is present in all four channel subunits. However, if even a single subunit contains an Asp at 431, then the channel remains relatively sensitive to toxin. The dominance of the toxin sensitive phenotype can be understood by considering the natural consequence of having an asymmetric ligand (the toxin) and a 4-fold symmetric receptor (the K^+ channel). A toxin can bind to a wild type channel with four equivalent configurations. If a D431N mutation in a single subunit excludes only one configuration, then channels with even a single wild type subunit should be sensitive (compared to channels with 4 mutant subunits) since one permissive configuration remains. This statistical explanation makes a strong testable prediction. The number of wild type subunits in a channel should influence the toxin association rate since the probability of successful encounter will be proportional to the number of permissive configurations. By measuring the kinetics of toxin blockade of wild type and wild type - D431N hybrid Shaker K^+ channels, we find that this prediction is satisfied. Moreover, once a toxin is bound, the dissociation rate is independent of the number of wild type subunits present.

W-Pos360

AN ALLOSTERIC MODEL FOR THE DELAYED RECTIFIER POTASSIUM CURRENT OF FROG SYMPATHETIC NEURONS. ((Kathryn J. Greene and Stephen W. Jones)) Department of Physiology & Biophysics, Case Western Reserve University, Cleveland, OH 44106.

The time course of whole-cell delayed rectifier current was not well fitted by an exponential raised to a power, for 70 ms depolarizations between -20 and +100 mV. Generally, n = 2 fit best, but did not produce enough delay at positive voltages; n = 3 could describe the delay but not the subsequent time course. The current at late times was well fitted by a single exponential, which reached a limiting $\tau \sim 5$ ms above +40 mV, possibly suggesting that a voltage-independent C - O transition became rate limiting. However, tail currents continued to accelerate with hyperpolarization, reaching τ = 1-2 ms at -110 mV. These results are not expected from usual models for gating of voltage-dependent channels, but can be described by a model (Jones and Marks, *J. Gen. Physiol.* 99:367-390, 1992) where channel opening is voltage-independent but favored by movement of allosterically coupled voltage sensors. During activation, all four postulated voltage sensors tend to 'open' before the channel opens, but upon repolarization 'closing' of 1-2 voltage sensors accelerates subsequent channel closing. Some quantitative discrepancies remain between the model and data, but currents simulated from the model systematically diverge from power law fits in the same way as the data.

W-Pos361

MODELS OF COUPLED INACTIVATION OF FERRET VENTRICULAR I_{TO}. ((R.L. Rasmusson, D.L. Campbell, Y. Qu and H.C. Strauss)) Biomed. Eng., Pharmacology and Medicine, Duke U. Med. Ctr., Durham, N.C. 27710

Advances in the study of K^+ channels have suggested that K^+ channel inactivation may be coupled, or partially coupled, to activation. Previous reports have indicated that the transient outward K^+ current (I_{TO}) measured in isolated single cells from ferret right ventricle show a sigmoid onset of activation which can be modelled by a series of closed states (Campbell et al. *Biophys. J.* 1992 61:252a). Whole cell recordings of I_{TO} yielded time constants of inactivation which are apparently voltage insensitive over the range +30 to +70 mV. Voltage insensitivity of inactivation was confirmed at the single channel level and appears to be due to channel inactivation and not delayed delivery of channels into the open state. Several partially coupled models involving voltage insensitive inactivation rate constants can reproduce the inactivation response to a depolarizing pulse. We have considered 3 alternatives: 1) An enabling model, in which activation enables a single inactivation process (interpretable as revealing a single binding site for the putative inactivation particle). 2) An additive model, in which the forward rate of inactivation increases linearly with the activation process (interpretable as increasing the number of sites as each subunit becomes activated). 3) A "push off model" in which there is energetic coupling between the activation "units" and the inactivation process. Models 1 and 2 fall short of reproducing the observed time constant of recovery from inactivation by an order of magnitude, indicating that energetic interactions similar to those described for TEA binding to K^+ channels (MacKinnon, *Nature* 1991 350:232-235) may also occur for inactivation of ferret I_{TO}.

W-Pos362

COOPERATIVE INTERACTIONS AMONG SUBUNITS OF A VOLTAGE-DEPENDENT POTASSIUM CHANNEL. ((R.S. Hurst, M.P. Kavanaugh, R.A. North and J.P. Adelman)) Vollum Institute, OHSU, Portland, OR 97201.

The gating current kinetics of the *Shaker* H4 channel suggest that individual subunits of the channel do not respond to voltage independently (Bezanilla et. al., *Science* 254:679, 1991). A concatenated potassium channel was generated to test interactions among subunits (domains); a single point mutation (L to I) was introduced into the S4 region of one or more domains (L305I in RBK1). The concatenated channel was produced by joining four cDNAs of a rat homolog of *Shaker* (RBK1; also called Kv1.1); the concatenated cDNA and channel are termed [4]RBK1. The L to I substitution in the wild-type RBK1 or in all four domains of [4]RBK1 caused a rightward shift in the midpoint of the conductance-voltage relation of approximately 50 mV with no apparent change in the slope. This substitution was then introduced into 1, 2 or 3 domains of [4]RBK1; each L to I substitution resulted in an approximately equal rightward shift along the voltage axis. Models of non-interacting domains predict a marked non-linearity. For example, the model of independent activation for channels containing 2 L and 2 I domains indicate that the normalized conductance should equal 0.5 at 23 mV; however, at 23 mV the experimentally observed value was 0.87. This corresponds to an energy difference of 4.6 kJ/mol; presumably this is supplied from cooperative interactions among the domains.

W-Pos363

REMOVING INACTIVATION OF AN A-TYPE CHANNEL (RHK1) POTENTIATES BLOCK BY 4-AMINOPYRIDINE (4AP). ((J.-A. Yao, G.-N. Tseng)) Dept Pharmacology, Columbia U., New York, NY 10032.

There have been controversies over whether 4AP blocks K channels from the rested or the open (O) state. We tested the effects of 4AP (10 uM-10 mM) on RHK1 (WT) and its mutant (DelA, whose N-type inactivation was disrupted) to see whether changing the time the channel stayed at the O state could influence 4AP block. Both channels were expressed in *Xenopus* oocytes and studied under 2-microelectrode voltage clamp. When examined using a long-depolarization pulse protocol (0.5 s for WT, 5 s for DelA), the peak current amplitude (Ipeak) induced by the 1st pulse after 4AP application ($\leq$ 1 mM) was comparable to that of control while the current decay was markedly accelerated. Ipeak started to decline at 2nd pulse after 4AP. Fewer pulses were needed to reach a steady state block in DelA (3-4) than in WT (4-5). Ipeak induced by the 1st pulse after extensive 4AP washout showed no significant recovery while the current decay was slowed. Ipeak started to recover subsequently. The concentration-response relationship of 4AP block could be fit with a 1 to 1 binding model for both WT (n=2) and DelA (n=6). The estimated binding constant (Kd) was higher for WT than for DelA (1.3 and 0.5 mM). The differences between WT and DelA in terms of rate and degree of 4AP block disappeared when examined using a short-pulse protocol (12.5 ms, allowing channel activation without inactivation). Furthermore when evaluated at the same amount of total depolarization time the short-pulse protocol induced more 4AP block than the long-pulse protocol. We conclude that, for RHK1, binding and unbinding of 4AP mainly occur at the O state.

W-Pos364

IDENTIFICATION OF NEGATIVELY CHARGED ELEMENTS OF THE VOLTAGE SENSOR IN A POTASSIUM CHANNEL. ((G.J. Gross, R.V. Lacro and D.E. Logothetis)) Children's Hospital, Harvard Medical School, Boston, MA 02115.

Mutations of the positively charged amino acid residues of the S4 region alter the gating characteristics of voltage-gated cation channels. These positive charges could interact with negative charges in adjacent transmembrane domains, thereby stabilizing the channel in either an open or closed conformation. The delayed rectifier RCK1 potassium channel has 2 negatively charged glutamate (E) or aspartate (D) residues in each of the putative transmembrane domains S2, S3, and S5. We constructed positively charged (lysine (K)), neutral (glutamine (Q) or asparagine (N)), and charge-conservative (E or D) mutants at the S2 sites E225 and E235, and at the S3 sites D258 and E272. Mutant mRNA was injected into Xenopus laevis oocytes and studied with 2 microelectrode voltage clamp. All mutations of D258 abolished ionic current. E272Q had gating valence (z) identical to control, but showed a 17 mV rightward shift of the 10% activation potential (n=8). E272K produced a 25% reduction in z and 18 mV rightward shift (n=12). E225 and E235 mutants are presently being studied. Mutants of the S5 residues D361 and D377 are being constructed. Similarly, double mutants of E272K and S4 positive to negative charge mutations will identify interacting charges. Our data suggest a role in voltage sensing for E272 and possibly for other negatively charged residues in non-S4 transmembrane domains.

W-Pos365

REGULATION OF AN INACTIVATING HUMAN K^+ CHANNEL BY PROTEIN KINASE C KT Murray, SA Fahrig, SS Po, NN Hu, DJ Snyders, MM Tamkun, and PB Bennett. Vanderbilt University, Nashville, TN

The transient outward current (I_{TO}), an important A-type repolarizing current in human heart, is inhibited by interventions which activate protein kinase C such as α-adrenergic stimulation. HK1 (Kv1.4) is a K^+ channel cloned from human heart which has phenotypic similarities to I_{TO}. We recently reported that HK1 current was suppressed by the phorbol ester phorbol 12-myristate 13-acetate (PMA, 50 ng/ml or 81 nM). Additional experiments were performed to investigate the mechanism of this effect. *Xenopus* oocytes were injected with *in vitro* transcribed cRNA encoding the HK1 channel. Current was expressed within 1-2 days and measured using the two electrode voltage clamp technique. At a concentration of 10nM, PMA caused a biphasic response with an initial increase (14 $\pm$ 4%, mean $\pm$ SEM) in the current which peaked in 14 minutes (n=10). This was followed by a significant reduction in the current with 39 $\pm$ 11% suppression at 30 min, 55 $\pm$ 11% suppression at 45 min. Although capacitance fell significantly during administration of PMA 81 nM (23 $\pm$ 9% decline at 30 min), there was little change using 10 nM (1 $\pm$ 2% decline at 30 min). 4-α PMA, the inactive stereoisomer of PMA, had no effect on HK1 current; as well, preincubation with the protein kinase inhibitor staurosporine 3μM blocked the effects of PMA (n=4 each). These results indicate that protein kinase C activation results in biphasic modulation of HK1 current and further suggests that this K^+ channel may represent an important component of I_{TO}.

W-Pos366

MODULATION OF SAK CHANNELS BY NEUROTRANSMITTERS VIA 2nd MESSENGERS? ((D.L. Small, X. Wan and C.E. Morris)) University of Ottawa, Neurosciences, Ottawa Civic Hospital, Ottawa, Ontario, Canada K1Y 4E9.

The physiological role of stretch-activated (SA) channels remains controversial. In molluscan neurons, SA K^+ channels are ubiquitous, but they seem not to be mechanotransducers. In *Aplysia*, we showed that the S-channel is a SA K^+ channel. Are all molluscan SA K^+ channels "S-like" in that their primary mode of regulation is by transmitters via the 2nd messengers, cAMP and arachidonic acid (AA)?

We looked at the effects of db-cAMP and AA on SA K^+ channels in unidentified *Lymnaea* neurons. Though db-cAMP and AA seemed to induce down and up regulation, respectively, in some cells, the results were highly variable. We therefore reject the simplest hypothesis, namely that all molluscan SA K^+ channels are S-like in the strictest sense (i.e. cAMP and AA sensitive). Variability may be due to cell heterogeneity and/or the involvement of diverse 2nd messengers. To test this, we have begun working on a homogeneous molluscan cell population, *Lymnaea* heart cells. These cells have SA K^+ channels which closely resemble those in molluscan neurons. In molluscan heart, 5-HT acts as an excitatory neurotransmitter via cAMP. 5-HT has been shown to decrease $^{86}Rb^+$ efflux in *Lymnaea* heart ($^{86}Rb^+$ is a K^+ analog). $^{86}Rb^+$ efflux in this preparation can be increased by osmotic swelling and decreased by the SA K^+ channel blocker quinidine. Possible links among these findings are being sought. Supported by NSERC, Canada.

W-Pos367

PHARMACOLOGICAL PROPERTIES OF K CURRENTS IN ISOLATED CANINE COLONIC MYOCYTES ((A.Carl, C.DU, J.L.Kenyon, K.D.Keef and K.M.Sanders)) Dept. of Physiology, University Nevada School of Medicine, Reno, NV 89557, USA, (supp. by NIH-DK41315)

The pharmacology of forskolin and lemakalim induced hyperpolarization measured with microelectrodes at the myenteric border of tissue strips from canine colon was investigated. Forskolin induced hyperpolarization was inhibited by TPeA (50 μM) and 4-AP (10 mM). Lemakalim induced hyperpolarization was inhibited by TPeA (50 μM), glibenclamide (10 μM) and PCP (100 μM). Neither response was inhibited by TEA (10 mM) or ChTX (100 nM).

A voltage ramp protocol was used to separate two clearly distinguishable components of outward current in voltage clamped freshly isolated myocytes from the circular layer of canine colon. Holding potential was changed from -100 mV to +100 mV, $dV/dt = 0.05\ Vs^{-1}$. The resulting current was compared with a standard IV curve obtained with 500 ms duration test pulses from a holding potential of -80 mV. The ramp current reflected the IV relationship at the end of 500 ms test pulses. The first current component (I_{dK}) activated at -40 mV and reached a max. value at +40 mV. Superimposed on this was a second noisy current component ($I_{K(Ca)}$) activating at +50 mV. ChTX (100 nM) selectively inhibited $I_{K(Ca)}$ while 4-AP (10 mM) selectively inhibited I_{dK}. TPeA (10 μM) and PCP (100 μM) selectively reduced I_{dK}. 3,4-di-AP (5 mM) inhibited both $I_{K(Ca)}$ and I_{dK}. Upon washout $I_{K(Ca)}$ was restored immediately while I_{dK} returned to control values within minutes, suggesting different binding sites for 3,4-di-AP on these channels. Both forskolin and lemakalim induced hyperpolarizations exhibit pharmacologically different characteristics than I_{dK} or $I_{K(Ca)}$.

W-Pos368

COMPARISON OF PERFORATED AND WHOLE-CELL RECORDINGS OF INWARDLY RECTIFYING K CURRENTS (I_{Ki}) IN MOUSE PERITONEAL MACROPHAGES: RESISTANCE TO H_2O_2 AND PMA STIMULATION, Susan I.V. Judge and Elaine K. Gallin, Physiology Dept., AFRRI, Bethesda, MD 20889

To examine if inhibition of I_{Ki}, the major conductance responsible for establishing the resting membrane potential, results in the depolarization previously noted to occur during the oxidative burst, I_{Ki} was monitored during exposure to either PMA (1 ug/ml) or H_2O_2 ($\leq$ 15 mM). However, in 21/23 whole-cell recordings I_{Ki} decreased within 10 min in untreated cells, regardless of whether the major anion in the pipette was Cl, methanesulfonate or glutamate. In contrast, in perforated patch studies (n=20) (pipette contained amphotericin B and in mM Kmethanesulfonate 115;KCL 23;NaCl 4.5;$CaCl_2$ 1.0;$MgCl_2$ 1.2;HEPES 10) I_{Ki} was stable for >60 min suggesting that retention of intracellular constituents (>200 D) prevented washout. If the access resistance of perforated patches $\leq$10Mohms, voltage-conductance curves and inactivation of I_{Ki} were similar to whole-cell data. Thus, perforated patches were used to evaluate the effects on I_{Ki} of PMA which mimics inflammatory agents by activating protein kinase C and H_2O_2 an oxidative burst product. Neither PMA (n=10) nor H_2O_2 (n=7) affected I_{Ki} amplitude or voltage-dependence.

W-Pos369

THE MECHANISMS OF ISOFLURANE- AND HALOTHANE-INDUCED DEPRESSION OF SINGLE K^+ CHANNEL CURRENT IN ISOLATED CORONARY SMOOTH MUSCLE CELLS. (J. Marijic, N. Buljubasic, J.P. Kampine and Z.J. Bosnjak) Departments of Anesthesiology and Physiology, Medical College of Wisconsin, Milwaukee, WI 53226 (Spon. Z. Bosnjak)

Volatile anesthetics are known to decrease K^+ current, however, the mechanisms of this decrease are not clear. Present study investigates the effects of isoflurane and halothane on a large-conductance (100 pS) K^+ channel using patch-clamp technique. Halothane (1.5%), but not isoflurane (2.6%), shifted the current-voltage curve to the left, and reversal potential changed toward more positive pipette potential (from -60 mV to -50 mV). Isoflurane and halothane decreased the probability of K^+ channel openings (P_o) from control of 0.53 ± 0.01% to 0.30 ± 0.01% and 0.18 ± 0.01% at resting membrane potential, respectively. Both anesthetics decreased the P_o at all voltages studied, as well as voltage-sensitivity of this K^+ channel. Halothane decreased the mean open time from 11 ± 1.5 ms to 8 ± 1.4 ms. Two exponentials with time constants of 0.987 ± 0.113 ms and 11.121 ± 2.041 ms were required to fit the distribution of open time in drug-free patches. The open times distribution in the presence of isoflurane and halothane were also described by two exponentials with time constants of 0.976 ± 0.082 ms and 11.001 ± 3.022 ms, and 0.754 ± 0.084 ms and 6.923 ± 1.831 ms, respectively. The fast component accounted for 25% of openings in control patches, and 24% and 21% of openings in patches exposed to isoflurane and halothane, respectively. This study suggests that halothane decreases K^+ current by decreasing unitary current, mean open time and the P_o, while isoflurane decreases only P_o of a large-conductance K^+ channel.

W-Pos370

TWO TYPES OF POTASSIUM CHANNELS IN ISOLATED CORONARY SMOOTH MUSCLE CELLS. (Z.J. Bosnjak, N. Buljubasic, J. Marijic and J.P. Kampine) Departments of Anesthesiology and Physiology, Medical College of Wisconsin, Milwaukee, WI 53226

Potassium channels play central role in regulating membrane potential of smooth muscle and therefore the cell excitation through modulation of Ca^{2+} channels. Multiple types of K^+ channels have been described in vascular smooth muscle, however characteristics, types and regulation of K^+ channels in coronary vasculature are not clear. The purpose of this study was to determine types and regulation of K^+ channels as well as their selectivity for K^+ in isolated canine coronary artery cells. A cell-attached mode was used to record single channel currents. In these cells, two types of K^+ channels were identified. The large conductance channel (98 pS) contributed 98% to total current while small conductance channel (6.3 pS) contributed only 2%. At resting membrane potential the mean amplitude of large conductance channel was 5.8±0.2 pA while small conductance K^+ channel had amplitude of 1.4±0.1 pA. Substitution of 145 mM K^+ with 145 mM Na^+ in the pipette decreased the large channel conductance to 17 pS, suggesting selectivity of these channels for K^+. Large conductance K^+ channel was Ca^{2+}-dependent and responded with a 2-fold increase in mean open time, 8.7-fold increase in probability of opening and no change in the current amplitude in the presence of Ca^{2+} ionophore. Two types of K^+ channels which have been demonstrated in this study may play important role in regulating smooth muscle cell excitability, as well as repolarization process of coronary smooth muscle cells.

W-Pos371

CALCIUM ACTIVATION AND PHARMACOLOGICAL CHARACTERIZATION OF BROWN FAT K CURRENT.

((S.I. Ortiz-Miranda and P.A. Pappone)) Dept. Animal Physiology, University of California, Davis, California 95616.

Adrenergic stimulation of cultured brown fat cells activates a transient, and in some cases, oscillatory K-selective current, $I_{K,NB}$. This conductance was shown to be sensitive to calcium-activated K-current blockers, suggesting it could be activated by intracellular increments in $[Ca^{++}]$. Using whole-cell patch clamp we have been able to activate $I_{K,NB}$ directly, with internal solutions in the pipette containing 1 to 4 μM free Ca^{++} buffered with EGTA or EDTA. $I_{K,NB}$ could also be activated using the ionophore A23187, but the level of activation was unstable. The current activated in this way is K-selective with a negative reversal potential near E_K, and displayed no voltage dependence. Pharmacological characterization of $I_{K,NB}$ showed it to be a distinctive type of Ca- activated K current in that it can be blocked by TEA, 4-AP, apamin, and charybdotoxin. A half maximum block was obtained with 2 mM TEA, ~100 μM 4-AP, or 6 nM apamin. Our results show that adrenergic activation of $I_{K,NB}$ in brown fat cells is mediated by increments in $[Ca^{++}]_i$, and that this current expresses a very distinctive pharmacology from any other Ca-activated K channel.

W-Pos372

EFFECTS OF PROPAFENONE ON $I_{K,s}$ AND $I_{K,r}$ IN GUINEA-PIG VENTRICULAR MYOCYTES. ((E. Delpón, C. Valenzuela, O. Casis, O. Pérez and J. Tamargo)) Dept. of Pharmacology, School of Medicine, Universidad Complutense 28040 Madrid, SPAIN. (Spon. by S. Sala)

The effects of propafenone (P) (5 μM) on I_K and its insensitive La^{3+} component ($I_{K,s}$) were studied in single guinea-pig ventricular myocytes using the perforated nystatin patch configuration of the patch clamp technique at room temperature (22°C). P inhibited I_K in a voltage-dependent manner (V_h= 21.0 ± 2.3 vs. 29.0 ± 3.7 mV. $p < 0.05$), this effect being more potent for short (0.5s) than for long (5s) pulses (59.7 ± 5.6 vs. 29.4 ± 3.6%. $p < 0.01$). In the presence of 30 μM La^{3+}, P inhibited $I_{K,s}$, and this effect was also more marked after short (0.1s) than after depolarizing pulses longer than 1s (56.1 ± 4.8 vs. 38.9 ± 1.6, $p < 0.05$). The effect of P on $I_{K,s}$ was not voltage-dependent (V_h= 30.2 ± 2.1 vs. 30.3 ± 1.9 mV). In the absence of La^{3+}, the activation kinetics of I_K were described as a biexponential process (τ_f= 0.64 ± 0.07 s, τ_s= 7.7 ± 2.0 s). In the presence of P this process was better described by a monoexponential function (τ= 2.2 ± 0.4 s). Under control conditions, the activation kinetics of $I_{K,s}$ were described as a monoexponential process. P had no effect on the time constant of activation (3.6 ± 0.3 vs. 3.1 ± 0.3 s) but delayed the activation of $I_{K,s}$ by 103.2 ± 21.7 ms. These results suggest that P preferentially blocks K_r-channels and that it binds to a closed state of K_s-channels. Supported by CICYT grant SAF92-0157.

W-Pos373

CAPSAICIN ON TYPE I K+CURRENT IN RABBIT SCHWANN CELLS. ((M.D. Baker & J.M. Ritchie)) Dept. of Pharmacology, Yale University School of Medicine, New Haven, CT 06510.

Capsaicin, a blocker of K^+ currents in myelinated nerve, blocks type I K^+ currents (Baker *et al., J. Physiol.* In Press) in voltage-clamped cultured Schwann cells. Type I current was reversibly suppressed by superfused capsaicin, the concentration dependence being well described by a rectangular hyperbola, K = 7.7 μM (data from 7 cells). Capsaicin induced 'inactivation' of the K^+ current, so in this respect the block resembled that produced in squid axon K^+ currents by internal quaternary ammonium (QA) compounds (Armstrong, *J. Gen. Physiol.* 58, 413-437, 1971). The action of capsaicin was voltage-dependent, the reduction of the peak current in the presence of the blocker being a minimum with pre-pulse potentials more negative than -65 mV and becoming maximal at about -30 mV (the same range over which the current activates). However, unlike the case with QA compounds, inward movement of K^+ ions (with high $[K^+]_o$) did not remove or reduce the block, suggesting that expulsion of the capsaicin molecule by inward ion movement does not occur. In contrast, 5-10 mM internal TEA produced marked inward rectification under the same conditions. As would be expected for an uncharged lipophilic molecule, block was not use-dependent (with cycle rates up to 5 Hz; holding potential -120 mV).We conclude that capsaicin blocks the open type I K^+ channel, the binding site apparently being unavailable when the channel is closed at hyperpolarized membrane potentials. Supported by USPHS grants NS12327 and NS08304.

W-Pos374

EFFECTS OF LP-805, A NOVEL VASODILATING AGENT, ON K CURRENTS IN CULTURED ENDOTHELIAL CELLS. ((M. Inazu, H. Zhang and E.E. Daniel)) McMaster Univ., Hamilton, Ont., Canada (Spons by Dr. Stephen Sims)

Vasodilatation by LP-805, a newly synthesized vasodilator, is related to its opening of K channels and/or release of endothelium-derived nitric oxide in artery. We investigated that the effects of LP-805 on membrane K currents in cultured bovine pulmonary arterial endothelial cells and human umbilical vein endothelial cells. Under whole-cell patch-clamp, LP-805 (1 to 100 uM) increased outward K currents in a dose dependent manner. The increase of this K current was inhibited by TEA (1 mM) and 4-AP (5 mM), and was partially recovered by wash. There effects were unaffected by up to 11 mM EGTA in the pipette. LP-805 decreased inward K current in a dose-dependent manner and this action was blocked by TEA (5 mM) and 4-AP (5 mM). LP-805 increased outward K current in human umbilical vein endothelial cells which was TEA sensitive. In cell-attached category, LP-805 (1 to 10 uM) activated an outward K conductance (60 pS) in a dose dependent manner. These results indicate that LP-805 inhibits inward but activates outward K currents in cultured endothelial cells. (Supported by MRC Canada and Pola Corporation. Japan)

W-Pos375

Effect of AL0671, a novel potassium channel opener, on potassium current in rat aortic smooth muscle cell

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We have evaluated the effects of AL0671(new K+channel opener) on K-current using patch clamp technique. Cultured smooth muscle cells from rat aorta were utilized in this experiment.

Under conditions of whole cell patch clamp, AL0671 (1–1000 μM) markedly increased potassium current with Hill coefficient of 2 and Kd of 1.5×10^{-4}M. The activation of this current was completely inhibited by 3 mM intracellular ATP administration.

Under conditions of inside-out patch, the gating behavior of ATP-sensitive K-channel (K_{ATP}) showed two different open states with fast time constant (τ_1)=0.67 ms and slow time constant (τ_2)=8.5 ms at HP=-40 mV. The channel conductance was 167 pS. Under this conditions, AL0671-modified channel showed prolongation of open state with τ_2 from 8.5 to 24.6 ms. Marked changes were not found in fast component of open time and close time constant.

These results indicated that: 1)new K+ channel opener, AL0671 activated K_{ATP} channel, causing the increase in open probability. 2) our results suggest that two drug molecules might bound to one K^+ channel.

W-Pos376

SIMILARITIES IN DYNAMIC I_K BLOCK BY COCAINE AND FLECAINIDE

Yiwang Chen, Christopher H. Follmer, Raymond L. Woosley and Richard A. Gillis. Dept. of Pharmacology, Georgetown University, Washington, DC

Cocaine-induced cardiotoxicity is a major public health problem. However, the cellular mechanism remain ill-defined. A role for I_K block is inferred from clinical and *in vitro* data. This was studies using isolated cat ventricular cells and suction pipette techniques (HEPES buffer; 0.2 mM Cd^{++} externally; T=30-32°C). Short voltage clamp steps (V_t, 750 ms) from -40 mV to +70 mV showed voltage-dependent block of I_K tails by cocaine (1, 3 and 10 μM; IC_{50} = 8.8 μM at V_t +20 mV, and 4.2 μM at V_t +60 mV), which was absent with long (5 sec) steps. IC_{50} for quinidine, flecainide and lidocaine were 0.2, 1.1, 110 μM, respectively (V_t = +60 mV). Envelope-tests confirmed cocaine-block followed I_K activation timecourse. As with flecainide, I_K tail deactivation (-40 mV, $\tau = 239 \pm 23$ ms) was slowed by cocaine ($\tau = 534 \pm 79$, n=3) which suggests slow relief of block. I_{K1} was unaffected by cocaine and flecainide. Thus, the similarity of activated channel block of I_K by cocaine and flecainide may be related to their common ability to induce arrhythmias.

W-Pos377

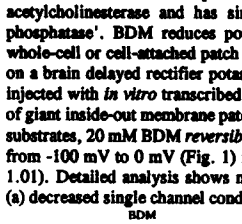
A MITOCHONDRIAL UNCOUPLER, FCCP, INCREASES K^+ CURRENT IN RAT PULMONARY ARTERIAL MYOCYTES ((X.-J. Yuan[1,2], T. Sugiyama[1], W.F. Goldman[1], L.J. Rubin[1,2] & M.P. Blaustein[1])) [1]Dept. of Physiol., [2]Div. of Pulmonary Med., Univ. of Maryland School of Medicine, Baltimore, MD 21201.

FCCP, carbonyl cyanide-*p*-trifluoromethoxyphenyl-hydrazone, a H^+ ionophore that uncouples oxidative phosphorylation, releases Ca^{2+} from mitochondria and non-mitochondrial stores and prevents Ca^{2+} uptake by dissipating the H^+ gradient across mitochondrial and sarcoplasmic reticulum membranes. We examined whether FCCP may affect K^+ channel activities by modulation of intracellular Ca^{2+} ($[Ca^{2+}]_i$) and ATP levels. Rat pulmonary arterial (PA) myocytes were primary cultured for 3-6 days before experiments. Whole-cell and single channel K^+ currents in cell-attached patches (symmetrical K^+) were recorded by patch clamp; $[Ca^{2+}]_i$ was measured with fura-2 and fluorescence image microscopy. The external solution (PSS) included (mM): 141 Na^+, 1.8 Ca^{2+}, 4.7 K^+; the internal (pipette) solution included (mM): 125 K^+, 5 ATP, 10 EGTA. FCCP (3-5 μM) in PSS reversibly raised $[Ca^{2+}]_i$ in a biphasic fashion: a rapid (<30 s) initial rise followed by a $[Ca^{2+}]_i$ reduction, and then, a second rise to a new, elevated plateau (≤500 nM). Similar effects were seen in the absence of external Ca^{2+}. This suggests that much of the FCCP-evoked rise in $[Ca^{2+}]_i$ was due to Ca^{2+} release from intracellular stores, and that extrusion of Ca^{2+} was inhibited. FCCP reversibly enhanced whole-cell K^+ currents evoked by test pulses of -40 to +80 mV (HP=-70 mV) in PA cells bathed in PSS or Ca^{2+}-free PSS. FCCP also reversibly increased single K^+ channel activity; this augmentation effect was not influenced by glibenclamide, an ATP-sensitive K^+ (K_{ATP}) channel blocker. Also, the response to FCCP was rapid and the pipette solution contained 5 mM ATP; thus, the enhanced K^+ channel activity is not mediated by K_{ATP} channels. Rather these channels possess the properties of Ca^{2+}-activated K^+ (K_{Ca}) channels ($g_K \approx 220$ pS). These results suggest that FCCP increases K_{Ca} channel activities by increasing Ca^{2+} release directly. Whether FCCP has a direct effect on K^+ channels is unknown.

W-Pos378

2,3 BUTANE-DIONE-MONOXIME (BDM) INHIBITS DRK1 POTASSIUM CHANNELS EXPRESSED IN *Xenopus* OOCYTES ((A.N.Lopatin and C.G.Nichols)). Dept.Cell Biol.and Physiol. Washington Univ.Sch. Med., 660 So. Euclid, St. Louis, MO 63110 (Spon. by J.A. Cooper).

BDM was developed as a tool against organophosphorus poisoning of acetylcholinesterase and has since been used as a membrane-permeant 'chemical phosphatase'. BDM reduces potassium currents in lymphocytes and heart cells in whole-cell or cell-attached patch experiments. We have examined the effects of BDM on a brain delayed rectifier potassium channel (DRK1) expressed in *Xenopus* oocytes injected with *in vitro* transcribed cRNA. BDM was applied to the cytoplasmic surface of giant inside-out membrane patches. In the absence of ATP or other phosphorylation substrates, 20 mM BDM *reversibly* decreases integral currents elicited by a voltage step from -100 mV to 0 mV (Fig. 1) in a dose-dependent manner (k1/2 = 10.7 mM, H = 1.01). Detailed analysis shows multiple actions on channel properties. 20 mM BDM (a) decreased single channel conductance by 10-20% in a voltage independent manner,



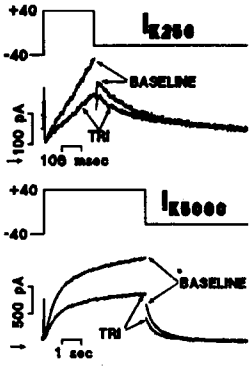
(b) reduced open channel probability at all potentials, (c) shifted the voltage dependence of activation to more positive potentials and (d) reduced the steepness of the activation curve. BDM slowed activation and increased the apparent rate of inactivation of integral currents. Under experimental conditions in which dephosphorylation dependent effects should be irreversible (inside-out configuration), BDM causes numerous *reversible* changes in channel gating and single channel current.

W-Pos379

CHARACTERIZATION OF THE INHIBITION OF THE DELAYED RECTIFIER (I_K) COMPONENTS BY TRIAMTERENE IN GUINEA PIG VENTRICULAR MYOCYTES.

((J. Turgeon and P. Daleau)) Quebec Heart Institute & School of Pharmacy, Laval University, Ste-Foy, QC, Canada.

The K^+-sparing diuretic triamterene (TRI) increases APD and protects against reperfusion-induced arrhythmias. In this study, we assessed effects of TRI on K^+ repolarizing currents. Cells were superfused at 30° with Cd^{2+}-containing solution to block I_{Ca}, held at -40mV to inactivate I_{Na} and currents measured in the whole cell configuration of the patch-clamp technique. While block of the slow component of I_K (I_{K5000}) was shown by a reduction of both activating and tail currents at high-voltage depolarizing long pulses, block of its rapid component (I_{K250}) was observed by a reduction of activating and tail currents at low-voltage depolarizing short pulses. Inward rectification of I_{K250} was also reduced. For test potentials above +30 mV, best fit during 1sec of I_K activation was changed from a bi- to a three-exponential curve. We have shown that I_{K250} and I_{K5000} were inhibited by TRI. Transformation of the activating current suggests an action on gating properties of I_K or inhibition of a third component.



W-Pos380

ACTIVATION OF SKELETAL MUSCLE K-ATP CHANNELS BY PROTONS AND LEMAKALIM : A COMPARATIVE STUDY

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The patch-clamp technique was used to examine and compare the action of intracellular acidosis and of the K-channel-opener, lemakalim (BRL38227), on skeletal muscle ATP-sensitive potassium channels (K-ATP channels). Inside-out patches were excised from sarcolemmal blebs which form without enzymatic treatment at the surface of a frog muscle fiber which has been split in half.

Raising cytoplasmic pH from 7.1 to 7.8 had little effects on K-ATP channel activity. Decreasing pH from 7.1 to 6 caused a rapid increase in channel activity with or without inhibiting concentrations of nucleotides. This effect resulted from a decrease in the channel sensitivity to ATP or ADP concomitant with an increase in the activity at all nucleotide concentrations.

Lemakalim at concentrations of 10 to 200 µM opened channels partially blocked by nucleotides by decreasing the sensitivity to ATP or ADP but, unlike acidosis, it reduced weakly the maximal activity recorded in the absence of nucleotides. Activation by lemakalim was much slower (several seconds) to establish and to reverse than activation by protons (<1 s). None of these effects required Mg^{2+} ions, thus ruling out a phosphorylating step.

A competitive interaction at the free nucleotide binding site of the K-ATP channel could explain the action of lemakalim but cannot account for the action of protons in the absence of nucleotides.

To further investigate these mechanisms, a study of the combined effects of the two activators, lemakalim and pH, was started. Preliminary results suggest unexpectedly that protons can block completely the lemakalim response. It remains to be seen if this is not due to a chemical interaction between pH and lemakalim rather than an interaction with the channel protein itself.

W-Pos381

ACTIVATION OF PROTEIN KINASE C INHIBITS OUTWARD POTASSIUM CURRENT IN CULTURED ENDOTHELIAL CELLS.

((H. Zhang, E.E. Daniel and B. Weir))McMaster Univ., Hamilton, Ont. L8N 3Z5.(Spon. by J.D. Huizinga)

A large inward and a small outward potassium currents were obtained by whole-cell patch-clamp from cultured bovine pulmonary endothelial cells. Activation of protein kinase C by phorbol myristoyl acetate (PMA) and phorbol 12,13-dibutyrate (PDBu) dose-dependently (0.3-10 uM) depressed the outward current but without effects on the inward current. The inactive analog phorbol 12-monomyristate (PMM) was devoid of any effects at same concentrations. The inhibitory actions of PMA and PDBu were blocked by kinase inhibitor H-7 (10 uM). Cyclopiazonic acid (CPA,3-20 uM), an inhibitor of the sarcoplasmic reticulum calcium pump, and LP-805 (1-100 uM), a novel potassium channel opener, increased the outward conductance which was sensitive to TEA (1-10 mM), 4-AP (5 mM) and charybdotoxin (100 nM). PMA and PDBu, but not PMM, reduced the outward conductance induced by CPA and LP-805. The mechanism for modulating potassium conductance by phorbol esters in endothelial cells needs to be clarified. Supported by MRC Canada.

W-Pos382

AGONIST AND ANTAGONIST EFFECTS OF ALMOKALANT ON THE DELAYED K^+ CURRENT IN CARDIAC CELLS. EVIDENCE FOR TRAPPING.

((E. Carmeliet)) Laboratory of Physiology, University of Leuven, Leuven, Belgium.

The mechanism of action of almokalant, a class III agent, on the delayed K^+ current, i_K, was studied in rabbit cardiac myocytes, using the whole cell patch clamp technique. Depolarizations of variable duration and amplitude were applied to single ventricular cells from a holding potential which was either -50 mV or -75 mV. Tail currents at -50 mV were taken as a measure of i_K. The results were as follows: 1) i_K was increased for short, small depolarizations and at low drug concentrations; the activation curve was shifted to negative potentials; 2) block of activated channels was preferentially seen for more depolarized and longer pulses and at higher drug concentration; 3) recovery from block was slower or absent at hyperpolarized levels; at -75 mV and in the absence of depolarizing clamp the block remained constant even during washout of the drug; stimulation resulted in a fast use-dependent unblocking. It is concluded that the drug exerts agonist and antagonist effects probably by binding to two different sites; closure of the activation gate traps the drug in the channel.

W-Pos383

ALTERATION OF VOLTAGE-ACTIVATED K CURRENT BY INTRA-CELLULAR PERFUSION OF cGMP IN BOVINE CHROMAFFIN CELLS.

((R.D. Shoop, R.A. Johns, and J.J. Pancrazio)) Depts. of BME and Anesthesiology, UVa Health Sciences Center, Charlottesville, VA 22908.

Cyclic GMP (cGMP) has been shown to affect ion channel activity in a variety of cell types. Utilizing the patch clamp technique, we investigated the effect of cGMP on voltage-gated currents of bovine adrenal chromaffin cells. Isolated chromaffin cells were affixed to poly-L-lysine coated coverslips and placed in a chamber over an inverted microscope at 22-24°C. The external bathing solution contained (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, pH 7.4. The internal pipette solution contained: 42 KOH, 100 KCl, 1 $CaCl_2$, 10 EGTA, 5 HEPES, 5 MgATP, pH 7.3 with and without 1 μM cGMP. For several experiments, K^+ was replaced with Cs^+ and TEA in the pipette solution to examine the effects of cGMP on inward I_{Ca}. In the absence of cGMP, cells displayed the "n-shaped" current-voltage (I-V) relation described by Marty and Neher (J Physiol 367: 117-141, 1985). The presence of 1 μM cGMP in the pipette linearized the I-V relationship, by increasing currents with large depolarizations (>+40 mV). In response to a step depolarization to +80 mV, outward current increased from 1284±229 pA (mean±SEM, n=13 cells) to 2504±361 pA (n=18). Examination of the tail currents elicited by repolarization to voltages ranging from -30 to -85 mV from a step depolarization to +60 mV indicated that the outward current induced by the inclusion of cGMP within the pipette is carried by K+ ions. No effect was observed on Ca^{2+} currents after replacement of intracellular K^+ with Cs^+ and TEA. Experiments are underway to examine the effects of known cGMP-liberating neurotransmitters/compounds, including atrial natriuretic peptide and sodium nitroprusside, on chromaffin cell K^+ current.

W-Pos384

EFFECT OF THAPSIGARGIN AND HEPARIN ON BARIUM ACTION POTENTIALS IN ISOLATED APLYSIA BAG CELL NEURONS.

((S. Levy, T.E. Fisher and L.K. Kaczmarek)) Dept. of Physiology, Boston Univ. Sch. Med.; Ctr. Res. Neurosci, McGill Univ, Montreal and Dept. of Pharmacology, Yale Univ. Sch. Med.

Initiation of the afterdischarge in *Aplysia* bag cell neurons is associated with a transient elevation of intracellular calcium (Ca_i). We have previously shown that activation of an afterdischarge in media in which calcium has been replaced with barium also results in an elevation of Ca_i, suggesting that the calcium transient at the onset of an afterdischarge may be due in part to a release of calcium from intracellular stores. When isolated bag cells are stimulated in barium-seawater, they undergo action potentials and a prolonged depolarization of 30 s or more and a robust Ca_i increase (Biophys. J. 59:588a, 1991). To characterize this Ba-induced increase in Ca_i, we have used thapsigargin, an inhibitor of the calcium pump; and heparin, an inhibitor of calcium release by $InsP_3$. Bath application of thapsigargin (1 μM) for 10 min or more decreased the prolonged depolarization to 30±10% of control (n=6). Preliminary measurements with Ca-sensitive electrodes showed that thapsigargin led to a gradual increase in resting Ca_i (see also Kao *et al.*, Soc. Neurosci. Abstr. 18:586, 1992) and to no increase in Ca_i following evoked action potentials. Intracellular pressure-injection of heparin (5 mg/ml in micropipette) had no significant effect on the length of the depolarization. The data suggest that evoked action potentials in barium induce the release of calcium from a thapsigargin sensitive intracellular store.

W-Pos385

RECEPTOR-ACTIVATED CALCIUM INFLUX IN HUMAN AIRWAY SMOOTH MUSCLE CELLS:COMBINED USE OF PERFORATED PATCH AND SINGLE CELL FURA 2 IMAGING TECHNIQUES. ((B.K. Fleischmann, R.K., Murray and M.I. Kotlikoff.)) Depts. of Medicine & Animal Biology, University of Pennsylvania, Philadelphia, PA 19104.

The relative contributions of voltage-dependent and voltage-independent calcium influx pathways to calcium homeostasis in human airway smooth muscle are not known. We have previously described sustained elevations in cytosolic calcium in cultured human ASM cells after stimulation with either histamine or bradykinin. These sustained elevations appear to be the result of activated calcium influx through a pathway other than dihydropyridine-sensitive VDCCs. To further characterize this influx pathway we measured cytosolic calcium in voltage-clamped, fura 2AM -loaded cells using the whole-cell, perforated patch technique (nystatin). Fura-2 loaded cells had a normal resting calcium (typically 150 nM) under conditions in which good electrical access was achieved (R_s < 40 mOhm). Voltage steps from -80 mV to 0 mV produced only small changes in $[Ca]_i$. By contrast, after cells were activated with histamine (which typically caused a transient rise in calcium followed by a sustained elevation), voltage steps produced large changes in $[Ca]_i$, which corresponded to E_{Ca}. Following activation, $[Ca]_i$ decreased with depolarizing, and increased with hyperpolarizing, voltage clamp steps. This agonist-dependent relationship between holding potential and $[Ca]_i$ was associated with calcium influx, since such changes were not seen in the absence of extracellular calcium. Histamine activates a calcium influx pathway in airway smooth muscle cells that is independent of voltage-dependent calcium channels.

W-Pos386

REDUCTION OF BOTH LVA- AND HVA- CALCIUM CURRENTS IN DRG NEURONS BY LEMS SERUM. ((Kelly D. Holmes*, Michelle Mynlieff#, John P. Walrond* and Kurt G. Beam#)). Depts. of *Anatomy & Neurobiology and #Physiology, Colorado State Univ., Fort Collins, CO 80523

Lambert-Eaton Myasthenic Syndrome (LEMS) is a human autoimmune disorder characterized by decreased quantal content at the neuromuscular junction. LEMS antibodies appear to affect voltage gated calcium channels since they decrease calcium currents in adrenal chromaffin cells and neuroblastoma cells. However, the identity of the affected calcium current(s) has not been well characterized. We have therefore been examining the effects of LEMS antibodies on murine dorsal root ganglion neurons, cells in which several types of Ca^{2+} currents have been well characterized. Acutely dissociated neurons were obtained from a mouse and were divided into two groups. One group was incubated overnight with serum from LEMS patients and the other group was incubated overnight with or without normal human serum. Calcium currents were measured with the whole-cell variant of the patch clamp method (10 mM Ca^{2+} as charge carrier). Currents were evoked by 300-ms depolarizations applied from a holding potential of -80 mV. The LVA currents and the transient and sustained components of HVA currents were measured in both control and LEMS-treated cells. Sera from two patients decreased the density of all three components of calcium current by ~50%, and serum from a third decreased the density of only the LVA current. These results suggest that LEMS antibodies affect a variety of channel types and that the different sera may recognize different epitopes. Supported by NIH grant NS 26416 to KGB.

W-Pos387

CAPACITANCE CHANGES ASSOCIATED WITH EXOCYTOSIS FROM MAMMALIAN PEPTIDERGIC NERVE TERMINALS PRODUCED BY RELEASE OF CAGED CALCIUM. ((N.I. Chernevskaya, R.S. Zucker* and M.C. Nowycky)) Dept. Anatomy & Neurobiol., Med. Coll. Penn., Philadelphia, PA 19129 and *Dept. Molec./Cell Biol., UC Berkeley, Berkeley, CA 94720.

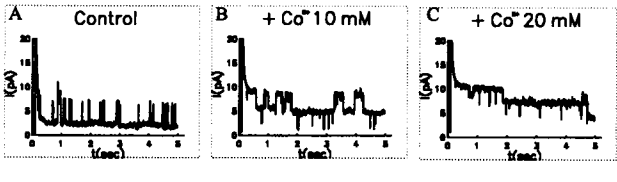
The coupling between secretion and intracellular calcium (Ca) concentration was studied in isolated neurohypophysial terminals using the photolabile Ca chelator, DM-nitrophen (DM-n). Secretion was monitored as changes in membrane capacitance using the phase detector technique (Joshi and Fernandez, 1988). DM-n 70% loaded with Ca was introduced to the terminal through the patch pipet. Photolysis of DM-n with steady UV light produces a small, slow increase in Ca concentration throughout the terminal (Zucker 1993). Capacitance increases were observed following ~620 ms of exposure to UV light, when Ca levels were estimated to be around 0.3 µM. The total increase in capacitance induced by DM-n photolysis did not exceed 100 fF. The results indicate that the Ca receptor(s) for secretion of large dense-cored vesicles can respond to low levels of Ca, provided that the elevations are maintained for sufficiently long periods of time. Photolysis of DM-n beyond 1270 ms led to a decrease in capacitance despite the continued rise of free Ca. This decrease may represent Ca-stimulated endocytosis or damage to the terminals produced by high global elevation of intracellular Ca.

In contrast, in the same terminals, large, brief Ca "spikes" produced by discharge of a high intensity UV flash lamp failed to elicit significant capacitance increases. The failure of Ca "spikes" to trigger secretion may indicate that the forward binding constant of the Ca receptor(s) are too slow to respond successfully to the 2 msec duration spike.

W-Pos388

Co^{2+} SLOWS DOWN INACTIVATION IN A SEA URCHIN SPERM Ca^{2+} CHANNEL. P. Labarca*, A. Liévano, A. García and A. Darszon. Instituto de Biotecnología, UNAM, Apdo.Postal 510-3, Cuernavaca, Morelos, México and *C.E.C.S & U. Chile

Sea urchin sperm channels are being studied by fusing sperm membranes into planar bilayers. Here we examine the effect of Co^{2+} on a Ca^{2+} -selective channel (*J. Gen. Physiol.* 95:273). At positive voltages (*cis* positive) the channel spends most of the time in the open state and inactivates at negative ones (A). Co^{2+}added to the *cis* side blocks the channel without affecting inactivation. In contrast, addition of mM Co^{2+} to the *trans* side slows down inactivation in a concentration dependent fashion (B and C). The results suggest the presence of an inactivation gate in the *trans* face of this Ca^{2+} channel where the occupation by Co^{2+} of a site impedes the closing of the gate.



W-Pos389

THE EFFECT OF 1,25(OH)$_2$ VITAMIN D$_3$ ON CALCIUM CHANNEL CURRENTS IN UMR-106 CELLS. ((B. Li and E. Karpinski)) Dept. Physiology, Univ. Alberta, Edmonton, Alberta, Canada

Bone is a target organ for Ca^{2+} regulating hormones such as 1,25(OH)$_2$ vitamin D$_3$ and parathyroid hormone. Since 1,25(OH)$_2$ vitamin D$_3$ is a steroid hormone, its action is usually via intracellular receptors. There is also evidence that 1,25(OH)$_2$ vitamin D$_3$ may induce short-term changes in cells by activating intracellular signalling pathways. In addition, there is evidence that 1,25(OH)$_2$ vitamin D$_3$ increases intracellular Ca^{2+} concentration and that this increase is dependent on extracellular Ca^{2+}.

The effect of 1,25(OH)$_2$ vitamin D$_3$ on the L-type Ca^{2+} channel was investigated in UMR-106 cells (osteogenic sarcoma) using the whole cell version of the patch clamp technique and Ba^{2+} as the charge carrier. UMR-106 cells express two types of Ca^{2+} channels in culture: T and L. After 48 hours in culture, the predominant channel is a dihydropyridine-sensitive channel (L-type). 1,25(OH)$_2$ vitamin D$_3$ increased the L-type Ca^{2+} channel current. The increase in the L-type current was concentration-dependent (10 nM-2 μM) and was reversible by washout. The peak of the IV relationship was shifted towards more negative potentials by 1,25(OH)$_2$ vitamin D$_3$. These results suggest that 1,25(OH)$_2$ vitamin D$_3$ activates an intracellular mechanism which modulates the L channel in UMR-106 cells.

Supported in part by a grant from Kureha Chemical Industry Co., Ltd.

W-Pos390

CALCIUM CHANNELS IN BOVINE PARATHYROID CELLS. ((M. Jia, M. Li, and G. Ehrenstein)) NINDS, NIH, Bethesda, MD 20892.

Calcium homeostasis is achieved by means of a feedback system that involves extracellular Ca concentration, the intracellular Ca concentration in parathyroid cells, and parathyroid hormone (PTH) secretion. The intracellular and extracellular Ca concentrations in parathyroid cells are known to be monotonically related, but the mechanism by which they are coupled has not been described. We have used single-channel patch clamp experiments to determine the properties of calcium-permeable channels that may provide this coupling. We found two types of Ca-permeable channel - a 12 pS channel that is selective to divalent cations and a 60 pS channel that is cation-selective. Both types are voltage-independent in the physiologically relevant voltage range, do not inactivate, and do not require agonists. With whole-cell recording, we found a cadmium-sensitive, voltage-independent current with a selectivity similar to that of the 60 pS channel. Because of the voltage independence of the calcium-permeable channels, the intracellular Ca concentration, and hence PTH secretion, responds to changes in the extracellular Ca concentration and not to changes in membrane potential. These properties are consistent with the function of parathyroid cells to maintain the requisite calcium concentration.

W-Pos391

GTP-DEPENDENT MODULATION OF T-TYPE CALCIUM CHANNELS IN BOVINE ADRENAL GLOMERULOSA CELLS. R.T. McCarthy*, C.M. Isales, and H. Rasmussen. *Miles Inc., West Haven, CT and Dept. of Med., Yale Univ., New Haven, CT.

With the use of whole-cell and single channel current recordings we have examined the action of angiotensin II (AII) on T-type calcium channels in bovine adrenal glomerulosa (AG) cells. AII (10 nM) enhanced whole-cell T-type current (V_t= -30 mV) and increased the activity of single T-type channels in cell-attached (CA) patch recordings. Enhancement of whole-cell T-type currents (n=8) by AII was dependent on GTP (40μM) in the pipette. The voltage-dependence of control T-type channel activation produced a midpoint ($V_{\frac{1}{2}}$) of -7.9 mV ±1.02(n=3). After the presence of AII, the voltage-dependence of T-type channel activation was shifted in the hyperpolarizing direction ($V_{\frac{1}{2}}$= -17.9 mV ± 3.23; n=3). This AII-induced shift in the voltage-dependence of T-type channel activation could be inhibited by saralasin (1 μM) and did not occur when GTP was omitted from the pipette. In the presence of internal GTPγS the voltage-dependence of T-type channel activation was shifted to more hyperpolarized potentials ($V_{\frac{1}{2}}$= -21.3mV;n=3) in the absence of AII. In CA patch recordings, AII more than doubled the probability of finding a T-type channel in the open state [P_o=.023; AII stimulated P_o= .052; n=4]. These results show that AII augments the T-type calcium channel current in AG cells.

W-Pos392

ROLE OF CALCIUM AND MEMBRANE POTENTIAL ON CATECHOLAMINE SECRETION FROM BOVINE CHROMAFFIN CELLS. ((S.Calvo,R. Granja, C. González-García and V. Ceña)). Departamento de Farmacología and Instituto de Neurociencias, Universidad de Alicante, Alicante, Spain.

Depolarization of chromaffin cells promotes opening of voltage-dependent Ca^{2+} channels, an increase in intracellular Ca^{2+} levels and catecholamine (CA) secretion. However, Ca^{2+} influx and CA secretion have not been studied under the same conditions in chromaffin cells. The resting membrane potential of bovine chromaffin cells, measured under current-clamp using the nystatin-perforated patch technique, was -55 ± 6 mV. Exposure to high extracellular K^+ (75 mM) depolarized the chromaffin cell membrane to +5 mV. This extracellular K^+ concentration induced in 5 minutes the release of about 15% of total norepinephrine and 8% of total epinephrine present in chromaffin cells. Single, long (10 seconds) depolarizing pulses from a holding potential of -55 mV to +5 mV induced an inward Ca^{2+} current that inactivated with a time constant of about one second and promoted the influx of about 10^{-15} moles of Ca^{2+} into the cell. High Mg^{2+} or nifedipine blocked K^+-induced CA secretion and, similarly, Ca^{2+} entry induced by a depolarizing pulse from -55 mV to +5 mV. These results suggest a direct relationship between Ca^{2+} entry and CA secretion in bovine chromaffin cells.

Supported, in part, by grants # PM88-0204 from DGICYT and SC1*-CT91-0709 from EEC.

W-Pos393

CALCIUM CHANNELS IN SMALL-CELL LUNG CANCER CELLS ARE INHIBITED BY ANTIBODIES AGAINST A P-TYPE CHANNEL PEPTIDE. ((R.L.R. Barry[1], M.P. Viglione[2], T.J. O'Shaughnessy[3], S.C. Froehner[3] and Y.I. Kim[3])) [1]Dept. of Pharmacology, Dartmouth Medical School, Hanover, NH 03755, [2]Dept. of Biomedical Engineering, University of Virginia, Charlottesville, VA 22908 and [3]Dept. of Physiology, University of North Carolina, Chapel Hill, NC 27599.

Small-cell lung cancer (SCLC) is frequently associated with paraneoplastic syndromes. Of particular interest is the Lambert-Eaton myasthenic syndrome, a paraneoplastic disorder involving the destruction of calcium channels by autoantibodies, thought to be produced in response to the presence of the tumor.

Polyclonal antibodies were raised to an acidic peptide, IDVEDEDSDEDEF, corresponding to a unique nucleotide stretch in a calcium channel clone (HumSCCLA) which was isolated from the SCLC H-146 cell line. Clone HumSCCLA is highly homologous (90% at the nucleotide level and 98% at the amino acid level) to the presumptive P-type calcium channel cloned and characterized by Mori *et al.* (*Nature*. 350:398, 1991). The unique acidic peptide is not found in any of the other classes of calcium channels which have been cloned to date (3 subclasses of L-type, or N-type). Peptide specific antibodies were purified by affinity chromatography on peptide coupled to solid resin.

Incubation of H-146 cells with the antibodies reduced the K^+-stimulated (80 mM K^+) increase in $[Ca^{2+}]_i$, as measured in single cells by fura-2, by 20%; control antibodies were without effect. Inhibition of voltage-dependent calcium channels was confirmed in whole-cell patch-clamp experiments of H-146 cells exposed to the peptide antibodies for 2 hours. After incubation, peak I_{Ca} evoked from V_h=-80 mV was reduced by 20 ± 6% (n=25 cells, $p < 0.025$) and the plateau I_{Ca} (measured at the end of a 350 msec pulse) was inhibited by 19 ± 7% (n=25 cells, $p < 0.025$), compared to cells exposed to control antibodies (n=33 cells). Sodium currents were not inhibited by the peptide antibodies. These data suggest that SCLC cells express P-type calcium channels.

W-Pos394

EFFECTS OF Ca CHANNEL ANTAGONISTS ON Ca LEVELS IN RAT CORTICAL SYNAPTOSOMES

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Synaptosomes were prepared from rat cortex using isotonic sucrose/discontinuous Percoll density centrifugation and were loaded with the fluorescent Ca indicator, fluo-3. Ca_i measurements were carried out in 96 well microtiter plates in a fluorescent plate reader (approx 80μg protein; approx 25 deg C; excitation 485nm emission 530nm). Basal Ca_i and K^+ stimulated Ca_i (that 20 secs after depolarisation with KCl, 50mM final) were 315.5 +/- 20.3 nM and 533.2 +/- 36.1 nM (mean +/- SEM, 19 determinations in triplicate). Ca_i in stirred suspensions of synaptosomes under the same conditions was similar.

IC_{50} values (i.e. conc. at which [K^+ stimulated Ca_i] - [Basal Ca_i] is reduced to half that in absence of compound) were determined. Three types of effect of calcium channel blockers were seen (1) Verapamil (a phenylalkylamine), nifedipine and nimodipine (dihydropyridines) had little effect. Dose-dependent decreases in basal and K^+ stimulated Ca_i were seen (IC_{50} values 100μM, > 100μM and 75 μM respectively). (2) Bepridil and flunarizine reduce K^+ stimulated Ca influx at low concentrations (IC_{50} 25μM and 10μM) without change in basal Ca_i. At > 10μM, basal Ca_i was increased and the reduction in K^+ stimulated Ca_i was reversed. (3) (S)-emopamil, (phenylalkylamine and $5HT_2$ receptor antagonist) caused no change in basal Ca_i but gave a dose dependent reduction in K^+ stimulated Ca influx (IC_{50} 10μM).

The observed differences in drug effects may indicate different sites of actions of these compounds.

W-Pos395

MODULATION OF MONOVALENT CURRENTS THROUGH N-TYPE CA CHANNELS IN BULLFROG SYMPATHETIC NEURONS

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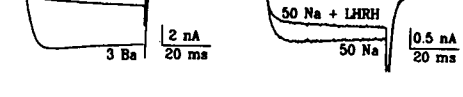
In bullfrog sympathetic neurons, most Ca current is through ω-conotoxin-sensitive N-type channels. In divalent-free external solutions, these channels are permeable to monovalent ions such as Na as reported before for L-type and T-type Ca channels. With 3 μM nimodipine to block L-type channels, 10 μM ω-conotoxin blocks most or all of the currents carried by Na, verifying that the currents are through N-type channels. The monovalent currents through N-type channels do not show the same pattern of modulation by transmitters as Ba currents. For example, LHRH is significantly less effective in inhibiting the whole-cell Ca channel currents with Na as the charge carrier than with Ba. When receptor-independent G protein mediated inhibition of Ca channels is induced by internal GTP-γ-S, Ba currents can be markedly enhanced by depolarizing prepulses (+110 mV), but the effect of prepulse is always much smaller for Na currents through these channels. The different modulation of divalent and monovalent currents suggests interactions between the ions in the pore and the modulating G protein. Such interactions may contribute to the overall voltage dependence of G protein inhibition of Ca channels.



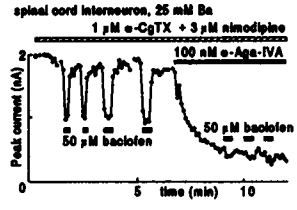
W-Pos396

GABA$_B$ RECEPTORS INHIBIT P-TYPE CALCIUM CHANNELS IN CENTRAL NEURONS.

((I. Mintz and B.P. Bean)) Harvard Medical School, Boston MA 02115.

Neurons possess a variety of high-threshold calcium channels, so far classified as ω-conotoxin (CgTX)-sensitive N-type channels, dihydropyridine-sensitive L-type channels and ω-Aga-IVA-sensitive P-type channels. Calcium channels are modulated by neurotransmitters and second messengers. Both N- or L-type calcium channels have been identified as the target of modulation in various neurons. We asked whether P-type calcium channels can also be modulated by transmitters.

GABA and the GABA$_B$ agonist baclofen inhibited ω-Aga-IVA-sensitive P-type current in cerebellar Purkinje and spinal cord neurons (see figure). Current kinetics and voltage-dependence were altered. GABA and baclofen induced a slow phase of current activation and a faster current deactivation. In addition, current inhibition was relieved by depolarizations.



G-protein activation mediates P-type current modulation by baclofen. Current inhibition produced by baclofen was irreversible in Purkinje cells dialysed with GTPγS. Current facilitation by big pre-pulses, which was induced by baclofen in cells with internal GTP, was mimicked by internal GTPγS alone.

W-Pos397

DYNAMICS OF MEMBRANE FUSION AND RETRIEVAL IN SINGLE SYNAPTIC TERMINALS OF A VERTEBRATE CNS NEURON.

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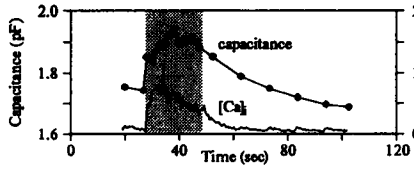
Type Mbl bipolar neurons from goldfish retina have a large synaptic terminal (8–12 μm diameter) suitable for direct studies of exocytosis in synaptic terminals of a CNS interneuron. Combined patch-clamp, fura-2, and capacitance measurements were made from single terminals. Intraterminal $[Ca]_i$ was elevated by a series of depolarizing pulses (shaded region in Fig.), and capacitance was measured during each interpulse interval. Increased $[Ca]_i$ was accompanied by a reversible increase in capacitance that had several phases: 1) a rapid initial jump in capacitance that may reflect fusion of a readily-releasable pool of synaptic vesicles (e.g., those docked to ribbons at the multiple ribbon-type synaptic sites in the terminal); 2) a slower rise to a steady state of elevated capacitance, which may represent the balance between arrival of new vesicles in the releasable pool (e.g., docking at ribbons) and a membrane retrieval process; and 3) a slow decline of capacitance presumably due to the retrieval mechanism acting in isolation. The peak increase in capacitance was typically ~200 fF, corresponding to the fusion of 2500 vesicles. *Fig. Capacitance measured just before each stimulus pulse using auto-compensation feature of EPC-9 amplifier. Depolarizing pulses from -60 to -10 mV elicited Ca current (not shown for simplicity). Shaded region: 250-ms pulses at 1/s; otherwise, 70-ms pulses at 1/10s. Pipette: Cs/TEA, ATP, GTP. 1 mM EGTA. 0.1 mM fura-2. External: 2.5-Ca Ringer.* *(Supported by NIH grant EY03821.)*



W-Pos398

A "NEURON-SPECIFIC" PEPTIDE CALCIUM CHANNEL BLOCKER REVERSIBLY BLOCKS THE CA CHANNELS OF AN INSULIN SECRETING CELL LINE.

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Voltage-gated Ca channels play a role in coupling glucose metabolism to insulin secretion in pancreatic islet B-cells. We proposed that slow inactivation of these channels may be essential for generating islet electrical bursting activity in elevated glucose (Cook, Satin and Hopkins, 1991; TINS 14:411-414). In order to develop new probes for identifying the role of Ca channels in bursting, we tested whether a synthetic neuronal Ca-channel blocking peptide, SNX-111(omega conotoxin MVIIa), also blocks the Ca current of clonal insulin-secreting HIT cells. HIT Ca current activates ~-50 mV, peaks ~ +10 mV (Satin and Cook, *Pflug.Arch* 411:401-409,1988) and has bi-phasic inactivation which is strongly reprimed by hyperpolarization (Satin and Cook, *Pflug.Arch* 414:1-10,1989). Standard whole-cell patch clamp methods were used to measure the Ca currents of single cultured HIT cells. Cytochrome C (0.1mg/ml) was included in control and SNX solutions to prevent non-specific adsorption of the peptide. Peak Ca current was inhibited within 2 minutes of SNX-111 application and recovered within 3 minutes of removal of the peptide. In 4 cells, 1 μM SNX-111 reversibly blocked 57.3 ± 13.4% of Ca current (X±s.e.m.). No marked changes in the shape of the Ca current-voltage relation were observed in SNX-111. In summary, it appears that non-neuronal Ca channels may have similiar toxin binding sites to those of neuronal Ca channels. M.R. was supported by an A.D.Williams Fellowship; support was also provided by a VCU Grant-in-Aid to L.S.

W-Pos399

LANTHANUM BLOCKADE OF N-TYPE CALCIUM CURRENT IN BULLFROG SYMPATHETIC NEURONS.

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Lanthanum (La^{3+}) blocked 50% of whole cell currents through N-type calcium channels at 30 nM (with 2 mM Ba^{2+} as the charge carrier). Large depolarizations partially and temporarily unblocked currents in a voltage dependent manner. Following 10 ms depolarizations to +120 mV, the current was 50% blocked at ~160 nM La^{3+}. Reblock during subsequent depolarizations to 0, +10, +20, or +30 mV was described by bimolecular kinetics with k_{ON} = 6 x 10^8 $M^{-1}s^{-1}$ and k_{OFF} = 17 s^{-1}. Closed channels reblocked slowly, τ >> 60 ms at 1000 nM La^{3+}, but steady state block reappeared after several seconds. Based on the fast kinetics of block and unblock in open channels compared to closed channels and the voltage dependence of unblocking, we concluded that the site of La^{3+} action was in the ion permeation pathway. Interestingly, block induced in La^{3+} persisted after free extracellular La^{3+} was washed out. Even after minutes of washing, which should have reduced free La^{3+} to negligible levels, unblocking and reblocking of the current was repeatedly observed. Washing with 0.1 mM EGTA in the extracellular medium removed all remaining La^{3+} block. This behavior suggested that after unblocking in control solutions, La^{3+} remained near the channel mouth, associated with the channel and/or other membrane structures, and was thus able to reblock the channel.

W-Pos400

THE ANTIAMOEBA GOSSYPOL MODULATES CALCIUM PERMEABILITY IN PARAMECIUM, Garcia, Eduardo; Calvillo, Mario and Bernal, Juan. Physiology Dept. University of Aguascalientes; 20100, AGS. Mexico.

Gossypol is a compound that has shown to be effective on killing amoeba at low concentration. With the aim to know how Gossypol affects protozoa, behavioral and electrophysiological experiments in the non-invasive protozoan Paramecium calkinsi were done. It was found that Gossypol increased the duration of Backward Swimming Behavior (BSB) of Paramecium in a dose-dependent manner. Thus, .1 uM, 1 uM and 10 uM increased BSB by 57.5%, 177 % and 989.4%, respectively. Intracellular recording experiments were done in current clamp conditions when cells were maintained in high calcium and sodium-free solution that contained potassium channel blockers. Calcium action potentials from Paramecium were measured in control conditions and when the cell was exposed to Gossypol. It was found that Gossypol at 4 uM increased the duration of calcium action potential by 159 + 13% S.D. n= 4 cells. This effect was onset within 1 minute and was maintained after 10 minutes. The reversible effect of Gossypol was observed after 20 min of washout. These results suggest that Gossypol may act at membrane level modulating the influx of calcium in protozoa.

W-Pos401

BIOCHEMICAL STUDIES OF THE DIHYDROPYRIDINE SENSITIVE RABBIT CARDIAC CALCIUM CHANNEL EXPRESSED USING THE BACULOVIRUS/INSECT CELL SYSTEM.

((Tipu S. Puri, D. Woodrow Benson Jr., and Marlene Hosey)) Department of Pharmacology, Northwestern University Medical School, Chicago, IL 60640

Biochemical studies of the cardiac L-type calcium channels have been difficult due to the low density of channels in cardiac tissue. In this study we have expressed a cDNA encoding a rabbit cardiac Ca^{2+} channel α_1 subunit using the baculovirus/insect cell system. Insect cells infected with the α_1 cDNA produce a protein of approximately 210-220 kDa as determined by SDS-PAGE and western blotting which is larger than the 185-190 kDa α_1 subunit protein previously purified from chick heart (Chang and Hosey, 1988). The expressed protein is recognized by antibodies generated against peptides from an internal region (loop between domain II and III) and the carboxy-terminal end of the predicted α_1 protein sequence from rabbit heart. The α_1 baculovirus construct has been epitope tagged at the amino-terminus with a sequence encoding the Glu-Glu peptide from medium T antigen. A mouse monoclonal antibody specific for the Glu-Glu peptide also recognizes the expressed protein. Thus immunoreactivity data indicate that the full length α_1 subunit protein is expressed by the insect cells. Our studies have shown that the expressed α_1 subunit protein alone fails to bind the dihydropyridine PN 200-110 or the phenylalkylamine LU49888 at drug concentrations of up to 10 nM. Biochemical characterization of the expressed α_1 subunit protein may facilitate a better understanding of calcium channel functions in cardiac tissue.

W-Pos402

TRANSIENT EXPRESSION OF SKELETAL AND CARDIAC L-TYPE CALCIUM CHANNEL SUBUNITS IN HUMAN EMBRYONIC KIDNEY CELLS. Chan Fong Chang and M. Marlene Hosey. Dept. of Pharmacology, Northwestern University Medical School, Chicago, IL 60611.

Full-length cDNAs of α_1 subunits of rabbit skeletal muscle and cardiac L-type calcium channels were transiently expressed in a human embryonic kidney cell line (HEK 293). A relatively high level of expression in HEK cells was achieved and provided sufficient amounts of expressed proteins for biochemical characterisation. The skeletal muscle α_1 subunit was expressed as a protein of 210 kDa, in comparison to the protein of 175-190 kDa that is detected in native skeletal muscle membranes. The expressed protein was immuno-reactive to peptide-specific antibodies raised against the predicted amino terminus, an internal peptide and the predicted carboxyl terminus. The results of binding assays with dihydropyiridines indicated that the expressed skeletal muscle α_1 subunit alone exhibited very little or no specific binding of dihydropyiridines. Immunoblotting with carboxyl terminal-specific anitibodies indicated that the cardiac α_1 subunit was expressed as a protein of ~240 kDa. This is similar to the size predicted by the cDNA but larger than the ~190 kDa protein characterized from native cardiac cells. Similarly, the expressed cardiac α_1 subunit did not exhibit any significant binding of dihydropyridines.

W-Pos403

THE PK OF ACIDIC SIDE CHAINS IN THE V.-GATED CATION CHANNELS. ((S. Bogusz and D. Busath)) Box G, Brown U., Providence, RI 02912

A beta-barrel model has been proposed for the pore of voltage-gated K+ channels (Bogusz, et al. Prot. Eng. 4:285,1992) and by analogy, for Na+ and Ca++ channels. The proposed K+ channel model has 4 Asp's in a ring near the external end of the channel. The homologous region in the Na channel has 2 Glu's, a Lys, and an Ala whereas the Ca channel has 4 Glu's (Heinemann et al. Nature 356:441). Do adjacent charges shift the pK of the acidic side chains enough to cause significant protonation of the acid? We used a model based on the Ca++ channel structure (the worst case because of the length and number of the charged side chains). The potential at one vertex of a 4.2 A square with negative charges at the other three vertices was calculated to be 115 mV assuming eps=80, 55 mV with a cation at the center of the square. Assuming a Boltzmann distribution of protons (Edmonds, Eur. Biophys. J 16:113,'89) in this electric field, we estimate that the pK of a Glu would be shifted from 4.4 to 5.4 in the presence of a bound ion, to 6.4 in the absence. More precise estimates of the water and bound ion interactions, as well as the Glu positions, using a full atomistic model, are underway.

W-Pos404

THE NB11 cDNA ENCODES THE β-SUBUNIT OF ω-CONOTOXIN-SENSITIVE N-TYPE CALCIUM CHANNELS. ((Oh-Joo Kwon, Hee-Moon Park, Hyung-Lae Kim, and Hemin Chin)) NINDS, NIH, Bethesda, MD 20892.

We have isolated a cDNA clone (NB11 cDNA) from NG108-15 cells which encodes a protein with 80% deduced amino acid sequence similarity to that of the previously cloned β-subunit of the rat brain L-type calcium channel. To identify and biochemically characterize the NB11 gene product , we have expressed NB11 cDNA in *E. coli* as a fusion protein. The fusion proteins, containing an unique portion of NB11 cDNA, were used to produce rabbit antisera. Western blot analysis showed that the rabbit polyclonal antisera obtained were highly specific for the fusion proteins. Further studies with one of the antisera (HC-9) indicate that NB11 encodes a β-subunit associated with ω-conotoxin (ω-CTX)-sensitive, N-type calcium channel. HC-9 antiserum specifically immunoprecipitated ^{125}I-ω-CTX-labeled N-type calcium channels from digitonin-solubilized rat brain membranes, whereas neither preimmune nor an irrelevant antiserum (HC-171) did. Furthermore, the NB11 fusion proteins inhibited concentration- dependently specific immunoprecipitation of the ^{125}I-ω-CTX-labeled N-type calcium channels by HC-9 antiserum. Thus, our results show that NB11 cDNA encodes the β-subunit of the ω-CTX-sensitive, N-type calcium channels.

W-Pos405

RATIO OF CA^{2+} AND CA^{2+}-ACTIVATED K^+ CURRENTS IN TURTLE HAIR CELLS. ((J.J. Art* & R. Fettiplace)) UW- Madison, Madison, WI 53706 & *U. of Chicago, Chicago, IL 60637. (Spon. by D. Hanck)

Turtle auditory hair cells are tuned to specific frequencies in the acoustic stimulus, a behavior thought to arise through the interplay of a voltage-dependent Ca^{2+} current and a Ca^{2+}-activated K^+ current. The amplitudes of the two currents were measured in isolated hair cells of known resonant frequency during superfusion with solutions containing normal (2.8 mM) and reduced (0.1 & 1 μM) Ca^{2+}. In low calcium the Ca^{2+}-activated K^+ current in most cells was abolished. The current flowing through Ca^{2+} channels was increased roughly five-fold, and had a reversal potential near 0 mV. This observation may be explained by the Ca^{2+} channels becoming non-selectively permeable to monovalent cations in low Ca^{2+} solutions. The size of the non-selective current increased systematically with the hair cells resonant frequency over the range from 10 to 320 Hz, without a concomitant increase in membrane area. This suggests that hair cells tuned to higher frequencies have a higher density of voltage-dependent Ca^{2+} channels. There was also a good correlation between the amplitudes of the non-selective current and the Ca^{2+}-activated K^+ current across cells. Using previous estimates for the size of the corresponding single channel conductances, the results suggest a constant (2:1) ratio of Ca^{2+} to K^+ channels. If these channels are localized at afferent synapses, and their density is constant within each site, the results would predict a correlation between the number of synaptic sites and the frequency of the hair cell, a notion consistent with previous anatomical studies (Sneary, 1988. *JCN* 276: 588).

W-Pos406

N-TYPE CHANNELS OF DORSAL ROOT GANGLION NEURONS INACTIVATE BY BOTH CA++-DEPENDENT AND VOLTAGE-DEPENDENT PROCESSES. ((D.H. Cox and K. Dunlap)) Departments of Physiology and Neuroscience, Tufts Medical School, Boston, MA 02111.

In order to better understand the variable nature and unusual holding-potential dependence of N type Ca^{++} current inactivation, the inactivation kinetics of N currents from chick dorsal root ganglion (DRG) neurons were studied with tight seal, whole cell recording. These cells, when grown in culture less than twenty four hours, exhibit only ω-conotoxin-sensitive, N-type current. When examined during a 1 s pulse to 0 mV from a holding potential of -80 mV (10 mM internal EGTA) these currents inactivated in a biphasic manner with time constants of ~0.1 and ~2.7 s producing a total of ~50% current decay. We tested the hypothesis that inactivation is current-dependent. The following three lines of evidence support this hypothesis: 1) In twin pulse experiments in which a variable amplitude 500 ms conditioning pulse preceded by 5 ms a test pulse to 0 mV, it was found that the conditioning pulse which produced the most inactivation of the test pulse current coincided with that at which inward current was a maximum. 2) Lowering the Ca^{++} buffer (EGTA) concentration inside the recording electrode from 10 to 0.1 mM greatly increased the rate and extent of Ca^{++} current inactivation, and 3) substitution of Na^+ for Ca^{++} as the charge carrying ion produced Na^+ currents which inactivated more slowly than Ca^{++} currents and in a monophasic rather than a biphasic fashion. Evidence was also found for voltage-dependent inactivation. Partial inactivation was produced by 500 ms prepulses to potentials at which very little Ca^{++} current was flowing into the cell. Taken together, these results indicate that the macroscopic properties of N-type Ca^{++} channel inactivation in chick DRG neurons are likely to result from a combination of both voltage-dependent and current-dependent processes.

W-Pos407

DESENSITIZATION OF MU-OPIOID INHIBITION OF CALCIUM CHANNEL CURRENT IN RAT DORSAL ROOT GANGLION NEURONS. ((K.Nomura and T.Narahashi)) Dept. of Pharmacol., Northwestern Univ. Med. Sch., Chicago, IL 60611.

A variety of neurotransmitters inhibit calcium channel currents through G protein-mediated pathways, and some of the inhibitory effects are known to wane over time in repetitive or continuous stimulation by the agonist. To elucidate the mechanism underlying this desensitization, whole cell calcium channel currents were recorded from acutely dissociated dorsal root ganglion (DRG) neurons from 5-10-day old newborn rats. DAGO (1 μM) inhibited high voltage activated (HVA) peak calcium currents in a voltage-dependent manner without affecting low voltage activated (LVA) currents. The inhibition was gradually relieved with continuous application of DAGO (44.5±10% recovery in 5 min). Pre-treatment for 20-30 min with DAGO (1 μM) before applying patch electrode greatly reduced the inhibition by DAGO and pre-treatment for 24 hr mostly eliminated the effect. Inhibition of HVA currents by norepinephrine (NE,10 μM) or baclofen (50 μM), was also desensitized after 5 min of application, and was faster than that by DAGO (70±10% for NE, 69±11% for baclofen). The inhibitory effect of subsequently applied DAGO was also attenuated. These results suggest that there may be a common factor in the receptor-G protein-calcium channel coupling pathway in rat DRG cells that is shared and desensitized by different receptor agonists.

(Supported by NIH grant NS14144.)

W-Pos408

P-TYPE CALCIUM CHANNELS EXPRESSED IN XENOPUS OOCYTES ARE REGULATED BY PROTEIN KINASE A AND C.

((Bourinet, E., Fournier (*), F., Nargeot, J., and Charnet, P.)) CNRS-CRBM, UPR 9008, F-34000 MONTPELLIER, and (*) Lab. Neurobiol. Cell. Univ. de Picardie, F-80039 AMIENS.

When injected with rat cerebellum mRNA, *Xenopus* oocytes expressed voltage-dependent calcium channels (VDCC) insensitive to DHPs and ω-conotoxin, but blocked by *Agelenopsis aperta* venom. These channels exhibited several similarities with VDCCs expressed in mammalian Purkinje neurons, characterized as P-type VDCCs [1], and recently cloned in rabbit and rat brain [2].

Our results show that expressed VDCCs activity can be potentiated by direct intraoocyte injection of cAMP, perfusion of forskolin, or application of isoprenaline, a β adrenergic agonist. These effects were attributed to activation of protein kinase A (PKA) since they were antagonized by injection of the peptidic inhibitor of PKA. The VDCC activity was also enhanced by protein kinase C activation using PMA or OAG but not 4αPDD. This PKC-induced potentiation was prevented by prior perfusion of H7 or staurosporine. Several phosphorylation sites for PKA and PKC are found in the primary sequence of the α1 and β subunits that could account for these effects. Investigations are under way to characterize similar regulations in rat Purkinje neurons.

[1] Llinas *et al*, (1989) Proc. Natl. Acad. Sci. USA, 89: 1689-1693
[2]Mori *et al.* (1991) Nature, 350: 398-402; Starr *et al.*, Proc. Natl. Acad. Sci. USA, 88: 5621-5625

W-Pos409

CHARACTERIZATION OF TWO FUNCTIONALLY DISTINCT HIGH-VOLTAGE ACTIVATED Ca^{2+} CHANNELS IN RAT AORTIC MYOCYTES.

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We have identified and characterized two types of DHP-sensitive high-voltage activated (HVA) Ba^{2+} current, referred to as $I_{Ba,HVA1}$ and $I_{Ba,HVA2}$, in whole-cell clamped rat aortic myocytes (primary culture). They were investigated in cells where no LVA current was detectable. They had clearly distinct waveforms. $I_{Ba,HVA1}$ has a slow monoexponential decay (tau = 1200 ± 454 ms, n = 8). In contrast, the decay of $I_{Ba,HVA2}$ was much faster and biexponential (tau_f = 93 ± 59 ms; tau_s = 560 ± 218 ms, n = 5). Moreover, $I_{Ba,HVA2}$ had more negative ranges of activation and steady-state inactivation than $I_{Ba,HVA1}$ and was more sensitive to the dihydropyridine antagonist nicardipine (IC50's 0.2 μM and 2 μM, respectively). When Ca^{2+} was used as the charge carrier, decay of HVA1 currents was not significantly changed while that of HVA2 currents was accelerated ((tau_f = 18 ± 3 ms; tau_s = 113 ± 38 ms, n = 4). In addition, the permeability ratios were as follows : Ba^{2+} (1.0) > Ca^{2+} (0.2 ± 0.1) for HVA1 currents, and Ba^{2+} (1.0) > Ca^{2+} (0.6 ± 0.1) for HVA2 currents. This study suggests the existence of two functionnally disctinct subtypes of the so-called 'DHP-sensitive L-type' Ca^{2+} channel in rat aortic myocytes. Our observations may established a link with a number of recent observations from molecular cloning. It is possible that the HVA2 Ca^{2+} channel described here is a cardiac-like Ca^{2+} channel.

W-Pos410

A SELECTIVE, DOSE- AND STATE-DEPENDENT INHIBITOR OF THE T-TYPE CA^{2+} CHANNELS IN GUINEA PIG ATRIAL CELLS. ((Xiaoping Xu, Esther W. Lee and Kai S. Lee)) Cardiovascular Diseases Research, Upjohn Laboratories, Kalamazoo, MI.

Many specific, clinically valuable L-type Ca^{2+} channels (I_{Ca-L}) blockers have been identified. However, only a few, often less selective blockers of the T-type Ca^{2+} channel (I_{Ca-T}) are known. We report a new compound, U-92032, an anti-lipid peroxidation agent and neuronal Ca^{2+} channel blocker, that blocks atrial cell I_{Ca-T} at low dose. Experiments were carried out using the suction pipette single atrial cell technique. Extracellular application of 1 μM U-92032 reduced I_{Ca-T} to half, with little effect on I_{Ca-L}. The block was a resting-state block as 90% of the current inhibition was reached at the first test pulse after a 3-minute drug exposure. Steady-state voltage-dependent activation and inactivation of I_{Ca-T} were not changed, nor was the time-dependent inactivation. At 0.1 Hz stimulus frequency, the block became slightly use-dependent. Peak T-currents were reduced to 49.4 ± 2.5 % and 45.4 ± 2.4 % of the controls respectively at the 1st and 6th test pulse. At 10 μM, U-92032 blocked I_{Ca-T} completely but also produced a 20% inhibition of I_{Ca-L}. The block of I_{Ca-L} by 10 μM U-92032 was strongly use-dependent even at 0.1 Hz stimulus frequency. Peak I_{Ca-L} were decreased to 80.8 ± 5.1 % and 44.2 ± 3.6 % of the controls respectively at 1st and 6th test pulse, and the inhibition was characterized by an acceleration of inactivation, suggesting open-channel block. These results demonstrate a clear separation of dose- and rate-dependent effect of U-92032 on cardiac Ca^{2+} currents: at low dose, it is a selective, primarily resting-state blocker of the T-type Ca^{2+} channels; at high dose, it produces a strong use-dependent block of the L-type Ca^{2+} channel as well.

W-Pos411

Hormone-Regulated Ca^{2+} Channel in Rat Hepatocytes Revealed by Whole Cell Patch Clamp

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It is clear that IP_3-generating hormones not only mobilize intracellular Ca^{2+}, but also facilitate Ca^{2+} influx into non-excitable cells. The protein responsible for regulated Ca^{2+} entry has not been identified. When hepatocytes were patch-clamped in the whole cell configuration, addition of 20nM vasopressin or of 100 μm ATP induced an outward current with a reversal potential -55 mV. This current was blocked by replacing K^+ with Cs^+ in the external media and in the pipette solution or by including 0.5 μM apamin (but not charybdotoxin) in the media, suggesting mediation by a Ca^{2+} sensitive K^+ channel.

In the presence of apamin, patch-clamped hepatocytes pretreated with vasopressin in a Ca^{2+}-free media reveal an inward current on addition of external Ca^{2+} (5 mM). No current is seen in the absence of vasopressin pretreatment. Initially, the inward current is ca. 200-300 pA, but it declines rapidly over 3 minutes to ca. 20 pA. The reversal potential of the current is +50 mV and it is not voltage-gated. Additions of 5 mM Mn^{2+} or 5 mM Ba^{2+} in place of Ca^{2+} produced no current. The data suggest that hormone-mediated Ca^{2+} entry into hepatocytes is mediated by a unique voltage-insensitive channel highly specific for Ca^{2+}. This is the first demonstration of such a channel in hepatocytes.

W-Pos412

INCREASE OF CALCIUM CURRENTS BY α_1-ADRENERGIC RECEPTOR STIMULATION IN CARDIAC MYOCYTES FROM CARDIOMYOPATHIC HAMSTER

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The cardiomyopathic strains of the Syrian hamster (Bio 14.6) exhibit an enhanced positive inotropic response to α_1-adrenergic receptor stimulation associated with an enhanced α_1-adrenergic receptor-mediated rise in cytosolic calcium. However, the underlying mechanism of Ca^{++} overload is not known. To elucidate the role of Ca^{++} channels in the enhanced α_1-adrenergic responsiveness, we studied the effect of α_1-adrenergic agonist and antagonist on whole-cell Ca currents in cardiac myocytes from 8 month-old myopathic and normal hamsters. Phenylephrine (in presence of propranolol) caused a greater increase in both T (+68±12%) and L (+36±9%) currents in myopathic cells than that in control cells (T: +19±5%, L: +32±7%, respectively, n=21, $p<0.01$). Prazosin fully antagonized the phenylephrine induced increase in I_{Ca} in both groups. When myopathic and normal cell pretreated with pertussis toxin (200 ng/ml for 2 hours), the enhancement of T current induced by phenylephrine was completely attenuated in both normal and myopathic cells. However, the α_1-adrenergic receptor-mediated increase in L type current was only slightly reduced in both groups. These results demonstrate that α_1-adrenergic receptor-mediated effects on T type calcium current appear to be mediated by a pertussis toxin-sensitive G protein. The alteration of T current and T channel mediation by α_1-agonist may be related to the pathogenesis of Ca^{++} overload in cardiomyopathy.

W-Pos413

INTERACTION OF SUBSTANCE P WITH NEURONAL NICOTINIC ACETYLCHOLINE RECEPTORS EXPRESSED IN XENOPUS OOCYTES.

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We and others have shown that the neuropeptide substance P (SP) may act as an inhibitory neuromodulator at the nicotinic acetylcholine receptor (nAChR). The possibility remains, however, that SP may be mediating its effect indirectly. We have used the Xenopus oocyte expression system to determine if SP interacts directly with nAChRs and to examine the role of subunit structure on this interaction. Defolliculated oocytes were injected with mRNA or DNA to direct the synthesis of neuronal nAChRs. We have expressed functional receptors using subunits α7, α4, β2, and β4 (α7 holomeric and α4β2 and α4β4 combinations). In voltage-clamp experiments inward currents were observed in response to acetylcholine. Simultaneous application of SP attenuated these currents in a dose-dependent manner. SP alone had little or no effect in oocytes injected with water or those expressing nAChRs. Thus we affirm that SP can interact directly with the nAChR. In addition, there appear to be differences in the IC_{50} values for the various subunit combinations. We are investigating the subunit specificity of inhibition to determine the structural requirements for SP interaction with the receptor.

W-Pos414

FUNCTIONAL PROPERTIES OF NEURONAL α7 ACETYLCHOLINE RECEPTOR EXPRESSED IN XENOPUS OOCYTES: A COMPARISON WITH NEURONAL α4β2 AND ELECTRIC ORGAN α1β1γδ ACETYLCHOLINE RECEPTORS.

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Messenger RNAs encoding α7, α4β2, or α1β1γδ nicotinic acetylcholine receptors(AChRs) were injected into oocytes. ^{125}I-α-Bungarotoxin(for α7 and α1β1γδ) and ^{125}I-mAb 299(for α4β2) were used for measuring the amount of AChRs. Inward current induced by ACh and nicotine was recorded using two-electrode voltage clamp. Results: 1) the surface membrane to total ratios of the three types of AChRs were similar; 2)the α7-mediated current amplitude was in the same range as α4β2, but 5 times less than that of α1β1γδ; 3) α7 was more sensitive to nicotine than ACh, α4β2 was as sensitive to nicotine as ACh, while α1β1γδ was more sensitive to ACh; 4)the rate of desensitization of α7 was much faster than of α4β2 and α1β1γδ; 5)the currents through α7 and α4β2 were strongly rectified, but α1β1γδ had a roughly linear I-V relationship;6) Ca^{++} permeability for α7 was 4 times larger than that of α4β2, and 10 times larger than α1β1γδ. In summary, we showed that α7 nAChR, as a homo-oligomer, has unique pharmacological and electrophysiological properties different from other typical neuronal and muscle-type AChRs.

W-Pos415

IDENTIFYING THE LIPID-EXPOSED REGIONS OF THE NICOTINIC ACETYLCHOLINE RECEPTOR.

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To identify regions of the *Torpedo* nicotinic acetylcholine receptor (AchR) interacting with membrane lipid, we have used three different hydrophobic photoactivatable probes: 1-azidopyrene (1-AP), 3-trifluoromethyl-3-*m*-[^{125}I]iodophenyl) diazirine ([^{125}I]TID) and 5-[^{125}I]iodonapthyl-1- azide ([^{125}I]INA). We have identified the [^{125}I]TID labeled residues in the hydrophobic segment M4 of the β-subunit (Tyr-441, Cys-447, and Ser-448), γ-subunit (Cys-451 and Ser-460) , δ-subunit (Ser-457 and Met-465) and based on earlier work, the α-subunit (Cys-412, Met-415, Cys-418, Thr-422, and Val-425; Blanton and Cohen, *Biochemistry* 31: 3738-3750.) The results establish a consensus view that the M4 region is α-helical in nature with a homologous "face" of the helix labeled in each subunit and secondly that M4 presents a rather broad face to membrane lipid. Interestingly, the residues labeled by [^{125}I]INA in the M4 region of the α-subunit are restricted to Cys-412 and Cys-418. This result is likely a consequence of the more limited reactivity of the photoactivated nitrene ([^{125}I]INA) than the carbene ([^{125}I]TID). [^{125}I]TID labeled residues in the hydrophobic segment M1 of the α-subunit have also been identified and include Cys-222, Leu-223, Phe-227 and Leu-228. In contrast [^{125}I]INA incorporates almost exclusively into Cys-222 and Leu-223. The pattern of [^{125}I]TID incorporation into α-M1 was the same both in the presence and absence of the agonist carbamoylcholine and in the presence of excess non radioactive TID and thus the labeled residues in M1 are likely to be at the protein-lipid interface. While the pattern of [^{125}I]TID incorporation into AchR M4 segments was consistent with the labeling of faces of α-helices, the pattern of incorporation of [^{125}I]TID into α-M1, which was restricted to the C-terminal half beyond Pro-221, was inconsistent with the pattern expected for labeling either of a face of an α-helix or of a β-sheet.

W-Pos416

MAPPING EPITOPES ON *TORPEDO* ACETYLCHOLINE RECEPTORS.

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Anti-acetylcholine receptor (AChR) monoclonal antibody (mAb) 383C completely blocks α-bungarotoxin binding to the AChR. Carbamylcholine or α-bungarotoxin treatment of AChR inhibits 383C binding. Western blots of AChR subunits with 383C highlight the α-subunit. Peptide scanning the α-subunit N-terminal 211 amino acids with 383C shows strong binding to peptide α-(187-199). Small angle X-ray diffraction of 383C complexed to AChR-enriched membranes places "extra" electron density at the top of the AChR synaptic head. Electron micrographs of negatively stained arrays of 383C/AChR complexes indicate two sites of stain exclusion on the AChR rosette: the location of α-(187-199) of each α-subunit. This localization along with Unwin and Stroud's 3-D reconstructions of AChR structure from electron micrographs of tilted arrays places the agonist binding site 50-60 Å above the ion channel (1988,J.Cell Biol.*107*:1123;1989,*ibid.109*:755).

W-Pos417

Atomic Force Microscope Imaging of The Nicotinic Acetylcholine Receptor

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Atomic Force microscopy was used to image the quaternary structure of the nicotinic acetylcholine receptor molecules (AChR) from the electric organ of *Torpedo californica*. The isolated nAChR-enriched vesicles were imaged as dried on a glass slip or in phosphate buffer (10 mM sodium phosphate, 2 mM $CaCl_2$, 1 mM EDTA, pH 7.0). The dried vesicles were flat and had a relatively large size distribution. The diameter varied from 0.2 to 1 μm with majority around 0.45 μm. The height was measured between 25 to 40 nm. The vesicles imaged in the phosphate buffer seem fairly homogenous in size with a 0.45 μm diameter and a height of 50 nm. The shape of the vesicles in phosphate solution was more oval than spheric, perhaps due to the electrostatic interaction between the vesicles and the glass slip on which the vesicles were imaged. Once a good vesicle was located, the scan area was narrowed to 50 nm x 50 nm on the surface of the dried vesicle. With this scan area the donut-shaped structure features became visible. As we further decreased the scan area to 20 nm x 20 nm, we could identify pentameric structures with central depressions. Of the five subunits of the receptor three of them appeared to be more bulky than the other two. The diameter of the structure varied between 10.7 nm to 11.9 nm and the diameter of the depression ranged from 3.5 nm to 4.0 nm. Both parameters were larger measured by this scanning technique than those reported for *Torpedo marmorata* from the EM imaging.

W-Pos418

LOCALIZATION OF THE ETHIDIUM BINDING SITE ON THE *TORPEDO* NICOTINIC ACETYLCHOLINE RECEPTOR: A FLUORESCENCE ENERGY TRANSFER STUDY.

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The localization of the ethidium high-affinity noncompetitive inhibitor (HNCI) binding site on the *Torpedo* nicotinic acetylcholine receptor (nAChR) was examined using fluorescence resonance energy transfer. Dansyl-C_6-choline, bound to the agonist/competitive antagonist binding sites, was used as an energy donor to ethidium bromide (acceptor) bound to the HNCI site. Site-specific monoclonal antibodies that selectively block ligand binding to either the "*A*" or the "*B*" acetylcholine (ACh) binding sites were used to direct dansyl-C_6-choline binding to the unblocked agonist/competitive antagonist sites. (The "*A*" and "*B*" ACh binding sites correspond to the high- and low- affinity d-tubocurarine binding sites, respectively.) Control experiments established that the monoclonal antibodies did not significantly affect the binding of the donor or the acceptor to the nAChR, and did not dramatically affect the energy transfer parameters. Measured as a reduction of the donor fluorescence lifetime, the calculated distances between the "*A*" and "*B*" binding sites and the ethidium HNCI binding site were not significantly different from one another (21–40Å), indicating that the ethidium binding site is located in the lumen of the nAChR or at least along a zone equidistant from the two agonist/competitive antagonist binding sites. (Supported by a grant (91-140) from the Am. Heart Assoc., Calif. affiliate.)

W-Pos419

QUINACRINE AND ETHIDIUM BIND TO DIFFERENT SITES ON THE NICOTINIC ACETYLCHOLINE RECEPTOR.

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Fluorescence spectroscopy was used to determine whether quinacrine and ethidium, two high-affinity noncompetitive inhibitors (NCIs) of the nicotinic acetylcholine receptor (AcChR) from *Torpedo californica*, bind to separate loci. The ability of three nitroxide spin-labels, 5-doxylstearate (5-SAL), spin-labeled androstane (ASL), and TEMPO, to quench receptor-bound quinacrine and ethidium fluorescence was measured. When bound to phencyclidine(PCP)-sensitive sites on the AcChR, quinacrine was 16.9 and 19 times more efficiently quenched than ethidium by highly membrane partitioning 5-SAL and ASL, respectively. TEMPO, which has a limited ability to partition into *Torpedo* plasma membranes (<1%), was only twice as efficient at quenching receptor-bound quinacrine than ethidium fluorescence. Additionally, the ability of a series of paramagnetic *n*-doxylstearates (*n*-SALs) to quench the fluorescence of receptor-bound quinacrine and membrane-partitioned octadecyl rhodamine B (C_{18}-Rho) was examined. The *n*-doxylstearate quenching experiments showed a rank order of 7-SAL > 5-SAL > 12-SAL > 16-SAL to quench receptor-bound quinacrine fluorescence. These results suggest that quinacrine and ethidium do not bind to the same PCP-displaceable loci and provide support for the existence of a non-luminal NCI binding sites located at the level corresponding to the C_5-C_7 segment of the phospholipid acyl chains. (Support: NSF grant BNS-8821357 and Am. Heart Assoc. grant 91-140)

W-Pos420

FAST-DESENSITIZING SINGLE CHANNEL CURRENTS CORRELATED WITH THE NICOTINIC WHOLE-CELL RESPONSE IN HIPPOCAMPAL NEURONS. ((N.G. Castro and E.X. Albuquerque)) Dept. Pharmacol. Exp. Ther., U. Maryland Sch. Med., Baltimore MD 21201

Fast-desensitizing inward whole-cell currents elicited by acetylcholine (ACh) and nicotinic agonists and antagonized by methyllycaconitine (MLA) and by α-bungarotoxin have been previously identified in cultured fetal rat hippocampal neurons (*Mol. Pharmacol.* 41:802-8, 1992). Using the same preparation, we recorded both whole-cell and single channel currents 25-29 days after plating the cells. Outside-out patches were excised from neurons which responded to ACh (0.1-1 mM) with peak whole-cell currents greater than 100 pA at −60 mV. During each 1-s ACh pulse, the frequency of events was high at the onset and decayed very fast, with the events occurring mostly isolated or in pairs. In addition to fast desensitization, the response exhibited a rundown after repetitive ACh application and was reversibly blocked by MLA (10 pM). To obtain estimates of conductance and mean channel lifetime, the recordings were analyzed with a 7 kHz bandwidth and the data were pooled from several cells. At least three unitary conductances were identified, yielding currents of 6.6, 4.3 and 2.5 pA at −80 mV. The two higher amplitude components together had a mean open time of 0.1 ms as measured by maximum likelihood fitting of a single exponential to log-binned histograms (0.11 ms dead time, no correction for missed events).

These single channel properties (brief open time, small number of events per burst and fast desensitization) can account for the kinetics of the predominant whole-cell current type. This MLA-sensitive nicotinic receptor-channel may be involved in fast synaptic transmission in the mammalian brain. Supported by NIH grant NS25296, CNPq and FINEP-Molecular Pharmacology Training Program/Brazil.

W-Pos421

USE-DEPENDENT INHIBITION OF NEURONAL NICOTINIC AChR BY TINUVIN® 770 (BIS (2, 2, 6, 6, - TETRAMETHYL-4-PIPERIDINYL) SEBACATE), A POSSIBLE ADDITIVE TO LABORATORY PLASTICS. Roger L. Papke Molecular Neurobiology Laboratory, Salk Institute, La Jolla CA. 92037

Tinuvin® 770, a sterically hindered amine light stabilizer, is manufactured by Ciba-Geigy Corporation for use as an additive to plastics. The ability of Tinuvin 770 to block neurotransmitter receptors was studied using *Xenopus* oocytes injected with cRNAs coding for receptor subunits. The heterologously expressed nicotinic AChRs are inhibited by Tinuvin 770 through a use-dependent noncompetitive mechanism.

Muscle type AChRs (α1β1γδ subunits) are inhibited by Tinuvin 770, but the inhibition is readily reversible, with full control responses observed after a 5 minute wash. This contrasts to the inhibition of neuronal-type receptors which have time constants of recovery on the order of 45 minutes to 4 hours. This implies that Tinuvin 770 would favor the equilibrium inhibition of neuronal nicotinic receptors over muscle-type receptors. Hybrids of muscle and neuronal subunits, which incorporate neuronal beta subunits (α1β2γδ and α1β4γδ), are blocked in a less reversible fashion than are normal muscle-type receptors, suggesting that there is a direct interaction between Tinuvin 770 and the neuronal AChR beta subunits. Non-NMDA type glutamate receptors (GluR3 and GluR6) are not inhibited by Tinuvin 770.

Tinuvin 770 is a symmetrical conjugate of methylated piperidines, which are themselves effective noncompetitive inhibitors of α3β4 receptors, showing however faster kinetics of inhibition and recovery than Tinuvin 770.

An agonist solution incubated for 10 minutes in a plastic syringe had an effect on the responses of oocytes injected with cRNAs for the α3 and β4 subunits that was indistinguishable from the effects of agonist solutions containing 2 μM Tinuvin 770. Since the use of plastic syringes to load or hold agonist solutions in patch clamp and two-electrode voltage clamp experiments is nearly universal, the presence of such inhibitory activity may have resulted in the appearance of closed states which have been misinterpreted in the past.

W-Pos422

PATCH-CLAMP RECORDING OF ACH/ATP AUTORECEPTORS IN A CHOLINERGIC PRESYNAPTIC NERVE TERMINAL. ((X.P. Sun and E.F. Stanley)) *Section on Synaptic Mechanisms NINDS NIH Bethesda Md 20892* (Spon. D. Gilbert).

There is much evidence indicating that neurotransmitter can act on presynaptic nerve terminals but receptors for these ligands have not as yet been demonstrated by direct recording techniques. We have used the large calyx-type presynaptic nerve terminal of the chick ciliary ganglion to search for presynaptic cholinergic receptors. Calyx terminals were acutely dissociated and were voltage clamped using whole-terminal patch clamp techniques (J. Neurosci. 11:985). A combination of ACh (100 uM) and ATP (10 uM) frequently (7/17) induced an inward current (>10pA) in these terminals whereas ACh (1/58) or ATP (1/8) alone were ineffective. Outside-out patch recordings exposed to simultaneous treatment with ACh and ATP exhibited single channel current transitions with a 44 pS conductance. This study represents the first direct demonstration of a current gated by a presynaptic receptor. The biological role of this channel may be to automodulate transmitter release from the calyx since ACh and ATP are known to be co-released from cholinergic vesicles.

W-Pos423

KINETICS OF THE INHIBITION OF ACH RECEPTOR CHANNELS BY VOLATILE ANESTHETICS AND ALCOHOLS. ((Y. Liu, H. Mody and J.P. Dilger)) Dept. of Anesthesiology, SUNY, Stony Brook, NY 11794.

Volatile general anesthetics and n-alcohols exert effects on single nicotinic acetylcholine receptor (AChR) channels that are consistent with drug blockade of both open and closed channels (J. Physiol. 437: 431, '91; Mol. Pharmacol. 41: 127, '92). To test this idea further, we measured current responses to rapid perfusion of mixures of ACh and drug onto multi-channel, outside-out patches from BC3H-1 cells. The drugs act quickly, so we selected patches having an extremely fast solution exchange time (≤200 μs). Perfusion with 10 mM ACh provided control current responses. Patches were then pre-incubated with drug (in mM, 1 isoflurane, 20 butanol, 0.5 hexanol or 0.025 octanol) and perfused with drug+ACh. Currents were reduced by about 50%, as expected from single channel measurements. Drug was then removed from the pre-incubation solution and the onset of current block was observed by perfusion with drug+ACh. Currents decayed from control values with time constants of ≤50 μs (butanol), 200 μs (hexanol) and 300 μs (isoflurane). These rapid kinetics are consistent with the speed of single channel flicker induced by the drugs. The decay time for octanol (≥2 ms) was slower than expected. Rates of recovery from block were also measured. The recovery time for isoflurane was 500 μs. Recovery was slower (and biphasic) for hexanol and octanol. Slow kinetics suggest the presence of a slowly equilibrating pool of drug in the membrane. Supported by GM42095 (JPD).

W-Pos424

KINETICS OF THE INHIBITION OF ACH RECEPTOR CHANNELS BY D-TUBOCURARINE. ((J. F. Roper, R.J. Bradley and J.P. Dilger)) Dept. of Pharmacology, UAB, Birmingham, AL 35294; Dept. of Psychiatry, LSU, Shreveport, LA 71130 and Dept. of Anesthesiology, SUNY, Stony Brook, NY 11794.

The kinetic mechanism of action of d-tubocurarine (curare) as a competitive antagonist at the nicotinic acetylcholine receptor (AChR) channel is not completely understood. We studied the action of curare by measuring the current response to rapid (≤1 ms) perfusion of mixtures of ACh and curare onto outside-out patches taken from BC3H-1 cells containing several hundred muscle-type AChRs. The current response (at –50 mV, 20-23°C) to perfusion of ACh (3 or 100 μM) was taken as the control. Patches were then pre-incubated with curare (50-1000 nM) and perfused with curare+ACh. Curare reduced currents from the control values; 90 nM curare reduced currents by 50%. Curare was removed from the pre-incubation solution and onset kinetics of curare action were measured by perfusion with curare+ACh (3 μM). With 500 nM curare, the current decayed from the control level with a time constant of 20 ms. Curare was again included in the pre-incubation solution and recovery kinetics of curare were measured by perfusion with 3 μM ACh alone. The current recovered to the control level with a time constant of 80 ms. These results provide estimates of 10^8/M/s and 10/s for the curare association and dissociation rates respectively.
Supported by GM44915 (RJB) and GM42095 (JPD).

W-Pos425

AMINO ACID SUBSTITUTIONS AT THE LIPID-PROTEIN INTERFACE OF THE ACETYLCHOLINE RECEPTOR (ACHR) ALTER ION CHANNEL KINETICS.

((S. Ortiz-Miranda, J.A. Lasalde, M.G. McNamee, and P.A. Pappone)) Dept. Biochemistry & Biophysics and Dept. Animal Physiology, University of California, Davis, California 95616.

M4 is one of the four homologous hydrophobic segments (M1-M4) present in all five subunits of the nicotinic AChR and is believed to be at the protein-lipid interface. We have recorded single channel activity of AChR's with different amino acid substitutions at CYS-418 in the M4 region of T. californica α subunit and at position CYS-447 of the β subunit using site-directed mutagenesis and expression in Xenopus oocytes. Wild type (WT) AChR (α CYS-418) showed a mean conductance (γ) of 65 pS and an open time (τ_o) of 0.6 ms at -80 mV. αTRP-418 mutants expressed a 20 fold longer τ_o. βTRP-447 substitutions showed an 8 fold increase in τ_o compared to WT channels. A change to αPHE-418 on the other hand showed a 2 fold increase in τ_o, an increase in channel γ to 84 pS, and a steeper τ_o dependency on voltage. αALA-418 and αGLY-418 substitutions had γ and τ_o values similar to WT. These results suggest that AChR domains in contact with membrane lipids are important for channel function and that substitutions in these regions dramatically alter channel kinetics.

W-Pos426

GATING OF NICOTINIC ACETYLCHOLINE RECEPTOR CHANNEL MAY INVOLVE ELECTRON TRANSFER BETWEEN TYROSINE AND A DISULFIDE BOND.

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Disulfide bond and aromatic amino acids are involved in many electron transfer reactions [1]. In the Torpedo nAChR, C192 and C193 are disulfide bonded. Mutation of either residues changes the agonist binding moderately, but eliminates channel activity entirely [2], suggesting their critical role in the coupling between agonist binding and channel's opening. Aromatic rings in tyrosines 190 and 198 are also important in the signal transduction [3]. These residues have been shown to be located near the agonist binding site. We propose that, upon agonist binding, another tyrosine near the extracellular end of transmembrane segments may donate electron to C192-193, with Y190 and Y198 as mediators. The tyrosine will become a radical, which may then induce conformational change of the pore. The electron transfer between tyrosine and disulfide bond may also play a role in the gating of glycine receptor channel, gap junction channel and eag potassium channel.

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2. Mishina et al., Nature, 313, 364 (1985).
3. O'Leary, M.E. and White, M.M., J. Biol. Chem., 267, 8360 (1992).

W-Pos427

CHARACTERIZATION OF ACETYLCHOLINE RECEPTOR DRUG BINDING SITES BY A COMPETITIVE PHOTOLABEL.

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3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine ([^{125}I]TID) is a sensitive probe of acetylcholine receptor (AChR) conformation. In this study, we determined the concentration dependence of drug inhibition of [^{125}I]TID photolabeling of affinity-purified AChR in the presence and absence of α-bungarotoxin (BgTx), both as a measure of the ability of these drugs to desensitize the AChR, and to characterize the [^{125}I]TID binding site. For each drug the calculated IC_{50} for blockage of [^{125}I]TID incorporation into each of the four subunits of the AChR was found to be similar, suggesting that for resting state AChR [^{125}I]TID preferentially binds to a single site. Procaine and chlorpromazine blocked [^{125}I]TID incorporation at slightly lower concentrations in the absence of BgTx than in its presence. However, BgTx dramatically raised the calculated IC_{50} values for lidocaine and phencyclidine, suggesting that at lower concentrations these drugs reduced [^{125}I]TID incorporation by binding to the agonist binding site and inducing desensitization, and only competed with [^{125}I]TID for the same binding site at higher concentrations. The effects of BgTx on phencyclidine binding are contrary to those observed in earlier studies, and may be due to different methods of AChR purification. Tetracaine and dibucaine blocked [^{125}I]TID incorporation competitively, suggesting that the high affinity [^{125}I]TID binding site is the non-competitive blocker binding site presumed to exist in the AChR ion channel. The demonstration that desensitization dramatically reduces the level of [^{125}I]TID incorporation into a site within the AChR ion channel provides good evidence that desensitization involves a structural change in the AChR ion channel region.

W-Pos428

EFFECT OF CHEMICAL MODIFICATION OF EXTRACELLULAR HISTIDYL RESIDUES ON THE CHANNEL PROPERTIES OF THE NICOTINIC ACETYLCHOLINE RECEPTOR.

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We have examined the effect of chemical modification with diethyl pyrocarbonate (DEP) on the properties of acetylcholine (ACh)-activated channels in the clonal muscle cell line BC3H-1. After protein modification, patch-clamp recordings showed alterations in the kinetics of the nicotinic acetylcholine receptor (AChR) channel. A major effect was observed in the channel mean open time, which was reduced up to 12-fold at 470 μM DEP. Consistent with an increase in the number of unprotonated histidine residues, this effect increased concomitantly with the pH of the reaction medium. Moreover, the modification was faster at pH 8 than at pH 6. The changes were time- and DEP concentration-dependent. Modified channels also showed an increase in the number of events per burst of openings together with a decrease in burst durations. The amplitude of the channel closed time component of about 1 ms increased with respect to the longest duration closed component. The number of α-bungarotoxin sites was slightly reduced after the modification, with no changes in the ligand binding affinity. The results suggest that the targets of the DEP modification are extracellular histidine residues involved in the ion translocation function of the AChR.

W-Pos429

EFFECTS OF THE HISTIDINE MODIFIER DIETHYL PYROCARBONATE ON THE SINGLE CHANNEL PROPERTIES OF NMDA-ACTIVATED ION CHANNELS.

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Diethyl pyrocarbonate (DEP) acts specifically to carboxymethylate histidine residues and has been shown to potentiate the whole-cell NMDA response (Traynelis, S.F., Cull-Candy, S.G., J.Physiol. 433:727-763, 1991). The effects of DEP on the single-channel characteristics of the NMDA receptor were investigated by treatment of the intracellular or extracellular surfaces of excised patches with 1-3 mM DEP. Patches were obtained from cultured rat cortical neurons and clamped at voltages between +50 and -70 mV. Channels treated with extracellular DEP for 1-5 min. showed a longer burst duration than unmodified channels, accounting for the increased open probability. Closed state analysis of both normal and modified channels revealed four shut states. DEP modification decreased the relative proportions of the two longer shut states, but had little effect on their lifetimes. Simple kinetic schemes can account for these effects if it is assumed that the sole effect of DEP is to alter the rate constant for entry into the longest shut state. The distribution of open intervals was similar to that of unmodified channels. Channels modified by DEP applied to the intracellular surface for 1-5 min. showed an increased frequency of transition to a high amplitude, long duration state. The apparently discrete kinetic effects of DEP provides information about the role of these amino acids in the conformational changes involved in transitions between kinetic states. Supported by NIH grant NS29881.

W-Pos430

ANALYSIS OF OPEN AND SHUT INTERVALS OF NMDA-ACTIVATED ION CHANNELS.

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Analysis of open and shut intervals of single ion channels provides useful kinetic information. NMDA-activated channels were studied in cell-attached patches of rat cortical neurons at three NMDA (1, 10, 30 μM) and one glycine (10 μM) concentration. The patch was held at -70 mV by first depolarizing the cell with potassium, and then adjusting the voltage across the patch. As expected, open probability was increased with NMDA concentration, a result of decreased duration of the longer shut intervals. Adjacent states analysis of the main conductance level openings (42 pS) indicated that short open intervals were associated with long shut intervals, and long opens with short shut intervals. Autocorrelation suggested that openings were correlated over at least 3 intervals. Likewise, shut intervals were correlated over at least 5 intervals. Burst analysis indicated that a large proportion of bursts existed as single openings of both short and long duration. Unlike open and shut intervals, burst durations themselves were not correlated. These results suggest that NMDA receptors that are saturated with glycine can be described by a model similar to the nicotinic acetylcholine receptor, with at least two open states associated with at least four closed states by two separate pathways. Supported by NIH grants NS08992 and NS29881.

W-Pos431

IONIC PROPERTIES OF GLUTAMATE RECEPTOR CHANNELS IN DROSOPHILA MYOTUBES. ((Ciani, S., Chang, H., and Kidokoro,Y.)). Dept. of Physiology, BRI and JLNRC, Univ. of California, Los Angeles, CA. 90024.

Inside-out patches were used to study the ion permeability of glutamate receptor channels in Drosophila myotubes. In gradients of NaCl, the reversal potential indicated almost ideal selectivity for Na^+. In symmetrical NaCl solution the single-channel I-V relationships were fairly linear, and the conductance dependence on $[Na^+]$ was a Michaelis-Menten curve with K_M=39 mM and G_{Max}=188 pS. The selectivity among alkali cations and NH_4^+ was low, the maximum value of P_{Na}/P_X being 1.75 for $X=Li^+$. External Mg^{2+} and Ca^{2+} shifted the reversal potential in a way that indicated their being more permeant than Na^+, but they also reduced the size of the current, suggesting strong binding within the channel and block of the Na^+ flux. The data could not be accounted for by the "constant field" theory, but were well fitted by an Eyring model for a "one-ion" channel with three energy barriers and two internal sites. From such model, the K_M and G_{Max} for Mg^{2+} were estimated to be: 0.26 mM and 22.5 pS, and for Ca^{2+}:0.14 mM and 19.2 pS.

W-Pos432

THE NEUROSTEROID PREGNENOLONE SULFATE POTENTIATES THE OPENING OF NMDA CHANNELS. ((M.R. Bowlby)) Department of Neurobiology, Harvard Medical School, Boston, MA 02115.

Steroid hormones exert profound influences on CNS pathways and behavior. The classical view is that effects on transcriptional regulation are key elements in these influences. Recently, however, brain metabolites of steroid hormones, called neurosteroids, have been found to exert important membrane-mediated modulatory effects on neuronal mechanisms. For example, the endogenous neurosteroid pregnenolone sulfate (PS) inhibits $GABA_A$ activated Cl^- currents and potentiates macroscopic NMDA activated cation currents, thereby possibly biasing CNS circuits in favor of excitation. To confirm and extend these findings, we recorded whole cell currents and single NMDA activated channels from cultured, dissociated hippocampal CA1-CA3 neurons. PS (50 μM) approximately doubled both the whole cell current flowing through the NMDA channels and the probability of opening of single NMDA activated channels in excised outside-out membrane patches. Two parameters of single channel kinetics were potentiated: the frequency of openings and the mean channel open time. PS did not enhance the single channel conductance in any patches. NMDA activated channels also were recorded in the cell attached configuration. A similar potentiation of opening frequency and mean channel open time was observed when PS was added to the solution bathing the cells. This suggests that PS can enter or pass through cell membranes to affect channels. (Supported by the Grass Foundation and NINDS)

W-Pos433

IDENTIFICATION OF NMDA RECEPTOR DOMAINS INVOLVED IN REGULATION BY PROTEIN KINASE C AND BY POLYAMINES. ((G.M. Durand, P. Gregor, G.R. Uhl, M.V.L. Bennett and R.S. Zukin)) Albert Einstein College of Medicine, Bronx, NY 10461. (Spon. by J. Saez)

Alternative RNA splicing generates at least six variants of the rat NMDA receptor subunit found initially, NR1a (Sugihara et al., BBRC 185: 826, 1992). We recently cloned two of these splice variants, NR1b and NR1c, (Durand et al., PNAS 89: 9359, 1992) and report here that NR1a-c differ in their functional properties. One variant, NR1b, differs from NR1a by the presence of a 21 amino acid insert near the N-terminal domain and by an alternate C-terminal domain in which the last 75 amino acids are replaced by an unrelated sequence of 22 amino acids. NR1c lacks the N-terminal insert like NR1a, but is identical to NR1b in its C-terminal domain. In *Xenopus* oocytes injected with NR1a RNA, NMDA responses show c. 50% potentiation by spermine. Responses of NR1c receptors show spermine potentiation and agonist affinity similar to those of NR1a receptors; NR1b receptors show reduced agonist affinity and greatly reduced or absent spermine potentiation. These findings suggest that the N-terminal insert affects binding of agonists and is involved with the action of polyamines. Responses of NR1a receptors showed c. 4 fold potentiation after 15 min treatment with the protein kinase C (PKC) activator, TPA. Both NR1b and NR1c receptors show c. 20-fold potentiation of NMDA responses by TPA, indicating that the C-terminus of NMDA receptor subunits is involved in regulation by PKC. Repeated test applications of NMDA during the TPA treatment reduce the maximal potentiation to about 10-fold. The onset of TPA potentiation is slow, making unlikely a direct action of TPA on the receptor itself.

W-Pos434

GABA-A RECEPTOR MEDIATED Cl^- FLUX INDUCES INTRACELLULAR CALCIUM INCREASE IN LHRH SECRETING NEURONAL CELL LINE. S. Yagodin, L.A. Holtzclaw, J.L. Barker and J. T. Russell LCMN, NICHD, and LN, NINDS.

In the immortalized GnRh secreting neuronal cell line (GT1-7), application of GABA (10μM) or the GABA-A receptor agonist, muscimol (5μM) evoked large increases (200 - 800 nM) in $[Ca^{2+}]_i$ in most cells. Three types of responses were observed: 1) a single spike like increase, 2) a single peak followed by a prolonged plateau, and 3) $[Ca^{2+}]_i$ oscillations. In contrast, baclofen (10 μM), the GABA-B agonist, had no effect on $[Ca^{2+}]_i$. The muscimol-induced calcium signals are inhibited by bicuculline (10 μM) and picrotoxin (10 μM) applied alone. When applied together, these antagonists completely abolished the response. Reduction of medium Cl^- concentration from 138 mM to 10 mM, reduced the amplitude of the muscimol evoked responses to 30% of control values, and complete replacement of Cl^- with gluconate abolished the signals. Similarly, the GABA-A response was abolished when $[Ca^{2+}]_o$ was 0 mM. Application of caffeine (10 mM), increased $[Ca^{2+}]_i$, presumably by release of calcium from intracellular stores. In normal medium ($[Ca^{2+}]_o$= 1.5 mM), pretreatment of cells with ryanodine (1μM) which depletes cellular calcium stores, reduced the amplitude of the $[Ca^{2+}]_i$ increase caused by muscimol. Taken together the data show that chloride influx through bicuculline- and picrotoxin-sensitive GABA-A channels induce calcium influx and calcium release from ryanodine-sensitive intracellular stores.

W-Pos435

LANTHANIDE MODULATION OF $GABA_A$ RECEPTOR SINGLE CHANNEL PROPERTIES. ((M.Yan, E.Reuveny and T.Narahashi)) Department of Pharmacology, Northwestern University Medical School, Chicago, IL 60611.

We have previously reported that lanthanides markedly potentiate the GABA-induced chloride current by acting at a distinct site on the $GABA_A$ receptor-channel complex (Yan and Narahashi, Biophys. J. 61(2):A105, 1991; Yan and Narahashi, Soc. Neurosci. Abstr. 18:249, 1992). These studies have now been extended to the single channel level and changes in gating kinetics of $GABA_A$ receptor currents caused by 100 μM terbium (Tb^{3+}) are reported. The currents were recorded from the outside-out membrane patches isolated from the rat dorsal root ganglion neurons in primary culture at a holding potential of -60mV. At least two conductance levels were recorded, a predominant one of about 26 pS and a minor one of about 19 pS. These two conductance levels were not changed by Tb^{3+}. The main conductance channel was further studied. The frequency histograms of durations of both GABA-induced opening and burst could be fitted by three exponential functions. The time constants of both opening and burst durations were not significantly changed by Tb^{3+}. However, Tb^{3+} increased the relative proportions of longer open time constants and longer burst time constants. Therefore, the enhancement of GABA receptor current by Tb^{3+} is due to an increase in the probability of openings with longer time constants of the $GABA_A$ receptor main conductance channel without altering its open time constants.

W-Pos436

AVERMECTINS POTENTIATE GABA-SENSITIVE CURRENT IN *XENOPUS* OOCYTES EXPRESSING CLONED $GABA_A$ RECEPTORS. ((J.P. Arena, P.J. Whiting*, K.K. Liu, J.F. McGurk, P.S. Paress and D.F. Cully)) Merck Research Laboratories, Rahway, N.J. 07065 and *Harlow, Essex, CM20 2PT, U.K.

Avermectins (AVM) are macrocyclic lactones with potent anthelmintic and insecticidal activity. AVMs have previously been shown to potentiate GABA-sensitive currents in *Xenopus* oocytes injected with chick brain mRNA. Oocytes were injected with rat brain mRNA (50 ng) or *in vitro* RNA encoding the bovine $GABA_a$ receptor subunits α1, β1, and γ2L (1 ng each). In oocytes injected with rat brain mRNA the response to 10 μM GABA was enhanced 6-fold by AVM (1μM), while the maximal current elicited with 300 μM GABA was unchanged. In oocytes expressing the α1, β1, and γ2L subunits AVM potentiated the response to 10 μM GABA 4.3 fold, and the maximal response was slightly increased. When the α1 and β1 subunits were coexpressed, AVM potentiated responses to 10 μM GABA only 1.6 fold and inhibited the maximal response by 22 %. With oocytes expressing only the β1 subunit AVM directly activated current in the absence of GABA. This effect was also observed with the α1 and β1 combination but not with rat brain mRNA or with coexpression of α1, β1, and γ2L. Channels consisting of only β1 subunits are directly gated by AVM suggesting the AVM binding site in on the β1 subunit. The data also suggest that the γ2L subunit is necessary for the potentiating effect of AVM.

W-Pos437

MUTATIONS WHICH EFFECT AGONIST-DEPENDENT GATING OF $GABA_A$-ACTIVATED CHLORIDE CHANNELS. ((J. Amin and D. S. Weiss)) Dept. of Physiol. and Biophyics, Univ. of S. Florida Coll. of Med., Tampa, FL 33612.

cDNAs encoding for the α_1, β_2, and γ_2 subunits were isolated from rat brain RNA using PCR. We have been investigating the effects of mutations in the α_1 and β_2 subunits on the sensitivity of these GABA channels to GABA using the two-electrode voltage-clamp technique.

The GABA channel, as well as other members of the receptor-operated channel superfamily (ACh and glycine- activated channels), contain a pair of highly conserved disulphide-bonded cysteine residues fifteen amino acids apart. It has been suggested that this cysteine loop, which is located in the N-terminal extracellular domain, is important for assembly and/or insertion of the subunits into the membrane. In support of this, when we prevent the formation of this cysteine loop in either the α_1 or β_2, by changing one of the cysteines to a serine, no GABA-activated currents are observed when coexpressed with wild type β_2 or α_1, respectively. Examination of the amino acid sequence of the β_2 subunit within the cysteine loop reveals two adjacent arginine residues which are absent in α_1 and γ_2. These positively-charged amino acids were replaced by asparagine and serine and the resulting mutant subunit was coexpressed with wild type α_1 and/or γ_2 subunits. The dose-response relationship for GABA demonstrated that the half-maximal concentration (EC_{50}) was shifted to the right (e.g., mean ± SD: 25.6 ± 4.4 to 161.7 ± 7.1 for coexpression of mutant β_2 with γ_2). The Hill coefficient was not altered (1.55 ± 0.11 and 1.55 ± 0.02). We are presently examining conservative substitutions of these arginines (to lysines) and mutations in neighboring regions to further elucidate the role this region plays in agonist-dependent gating. (Supported in part by ADAMHA R29 AA09212.)

W-Pos438

RECEPTOR SUBUNIT DEPENDENT ETOMIDATE POTENTIATION OF $GABA_A$ CURRENT ((I. Uchida, G. Kamatchi, D. Burt, J. Yang)) Dept Anesthsiology and Pharmacology, U.Maryland Sch Med, Baltimore, MD 21201.

Functional studies of expressed $GABA_A$ receptors with and without the $\gamma 2$ subunit suggest that this subunit is required for the benzodiazepine potentiation of GABA gated currents. In contrast, barbiturate potentiation of I(GABA) does not require this subunit. Etomidate is a non-benzodiazepine, non-barbiturate general anesthetic which potentiates I(GABA). Therefore, we studied the effect of $\omega_6\gamma 2$ subunit on etomidate potentiation of I(GABA). Murine $GABA_A$ receptor $\alpha 1$, $\beta 1$, and $\gamma 2$ subunit cDNAs sub-cloned in pcDNA mammalian expression vector were expressed in H293 cells by transient transfection. After 48 - 72 hours, whole cell patch clamp currents gated by puffer application of GABA were characterized. Etomidate, at clinical concentrations, increased the I(GABA) peak response with or without the presence of $\gamma 2$. In contrast, the $\gamma 2$ subunit was necessary for the prolongation of the current decay. Flumazenil (1 μM) did not prevent the effect of etomidate on current decay. The $\gamma 2$ subunit appears to mediate drug-receptor interaction for non-benzodiazepines as well.

W-Pos439

FRACTIONAL CA^{2+}-PERMEABILITY OF NMDA AND AMPA/KAINATE RECEPTORS IN FOREBRAIN MEDIAL SEPTUM NEURONS ((R. Schneggenburger, Z. Zhou, A. Konnerth & E. Neher)) Max-Planck-Institut für biophysikalische Chemie, 3400 Göttingen, Germany.

The standard method for determining the Ca^{2+}-selectivity of glutamatergic cation channels, such as the NMDA receptor channel (NMDA-R) and AMPA/kainate receptor channel (A/K-R) is the measurement of permeability ratios P_{Ca} / $P_{monovalent}$ from reversal potentials. This, however, may not be appropriate for estimating physiologically relevant flux ratios at negative membrane potentials (V_m). Ca^{2+}-flux can, however, be determined quantitatively by simultaneous fura-2 and whole-cell recordings. To do so, we have loaded neurons of the medial septal nucleus from slices of rat forebrain with 1 mM fura-2, a concentration at which the indicator dye effectively competes for the binding of Ca^{2+}-ions with the endogenous Ca^{2+}-buffers. Therefore, changes in the fluorescence at 380 nm (F_{380}) directly reflect Ca^{2+}-fluxes. Agonist-induced inward currents, elicited by short ionophoretic applications of the selective agonists NMDA, AMPA and kainate, were accompanied by decreases in F_{380} when Ca^{2+} (2mM) was present in the extracellular solution. We determined the fraction f of fura-2 detected Ca^{2+} over total charge of the agonist induced currents and related it to the same fraction (f_{max}) for voltage-activated Ca^{2+}-currents. We define as 'fractional permeability', P_f, the ratio f/f_{max} which represents the Ca^{2+}-fraction of the total agonist-induced cation current. For the NMDA-R, a P_f-value of 6.8 ± 1.3 % (average ± S.D., n=13 cells) was obtained at V_m = -80 mV. Such a value would be expected at sufficiently negative V_m, using GHK assumptions, for a very moderate Ca^{2+}-selectivity (permeability ratio based on ionic concentrations: P_{Ca} / $P_{monovalent}$ = 1.2). Surprisingly, the linearly conducting A/K-R found in these neurons was also significantly permeable to Ca^{2+}, yielding a P_f-value of 1.4 ± 0.6 % (n=10 cells) at V_m = -80 mV.

W-Pos440

CLONING OF TWO KAINATE RECEPTOR SUBUNITS FROM GOLDFISH BRAIN. ((Z. Galen Wo & Robert E. Oswald)) Dept. Pharmacology, Cornell University, Ithaca, NY 14853 USA.

Kainate receptor subunits of 41 and 45 kD have been isolated from goldfish brain and characterized biochemically (C.J. Ziegra *et al., Mol. Pharmacol.* 42:203-209, 1992). Using two 600 bp DNA fragments amplified by PCR with synthetic degenerate oligonucleotide primers, we screened a goldfish brain cDNA library. Two types of full length cDNA clones encoding putative kainate receptors have been cloned. Both types, α and β, have an open reading frame for a protein with calculated molecular mass of approximately 40 kD. Their amino acids sequences share approximately 80% homology. Both are about 65% homologous to the kainate binding protein of frog and chicken brain. The mRNA of subunit β is 5-fold more abundant than that of the α subunit in goldfish brain. A 17 amino acid segment near the N-terminal end of subunit β is identical to a peptide fragment derived by proteolysis and sequenced from the 41 kD goldfish kainate receptor (Ziegra *et al.*, 1992). The ligand binding profile of these two putative kainate receptor subunits will be characterized by transient expression. It is of great interest to determine if the kainate receptor subunits α and β can form a functional ligand-gated ion channel when co-injected into *Xenopus* oocytes.

W-Pos441

COMPUTATIONAL ANALYSIS OF TETRAMETHYLAMMONIUM-BENZENE INTERACTIONS IN WATER ((Jiali Gao, Lawrence Chou, and Anthony Auerbach)) Dept. Chemistry and Dept. Biophysical Sciences, SUNY, Buffalo, NY 14214 (Spon. by M. Slaughter).

Interactions between cations and aromatic residues in proteins are of considerable biological importance. We have used an approach that combines semi-empirical quantum mechanical calculations (AM1 theory) and molecular mechanical Monte Carlo simulations to elucidate the free energy of tetramethylammonium (TMA) and benzene in water. The distance between the nitrogen and the plane of the benzene ring (constrained to be perpendicular to the reaction coordinate) was varied between 3.5 and 10 A in 0.1 A increments, with 1.5 million configurations sampled at each distance. Two energy minima are apparant at 4.7 A (-0.69 kcal/mol) and 7.5 A (-1.64 kcal/mol), separated by a barrier centered at 6.1 A (0.06 kcal/mol). Both wells and barriers are broad and span about 1 A at half-height, presumbly because of TMA rotation. The well depth at the contact minimum is in good agreement with recent experimental evidence for cation-aryl interactions in water (about 0.5 kcal/mol; Schneider *et al*, J. Am. Chem Soc. 114:7698, 1992). The AM1 theory predicts an interaction energy of 4 kcal/mol in the gas phase, which is largely damped out by competition with solvent and entropic effects due to configurational averaging. Other combinations of cation-solvent-aromatic are under investigation.

W-Pos442

PURINERGIC P_2 RECEPTOR CHANNEL COUPLING IN COELIAC GANGLIA NEURONS. ((Victor Derkach & A. Surprenant, Vollum Inst., Oregon Hlth Sci Univ, Portland, OR. 97201 (Sponsored by M. Forte))

Neuro-neuronal fast ATP-mediated synaptic transmission occurs in guinea-pig coeliac ganglion neurons grown in tissue culture (Evans et al. *Nature*, 1992, 357). In this study we measured whole cell and single channel currents in response to ATP and ATP analogues in these neurones. At -80 mV inward currents were evoked in response to agonists with the following order of potency: 2-MeSATP > ATP > α,βmetATP ≈ DADPP > β,γATP >> ADP > AMP > adenosine, thus confirming this response as mediated by P_2 purinoceptors. α,βmetATP produced a desensitizing response and either inhibited (n=2) or potentiated (n=4) the ATP current; the P_2 receptor blocker suramin also either inhibited (n=5) or potentiated (n=4) the current. Unitary conductances showed a trimodal distribution of 10.5 ± 1, 14 ± 1.5 (n=5) and 19.1 pS (n=2). ATP, but not α,βmetATP produced super-long bursts of channel activity (144 ± 26 ms at -70 mV, n=5). A briefer burst duration (10.7 ± 1 ms, n=6) was similar to the time constant of decay of the ATP synaptic current (13 ± 1 ms, n=4). We conclude these neurones possess at least two pharmacologically distinct ATP receptor ion channel complexes; apparently only one is activated by synaptically released ATP.

W-Pos443

SYNAPTIC EFFECTS OF 2,3-BUTANEDIONE MONOXIME (BDM) ANALOGUES AT THE RAT NEUROMUSCULAR JUNCTION. ((S.P. Aiken, D.L. Becker, L.C. Sellin* and J.J. McArdle)) Dept. of Pharmacology & Toxicology, N.J. Med. Sch.-UMDNJ, Newark, NJ and *Astra-Finland, PL 6, Masala, Finland. (Spon. by E. Stephenson)

We have previously shown that BDM increases quantal release at the neuromuscular junction, and prolongs the decay of endplate currents (EPCs) in a fashion suggesting asynchronous transmitter release. A series of five BDM analogues have been tested at the rat neuromuscular junction *in vitro* using a two electrode voltage-clamp technique. Three oxime compounds were found to have no obvious effects on transmission. One oxime, salicylaldoxime, enhanced quantal release in a similar manner to BDM, but was active at 10μM. This is roughly three orders of magnitude more potent than BDM. Prolongation of the EPC decay and "flattening" of the peak were seen with salicylaldoxime, at 100μM. However, another analogue, anti-(1S)-(-)-camphorquinone-3-oxime, inhibited release at 10μM to 1mM, and also reduced miniature endplate current amplitude, suggesting a post-synaptic effect. The decay time constant (τ) was markedly shortened at 1mM, and two-component exponentials were observed. It is suggested that investigation of BDM analogues may help elucidate the mechanism of neurotransmitter release, and may provide new targets for drug action. Supported by NS 31040.

W-Pos444

THE RATE CONSTANTS IN THE KINETIC MODEL: C_1-C_2-C_3-O_4 CAN BE SOLVED ANALYTICALLY AS FUNCTION OF TIME CONSTANTS AND THEIR FRACTIONS

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The rate constants, k_{ij}, except k_{43}, can be determined from the closed times (τ_i , i=1,2,3) and their fractions (f_i, i=1,2,3 and $f_1+f_2+f_3=1$) by solving the following equations (see equations (3.65)-(3.67), (3.70) and (3.75) in [1]):

(1) $k_{34}(\lambda_1-y)=f_1(\lambda_1-\lambda_2)(\lambda_1-\lambda_3)-\lambda_2\lambda_3=a_1$

(2) $k_{34}(\lambda_2-y)=f_2(\lambda_2-\lambda_1)(\lambda_2-\lambda_3)-\lambda_1\lambda_3=a_2$

(3) $k_{32}+y+k_{34}=\lambda_1+\lambda_2+\lambda_3=b$

(4) $zk_{34}=\lambda_1\lambda_2\lambda_3=d$

(5) $k_{32}w+z+yk_{34}=\lambda_1\lambda_2+\lambda_2\lambda_3+\lambda_3\lambda_1=c$,

where $w=k_{12}+k_{21}$, $y=k_{12}+k_{21}+k_{23}=w+k_{23}$, $z=k_{12}k_{23}$, and eigenvalues $\lambda_i=1/\tau_i$ (i=1,2,3). τ_i and f_i can be obtained by fitting the closed time histogram. This set of nonlinear equations has been solved numerically [2,3]. Here, we give the analytical solution for this problem. We first solved for k_{34} and y from (1) and (2), then solved for k_{32} and z from (3) and (4), finally solved for w from (5). The explicit expressions are: $k_{34}=f_1\lambda_1+f_2\lambda_2+f_3\lambda_3$, $y=\lambda_1-a_1/k_{34}=\lambda_2-a_2/k_{34}$, $k_{32}=b-(y+k_{34})$, $z=d/k_{34}$, and $w=(c-z-yk_{34})/k_{32}$. From the definitions of w, y and z, we have $k_{23}=y-w$, $k_{12}=z/k_{23}$, and $k_{21}=w-k_{12}$. This model has been used to describe kinetics of muscle AChR [1], stretch-activated channels [2], and voltage-gated K^+ channels [3]. Since $k_{12}=2k_1x_A$ and $k_{23}=k_2x_A$ for AChR [1], the rate constants $k_1$and k_2 can be extracted from k_{12} (or k_{23})-x_A plot (x_A is agonist concentration).

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W-Pos445

RELEASE PROBABILITY AT EXCITATORY SYNAPSES ON CULTURED HIPPOCAMPAL NEURONS (C.Rosenmund, J.D. Clements and G.L.Westbrook). Vollum Institute, Oregon Health Sciences University, Portland, OR 97201.

The probability of vesicular release (P_r) is a primary determinant of synaptic efficacy. Changes in P_r underlie, at least in part, synaptic plasticity at central excitatory synapses. Previously, P_r has been estimated using quantal analysis of fluctuating synaptic potentials. These estimates require knowledge of the number of release sites (N) and the mean quantal amplitude (Q). In the present study we describe an approach for estimating Pr that does not require knowledge of N or Q. Whole-cell recordings were made from single hippocampal neurons that formed autapic synapses in 'microdot' cultures. NMDA receptor mediated EPSCs were recorded under control conditions and in the presence of the irreversible open channel blocker MK-801. The time course of the synaptic current was faster in the presence of MK-801. The change in time course was used to determine the open probability (P_O) of the synaptically activated NMDA channels, based on a simple kinetic model. The average open probability of the NMDA channel at the synapse was ≈ 5%, consistent with whole-cell measurements of currents evoked by application of NMDA, but lower than in outside-out patches (Jahr, Science 255:470, 1992). Assuming that NMDA receptors are saturated (Clements et al., Science, 1992, in press), P_r can be determined by comparing the reduction of the EPSC amplitude predicted from the model with that measured during a series of EPSCs in MK-801. Preliminary results suggest that P_r is approximately 0.2 in the presence of 2 mM [Ca]o. This provides a tool for evaluating whether alterations in synaptic efficacy result from changes in P_O or P_r. Supported by USPHS grants NS26494 and MH46613.

W-Pos446

STIMULATION OF ADENOSINE 5' TRIPHOSPHATE (ATP) RECEPTORS INDUCE INTRACELLULAR CALCIUM ($[Ca^{2+}]_i$) CHANGES AND INWARD CURRENTS IN MOUSE LEYDIG CELLS.

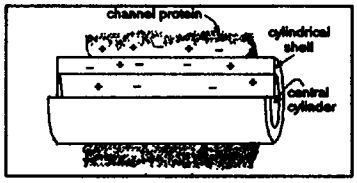
((A. Nadal, E. Fuentes, D. Spray*, M.V.L. Bennett* and M. Pérez-Armendariz*.)) Dept. of Neuroscience, Albert Einstein College of Medicine*, U.S.A. and Dept. of Physiology and Institute of Neuroscience, University of Alicante, Alicante, Spain. (Spon: Esperanza Recio).

External ATP, activates cationic currents and increases $[Ca^{2+}]_i$ in a number of cell types. Leydig cells were obtained from mouse testes and cultured as described (Kawa, J. Physiol 1985). $[Ca^{2+}]_i$ changes and membrane currents were recorded within 12-24 hrs after cell dispersion. $[Ca^{2+}]_i$ was recorded in Fura-2AM (5 uM) loaded Leydig cells with digital ratio imaging in 145 mM NaCl, 5 mM KCl and 1 mM $CaCl_2$, 10 mM Hepes, pH 7.4. Whole cell currents were recorded using patch pipettes, containing: 150 mM KCl, 0.5 mM EGTA, 10 mM Hepes, pH 7.16. In 85% (n=70) of the cells, ATP induced a biphasic response in $[Ca^{2+}]_i$: $[Ca^{2+}]_i$ rose to a peak within sec and decreased to a plateau level within 5 min. ATP applied in 1 mM EGTA depressed the plateau $[Ca^{2+}]_i$ response; thus ATP evoked Ca^{2+} release from intracellular stores as well as Ca^{2+} influx from extracellular medium. Peak $[Ca^{2+}]_i$ response increased in a dose dependent manner with ATP from 1 to 1000 uM (n=8). The increase in $[Ca^{2+}]_i$ desensitized on repetitive application of ATP (5 uM) at 2 min interval, but not at 5 min intervals (n=8). Microperfusion of ATP (0.5 mM) by pressure through a patch pipette, induced transient (40 sec) inward currents (of about 330 pA; n=7) in Leydig cells held at -40 mV. Repetitive stimulation with ATP decreased the current, this depression was reversible after 4-5 min rinse (n=5). The ATP induced transient $[Ca^{2+}]_i$ and inward currents in Leydig cells appear to be receptor mediated. Ionic dependence of inward currents, mechanisms of regulation $[Ca^{2+}]_i$ and effect of ATP on testosterone release are currently under study.

INTRACELLULAR CHANNELS

W-Pos447

A NOVEL DESCRIPTION OF ION FLOW THROUGH LARGE CHANNELS. ((E. Brygida Zambrowicz and Marco Colombini)) Dept. Zoology, Univ. Maryland, College Park, MD 20742. (Spon. Lynn Amende)



Ion flow through large channels is a complex process because both cations and anions can penetrate and multiple ions can be in the pore at the same time. A theory was developped in which the channel is divided into two compartments: a relatively-charged cylindrical shell of solution adjacent to the wall of the pore with thickness of 1 Debye length and a relatively neutral central cylinder of solution. The fixed-charge membrane theory of Teorell was used to describe the ionic concentrations and potential through the pore. According to the theory, the zero-current (reversal) potential should drive current flow in opposite directions in these two compartments resulting in unusual phenomena. The theory was tested with the mitochondrial channel, VDAC (isolated from *N. crassa*), reconstituted into planar phospholipid membranes. The variation of the observed reversal potential with transmembrane activity ratio, ionic strength, ion mobility ratio, and net charge on the wall of the pore are accounted for reasonably well by the theory. Classical Goldman-Hodgkin-Katz theory totally fails to account for the observations. (supported by ONR and NIH)

W-Pos448

CARDIAC MITOPLAST ANION CHANNELS WITH VOLTAGE-DEPENDENT SUBSTATE BEHAVIOUR. ((R.H. Ashley and K.A. Hayman)) Dept. of Biochemistry, University of Edinburgh, Scotland EH8 9XD, UK.

Mitoplast membrane vesicles isolated from sheep cardiac mitochondria were incorporated into voltage-clamped planar lipid bilayers by additions to the *cis* chamber. We observed several channels, including intermediate- and low-conductance anion channels. These were clearly distinct from the voltage-dependent anion channel (VDAC) of the outer mitochondrial membrane. One anion channel (g=100 pS in 300 mM KCl, 40 pS in 300 mM choline Cl, P_{Cl}:P_{X^+}=7:1, relative anion permeabilities $Cl^->Br^->I^->NO_3^->SCN^-$) had prominent substates at ~25% and ~50% of the fully-open level. It rectified strongly in charged bilayers at negative (*cis-trans*) potentials. This was removed (titrated away) by divalent cation screening of *cis*-facing negative lipid surface charge, indicating that one face of the channel lies within a few Å of the membrane surface. Channel activity and substate behaviour appeared to be unaffected by pH between 6.5 and 8.8, but the substates were voltage-dependent, with an abrupt change in the pattern of gating transitions around 0 mV. Channel gating could be well-described by a correlated binomial model with cooperative gating between 4 protomers in a multibarrelled pore.

Supported by the British Heart Foundation.

W-Pos449

THERMAL SENSITIVITY OF A PLANT VOLTAGE-GATED CHANNEL AND A LIGAND-GATED NEUROTRANSMITTER RECEPTOR CHANNEL. ((**Laura P. Zanello, E. Aztiria and F.J. Barrantes**)) Instituto de Investigaciones Bioquímicas, Arg. Scientific Res. Council and Universidad Nac. Sur, 8000 Bahía Blanca, Argentina.

The effect of temperature on the activity of a) the voltage-gated K^+ channel of cytoplasmic droplets from the algae *Chara;* b) the ACh-gated nicotinic receptor (AChR) channel from mouse embryonic muscle of BC3H-1 cells and c) the same mouse muscle AChR heterologously expressed in transfected CHO cells has been characterized by means of the patch-clamp technique. The activity of these channels was recorded in inside-out patches over a range of temperatures (3°C to 25°C for the plant cell ion channel, and 5°C to 40°C for the AChR). We found that the conductance of the channels increased as temperature augmented in all cases, with Q_{10} values of 1.2-1.4. Mean open durations of the channels diminished as the temperature was raised, yielding Q_{10} values of 1.9-2.0. A thermodynamic analysis using transition rate theory was carried out for the processes of ion conductance and kinetics of the channels. The values obtained for the activation enthalpy and entropy were compared to those reported for other types of channels from animal cells. The relative thermal insensitivity of the channel conductance suggests that ions traverse the pore by diffusion. The activation entropy found for the *Chara* K^+ channel closing rate α (-11.93 cal.mol^{-1}.°K^{-1}) would provide the driving force for stabilizing the closed channel configuration as the temperature increases.

W-Pos450

KINETICS OF ACTIVATION OF VOLTAGE DEPENDENT CHANNELS IN RADISH ROOT VACUOLES

((F. Gambale, A. M. Cantu', A. Carpaneto and B.U. Keller*)) Istituto Cibernetica e Biofisica, Genova, Italy and *Max-Planck Institut fur Biophysikalische Chemie, Gottingen, Germany (Spon. S. Firenstein)

Voltage dependent opening and closing (gating) of ion channels is a well studied phenomenon in a variety of animal and plant cells. However, it has been particularly difficult to monitor channel openings in animal cells as they are characterised by fast time constants and often masked by simultaneous channel inactivation. We studied by the patch-clamp technique the opening of voltage and calcium activated "slow vacuolar" channels (Hedrich and Neher, Nature, 329, 1987) in a new preparation from plant cells, the radish root vacuole. These channels are characterised by activation times in the order of hundreds of milliseconds. We have shown that they have also another kinetically distinct activation mode which is characterised by even slower activation times. Different membrane potential protocols allowed to switch between the fast and the slow mode in a controlled and reversible manner. This process may represent a modulator of voltage dependent ion channels in other plant and animal systems.

W-Pos451

PROPERTIES OF ION CHANNELS IN VACUOLES FROM SUGAR BEET

((F. Stragapede* and F. Gambale)) Istituto di Cibernetica e Biofisica, Genova, Italy *Eridania ZN fellowship, Genova,Italy. (Spon. C. Marchetti)

The slow activating (SV) channel present on the vacuolar membrane of sugar beet main root has been studied by the patch-clamp technique. In the sugar beet tonoplast a large component of the ionic current is mediated by a slow activating rectifying channel which opens only at hyperpolarizing voltages and micromolar cytoplasmic calcium concentrations (Hedrich and Neher, Nature, 329, 1987). The single channel current has a reversal potential of about +17 mV in 200 mM cytoplamic KCl and 100 mM internal KCl. Thus, in our experimental conditions, the channel is cation selective. This is also confirmed by substituting the external potassium with a larger cation, e.g. TMA (tetramethylammonium); a decrease of the single channel conductance is observed. The single channel conductance shows a linear relationships as a function of the transmembrane voltage in symmetric potassium concentrations ranging from 50 to 500 mM. Experiments are in progress to design a model which would describe these and other properties of the SV type channel.

W-Pos452

STUDY OF CHLORIDE CHANNELS OF LIVER ENDOPLASMIC RETICULUM (ER) MEMBRANE. ((Ashutosh Tripathy and H. Ti Tien)) Dept. of Physiology, Michigan State University, East Lansing, MI 48824. (Spon. by Dr. Ronald A. Meyer).

Rat liver rough ER vesicles were fused to a planar bilayer membrane (BLM) with an aim to reveal the chloride channels present in the ER membrane. BLMs were made in symmetrical low ionic strength solution of 10mM Tris-HCl (pH -7.4) and fusion was achieved by adding potassium chloride to the cis side as osmoticant. Macroscopic current- voltage measurements of a reconstituted BLM showed that the current is carried by chloride ions. About 50% of the chloride current can be blocked by DIDS and the rest by Zn. The permeability ratio of chloride to potassium is about 12 and measurement under near bi-ionic conditions displays the following sequence of anion permeability: $SCN^- = I^- > Br^- > Cl^- >> $ gluconate. At the microscopic level, a single chloride channel most frequently seen, has the conductance of 550pS in 363 mM symmetrical KCl solution. The conductance saturates with increasing Cl^- concentration in a Michaelis-Menten fashion with a maximum conductance of 946pS and a K_m of 260mM. The channel has many sub-conductance states. The gating of the channel is weakly voltage-dependent and at higher positive or negative voltages the channel prefers the low sub-conductance states.

W-Pos453

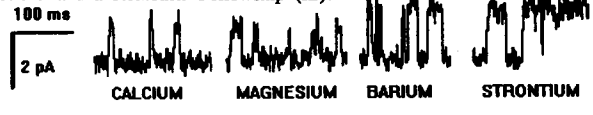
SPATIO-TEMPORAL CHARACTERISTICS OF AGONIST-INDUCED CALCIUM WAVES IN ASTROGLIA. S. Yagodin, L.A. Holtzclaw, and J. T. Russell LCMN, NICHD, and LN, NINDS.

Neurotransmitters such as norepinephrine and glutamate induce intracellular calcium signals primarily by release of cellular stores in rat cortical astrocytes. This calcium release occurs by ligand-receptor interactions resulting in IP_3 formation. We have studied these agonist-induced calcium signals using video imaging techniques with high temporal (0.25s) and spatial (1 - 5 μm) resolutions. Calcium signals were found to propagate as waves within individual astrocytes at a rate of 15 to 25 μm/s. The waves consistently originated from a single locus within any astrocyte, and this locus remained invariant for subsequent challenges with the same and other agonists that are coupled to IP_3-generation. From the locus of origin, calcium waves propagated by a process of diffusion and were regeneratively amplified at one or more predestined cellular loci. The primary locus and the secondary loci possessed independent oscillatory properties. The specific cellular location and the independent properties of the cellular loci enabled the cell to respond in identical spatio-temporal patterns for repeated stimulations. After an initial wave has passed through the cell, other waves could originate at secondary loci and propagate in opposite directions and collisions of waves resulted in annihilations. It is hypothesized that multiple sites exist within astroglia with different thresholds of activation for calcium release in a hierarchical manner, such that the low threshold centers initiate waves, and the higher threshold centers provide for regenerative amplification. In this way, since diffusion of IP_3 in the cytoplasm is rapid, wave propagation could proceed with IP_3 channel openings alone.

W-Pos454

DIVALENT CATION CONDUCTION OF THE INOSITOL 1,4,5-TRISPHOSPHATE GATED CALCIUM CHANNELS OF CANINE CEREBELLUM. ((Ilya Bezprozvanny and Barbara E. Ehrlich)) Depts. of Medicine and Physiology, Univ. of Connecticut, Farmington, CT 06030.

The conduction of alkaline earth divalent cations through inositol 1,4,5-trisphosphate ($InsP_3$)-gated calcium channels of canine cerebellar endoplasmic reticulum was determined after reconstitution into planar lipid bilayers. Channel currents were recorded after addition of 2 μM $InsP_3$ and 0.5 mM ATP to the cytoplasmic side; the intraluminal chamber contained 55 mM calcium (Ca), magnesium (Mg), barium (Ba) or strontium (Sr) dissolved in HEPES. The single channel conductances of the main conductance state of the channel follow the sequence Ba (93 pS) > Sr (72pS) > Ca (60pS) > Mg (32pS). The same order of permeabilities was previously reported for the cardiac ryanodine receptor (Tinker and Williams, J. Gen. Physiol., 1992, 100:495). Interestingly, the mean open time of the channel varied depending on the ionic species: Ca (2.2 ms) < Mg (4.5 ms) ≤ Ba (5.5 ms) < Sr (12 ms); this result could be explained if increased divalent cation concentration in the vicinity of the channel pore on the cytoplasmic side rapidly inhibits the $InsP_3$-gated channels. According to this model, differences in open times reflect binding selectivity among the divalent cations to an inhibitory site. HL33026, GM39029 and a Steinbach Fellowship (IB).



W-Pos455

PHARMACOLOGICAL CHARACTERIZATION OF CA^{2+}-REGULATED IP_3-DEPENDENT CA^{2+} RELEASE. ((E. A. Finch[1,3] and S. M. Goldin[2,3],)) [1]Program in Neuroscience and [2]Biol. Chem. Dept., Harvard Med. Sch., Boston, MA; [3]Cambridge NeuroScience, Inc., Cambridge, MA.

We have previously reported that extravesiclular Ca^{2+} acts as a coagonist with IP_3 to rapidly potentiate and more slowly inactivate IP_3-induced $^{45}Ca^{2+}$ release from brain microsomal vesicles (Finch, et al., Science 252:443, 1991) and that ATP further potentiates this Ca^{2+} release. These findings suggest funtional homology with the stucturally homologous ryanodine receptor. Thus, clarification of the specificity of pharmacological agents which act on release channels and Ca^{2+} stores would be useful in interpreting their effects on Ca^{2+} homeostasis.

Thapsigargin, which inhibits Ca^{2+}-ATPases of ER and SR, inhibited most of the ATP-dependent $^{45}Ca^{2+}$ uptake. In vesicles preincubated with 30nM thapsigargin prior to $^{45}Ca^{2+}$ loading, 50% of $^{45}Ca^{2+}$ uptake was inhibited whereas the majority of IP_3-mediated $^{45}Ca^{2+}$ release was inhibited. In contrast, the $^{45}Ca^{2+}$ taken up by vesicles loaded under control conditions for 1 min., which loaded vesicles to the same extent as thapsigargin pretreatment, was as releasable by IP_3 as the under control conditions. After thapsigargin preincubation, the residual IP_3-mediated $^{45}Ca^{2+}$ release was modulated by extravesicular Ca^{2+} in its magnitude and time course in a manner similar to release under control conditions. Caffeine directly inhibited IP_3-mediated $^{45}Ca^{2+}$ release in our vesicle preparation.

These data demonstrate that the Ca^{2+}regulated IP_3-dependent Ca^{2+} release activity from brain microsomal vesicles, which we have functionally characterized in vitro, exhibits pharmacological characteristics which have been demonstrated in vivo, and suggests this system should prove useful in further pharmacological characterization of Ca^{2+} release.

W-Pos456

BIOCHEMICAL CHARACTERIZATION OF TWO DISTINCT ISOFORMS OF RAT TYPE 1 RECEPTORS FOR INSOITOL-1,4,5-TRISPHOSPHATE STABLY EXPRESSED IN HUMAN EMBRYONIC KIDNEY CELLS. ((D.M. Lin and W.S. Agnew)) Interdepartmental Neuroscience Program and Dept. of Cell. & Mol. Physiology, Yale University School of Medicine, New Haven, CT 06510

Inositol-1,4,5-trisphosphate (IP_3) receptors serve as a major gateway for intracellular calcium release in many different cells in response to extracellular stimulation by growth factors, hormones and neurotransmitters. Recently several labs have reported the existence of multiple genes for IP_3 receptors, each varying in tissue distribution. The Type 1 receptor was first cloned from mouse cerebellum. The gene itself is subject to alternative-splicing to yield up to eight different isoforms, suggesting that biochemical properties of Type 1 proteins may be diverse. Biochemical studies performed in our lab on receptors in solubilized rat cerebellar membranes have revealed a mixed population of (separable) IP_3 binding sites, including one population with high affinity IP_3 binding ($K_{d,H}$ = 4 nM, B_{max} = 8-12 pmol/mg) and one of low affinity ($K_{d,L}$= 120 nM, B_{max} = 20-30 pmol/mg protein), exhibiting different responses to temperature and changes in free calcium levels. As a first step in examining the functional complexity of Type 1 receptors, we report the functional expression of two Type 1 isoforms in stably transfected cell culture lines and their preliminary biochemical characterization. The two isoforms correspond to alternatively spliced variants of Type 1 differing in the presence or absence of a stretch of 15 amino acids near the N-terminus of the protein subunit. This 15 aa insert has a sequence motif resembling part of an EF hand, but whose functional role is unknown. Immunochemical assays detected low levels of endogenous receptor in untransfected cells which were undetectable in direct [^{3}H]-IP_3 binding assays. However, with stably transfected cell lines produced from both constructs, large homogenous populations of receptor were detected. Preliminary data indicate that both constructs possess high affinity binding sites (K_d = 5-15 nM) and specific activities (B_{max} = 20-40 pmol/mg protein) exceeding those in the richest known tissue source, cerebellum (B_{max} = 8-12 pmol/mg protein); this level of expression is sufficient for simple biochemical purification protocols. The expression of single, homogeneous preparations of identified Type 1 receptor isoforms will facilitate biochemical and biophysical structure-function studies on the effects of ligand binding, elevated calcium, modulation by small molecules, by second messenger dependent phosphorylation, and by interactions with other protein components of the cell.

W-Pos457

MOLECULAR CLONING AND CHARACTERIZATION OF THE INOSITOL 1,4,5-TRISPHOSPHATE RECEPTOR FROM HUMAN T CELLS

((T. Jayaraman, Loewe Go, and Andrew R. Marks)) Molecular Medicine Program, Dept. of Medicine, Mount Sinai School of Medicine, New York, NY 10029

Inositol 1,4,5-trisphosphate (IP_3) is a second messenger involved in signal transduction during T cell activation. IP_3 is generated during activation of the human T cell line Jurkat. T cell activation by phorbol myristate acetate (PMA) and CD3 is associated with a rapid rise in cytoplasmic calcium. We have previously cloned a vascular smooth muscle IP3 receptor. The IP_3 receptor in smooth muscle is an intracellular calcium release channel on the endoplasmic reticulum. We used rat smooth muscle IP_3 receptor cDNA clones to screen a PMA-stimulated Jurkat cDNA library. Multiple overlapping cDNAs were isolated. Sequencing revealed substantial homology to the smooth muscle and brain forms of the IP_3 receptor. A 10 kb mRNA was identified in Jurkats. An antibody directed against a synthetic peptide based on the deduced amino acid sequence of the IP_3 receptor recognized a ~300 MW protein in Jurkats on immunoblots. IP_3 receptor mRNA was induced during PMA activation of Jurkats within 2 hours. Cloning of the human T cell IP_3 receptor provides the basis for further elucidation of its role in modulating T cell activation. Supported in part by NIH grant NS29814 to A.R.M.

W-Pos458

PROPERTIES OF INTRACELLULAR CALCIUM RELEASE IN RAT CEREBELLAR PURKINJE NEURONES BY $InsP_3$.

Kamran Khodakhah and David Ogden. ***National Institute for Medical Research, Mill Hill, London NW7 1AA, U.K.***

Cerebellar Purkinje neurones contain a very high density of $InsP_3$ receptors when compared to peripheral tissues. The functional characteristics of $InsP_3$ evoked Ca release were studied in single Purkinje cell soma in slices, with flash photolysis of caged $InsP_3$ and microspectrofluorimetry of Fluo-3 or Furaptra. Marked differences were found from Ca release in liver and astrocytes. The concentrations of $InsP_3$ estimated for minimal Ca release are more than ten fold higher, 9 μM in PCs and 0.2-0.5 μM in liver and cerebellar astrocytes. The peak cytosolic [Ca] is estimated to be more than tenfold higher at high $InsP_3$, and the [Ca] rises more quickly and with shorter latency in PCs. Evidence was found of Ca induced Ca release at high $InsP_3$ concentrations in PCs. Comparison of data obtained with Fluo-3 (200-1200 μM, K=0.5μM, Ca buffered >200:1) and Furaptra (500μM, K=44μM, Ca buffered approx 10:1) suggest that neither exogenous nor endogenous Ca buffers can account for the difference between PCs and liver cells in $InsP_3$ concentrations needed to release Ca. The speed of response to $InsP_3$ when compared to peripheral tissues may represent a physiological adaptation in Purkinje cells.

W-Pos459

Ca^{2+} BINDING AND Ca^{2+} REGULATORY SITES IN THE RYANODINE RECEPTOR. **((S. R. Wayne Chen, Lin Zhang and David H. MacLennan)) Banting and Best Department of Medical Research, University of Toronto, Toronto, Canada M5G 1L6.**

In order to determine potential Ca^{2+} binding and Ca^{2+} regulatory sites in the ryanodine receptor, we have measured $^{45}Ca^{2+}$ and ruthenium red binding in bacterially expressed fusion proteins, representing about 90% of the molecule. Ca^{2+} binding was mapped to three sequences, 22($13b_1$), 36($13c_1$) and 35($13c_2$) amino acids long, each encompassing a predicted Ca^{2+} binding site. Polyclonal antibodies were raised against these short sequences and purified on antigen affinity columns. Anti-$13c_2$ antibody, against amino acid residues 4478-4512, reacted specifically with ryanodine receptors in sarcoplasmic reticulum vesicles, showing cytoplasmic surface exposure of the epitopes. The antibody (*cis*) increased the Ca^{2+} sensitivity for activation of single channels incorporated into planar lipid bilayers. The antibody bound channel was still regulated by ATP, Mg^{2+}, ryanodine or ruthenium red. A minor subset of the anti-$13c_2$ serum was isolated on a peptide column of the sequence PEPEPEPEPE, corresponding to part of the $13c_2$ sequence. This antibody(*cis*) specifically inhibited Ca^{2+} and caffeine activation of single channels. ATP activation was retained and was further modulated by Mg^{2+}, ryanodine and ruthenium red. These results suggest that the $13c_2$ sequence is involved in the formation of the Ca^{2+} activation site or in the Ca^{2+} activation pathway.

W-Pos460

EXPRESSION OF RYANODINE RECEPTORS IN NON-EXCITABLE TISSUES.

((Richard E.A. Turwell and F. Anthony Lai))
MRC National Institute for Medical Research, Mill Hill, London, UK.

Calcium release from intracellular stores in response to hormones and neurotransmitters is important for the regulation of a wide variety of cell-specific responses. The control of calcium release from these stores has been shown to be mediated by two classes of intracellular calcium channels which may coexist or be segregated into distinct stores. One class, the inositol 1,4,5-trisphosphate (IP_3)-gated calcium channels, has been shown to be widely distributed, whereas the ryanodine-sensitive class of channel has only been demonstrated in cardiac, skeletal and recently brain tissues. Although there is little direct evidence to confirm that ryanodine-sensitive channels are ubiquitous, electrophysiological and calcium flux studies on IP_3-insensitive stores suggest that these receptors are also widely expressed. We have used a variety of molecular biology techniques including the polymerase chain reaction (PCR) with isoform-specific oligonucleotide primers to investigate the expression of these ryanodine-sensitive channels. PCR using cardiac-specific primers on reverse transcribed mRNAs has yielded a nucleotide sequence common to many tissues whereas skeletal-specific primers only gave products for mRNAs isolated from skeletal muscle. This data has enabled the development of isoform-specific antibody probes to investigate the expression of protein in non-excitable tissues.

W-Pos461

ω-CONOTOXIN SENSITIVITY AND VOLTAGE-DEPENDENCE IN A BRAIN MICROSOMAL CALCIUM CHANNEL. ((C. Martin and R.H. Ashley)) Dept. of Biochemistry, University of Edinburgh, Scotland EH8 9XD, UK.

High-conductance calcium channels (g=100 pS in 50 mM $CaCl_2$, 500 pS in 50 mM CsCl) were incorporated into voltage-clamped planar lipid bilayers from rat brain microsomal membrane vesicles. Channel activity was unaffected by ryanodine or $InsP_3$ but was markedly voltage-dependent. Activation (apparant gating charge -3) was half-maximal at -50 mV (*cis-trans* potential) and appeared to follow a bell-shaped curve centered on 0 mV (where Po = 1.0). Rapid inactivation limited observations at positive holding potentials. Lifetime analysis at -60 mV in CsCl revealed a minimal C-C-O gating scheme with time constants (in ms) of 37±17, 0.46±0.12 and 5.7±4.7 respectively (all means ± SD, n=7). The 4 rate constants were subsequently determined by burst and Q matrix analysis. 5-40 μM ω-conotoxin GVIA, known to inhibit neuronal plasma membrane Ca^{2+} channels, blocked the open channel on an intermediate time-scale, with an on-rate of $7.1x10^7$ $M^{-1}.s^{-1}$ (measured at -40 mV). Although this microsomal Ca^{2+} channel is voltage-dependent and ω-conotoxin-sensitive, it appears to be distinct from known surface-membrane Ca^{2+} channels, but similar to voltage-dependent Ca^{2+} channels found by patch-clamping pancreatic ER (Schmid *et al.*, 1990, Nature **346**, 374-376).

Supported by the Wellcome Trust.

W-Pos462

ANALYSIS OF MYOMETRIAL CONNEXIN 43 TRANSCRIPTS DURING PREGNANCY. ((M. Ali, M. Byam-Smith, T. Tabb, E.A. Thompson and R.E. Garfield)) The University of Texas Medical Branch, 301 University Boulevard, Galveston, Texas 77555.

Previous studies have shown that myometrial gap junctions (GJ) and their proteins (connexin 43, Cx43) increase at the end of pregnancy and provide the basis for synchronous contractility. It has been proposed that the rise in GJ-Cx43 is regulated by genomic mechanisms. However, there are contradictory studies which describe the corresponding increase in mRNA Cx43 levels during gestation, possibly because the transcripts were normalized to products which may also change during pregnancy. In this study we analyzed the expression of rat uterine connexin 43 transcripts throughout gestation employing polymerase chain reaction (PCR). In order to overcome prior problems we developed an internal standard. For this study we used the following Cx43 primers:

5' ACAGCTGTTGAGTCAGCTTG 3'
5' ACGAATATGATCTGAAGGAC 3'

We also designed another set of primers which contain a specified sequence of plasmid PBS flanked by the Cx43 primers (above). We used these primers to amplify a piece of PBS^+ plasmid. This PCR product was cloned into the PCR II vector (InvitroGen) which was amplified and subsequently transcribed *In vitro* to RNA. This RNA, added as an internal standard in every PCR tube with myometrial RNA, was reversed transcribed and PCR performed on the cDNA using the Cx43 primers. The larger fragment, representing the internal standard, was used to normalize the PCR fragment from the mRNA of myometrial cells at various times of gestation by densitometry. The results confirm that mRNA abundance of Cx43 rises at term and support the concept of genetic regulation of myometrial gap junction formation at the end of pregnancy. The methods used in this study will be useful to evaluate the abundance of other transcripts.

W-Pos463

Voltage Clamp of the Nuclear Envelope. L.J. DeFelice, B. Dale, K. Kyozuka, L. Santella, E. Tosti. Stazione Zoologica, 80121 Naples, Italy, and Anatomy and Cell Biology, Emory University, Atlanta, GA 30322

We have used the whole-cell recording technique to voltage clamp the nucleus. After dissecting nuclei from starfish oocytes in the germinal-vesicle stage, we recorded currents through the nuclear envelope in four electrode/bath solutions: C/C, C/S, S/C, S/S, where C = 200 K2SO4, 20 NaCl, 200 sucrose, 10 EGTA, pH 7.4, and S = 10 KCl, 500 NaCl, 10 CaCl2, pH 8.2; in mM, at room temperature. All conditions but S/S show time-variant currents after applying 500 msec voltage steps from a holding potential of 0 mV to test potentials between +/-100 mV. With C inside the whole-cell electrode (inside the nucleus), the steady-state resistance of the nuclear envelope had a value close to 100 megohm (roughly 50,000 kilohms x cm2). With S inside the nucleus, the resistance drops to about 35 megohm. *In situ*, the nuclear envelope has a rest potential between 4 and 30 mV, positive with respect to the cytoplasm. Electron micrographs of dissected oocytes show nuclear pores of outer diameter about 1 um and density between 65-110 per um2. Each pore has the *8 + 1* structure. Annulate lamellae do not fold over the nuclear membrane following dissection. Prior to rupturing the patch, we observe channels like those seen in mouse germinal-vesicle stage nuclei (Mazzanti et al., J. Memb. Biol. 121:189, 1991). The largest sub-conductance state has a value near 200 pS, and we have postulated previously that the nuclear pores themselves act as gated channels. Taken together, these data suggest that the nucleus establishes a distinct electrical compartment within the cell capable of segregating charged molecules. NATO grant CRG 910025 to BD and LJD supports this work.

ION-MOTIVE ATPASES

W-Pos464

THE ROLE OF THE TRANSMEMBRANE DOMAIN OF THE β SUBUNIT OF H,K ATPASE IN ASSEMBLY WITH THE α SUBUNIT OF NA,K ATPASE. ((K. A. Eakle and R. A. Farley)) Dept. of Physiology and Biophysics, USC School of Medicine, Los Angeles, CA 90033. (Sponsored by D. Marsh)

Heterologous expression in yeast of the β subunit of H,K ATPase (HKβ) with α subunits of Na,K ATPase (NKα) leads to the formation of ouabain binding complexes implying assembly of the two subunits into active sodium pumps. (K.E. et al, PNAS 89:2834 1992) Ouabain binding by these complexes requires phosphoenzyme formation, either by ATP in a Na^+ dependent fashion, or Mg^{++} and P_i in a reaction which can be inhibited by K^+. Structural differences in the HKβ subunit have little effect on the affinity of complexes for ouabain, as compared to complexes with the Na,K ATPase β subunit (NKβ). However, NKα/HKβ complexes require higher concentrations of K^+ to inhibit ouabain binding by Mg^{++} and P_i, suggesting that the structure of K^+ binding sites has been affected. NKα/HKβ complexes are also significantly less stable when membranes are extracted by SDS. We have created hybrid cDNAs which swap the transmembrane domains between NKβ and HKβ. Expression of these constructs in yeast will allow us to look at the role of the transmembrane domain in the stability of αβ complexes. Functional differences in the interactions with different cations are also currently under investigation. Supported by AHA-GLAA Initial Investigator Award 983 F1-1 to K.A.E. and US Public Health Service Grants GM2873 and HL39295 and NSF Grant DMB-8919336 to R.A.F.

W-Pos465

EXPRESSION OF THE ATP-BINDING DOMAIN OF NA,K-ATPASE IN BACTERIAL CELLS.
((R.A. Farley, B.S. Gordon, E.E. Huston, K. Wang, B.D. Caldwell, and C.M. Grisham)) University of Southern California, Los Angeles, CA 90033 and University of Virginia, Charlottesville, VA 22901.

Amino acids between M344 and E779 of Na,K-ATPase were expressed in bacterial cells, either as an isolated domain (DP47) or as a fusion protein with glutathione-S-transferase (DP47f). After partial purification on a column of QAE-Sepharose, approximately 50% of DP47 was retained on a column of Blue Sepharose. The DP47 was eluted from the Blue Sepharose by ATP, ITP, and UTP, with decreasing efficiency, or with high salt concentrations. The DP47 that was eluted from the column could be re-bound and eluted again with the nucleotides or salt. The DP47 that did not initially bind to the Blue Sepharose did not bind to the resin under any conditions tested. The DP47f fusion protein was purified by affinity chromatography. The binding of CrATP to DP47f and to purified glutathione-S-transferase was measured by paramagnetic enhancement of water proton relaxation rates. CrATP binding to DP47f, but not to glutathione-S-transferase, was observed, indicating that the nucleotide binds to the DP47 region of the fusion protein. These data indicate that the ATP binding site of Na,K-ATPase is located between M344 and E779. (Supported by NIH GM28673).

W-Pos466

SITE-DIRECTED MUTAGENESIS OF AMINO ACIDS LOCATED WITHIN THE ATP BINDING DOMAIN OF NA,K-ATPASE.

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Amino acids predicted to be in the ATP binding site of Na,K-ATPase were changed by site-directed mutagenesis. Wild type and mutant molecules were expressed in yeast cells, and reactions of the pump were compared in yeast membrane fractions. Apparent affinities for ATP and phosphate, and steady-state phosphoenzyme abundance, were estimated from the ligand-dependent binding of ^{3}H-ouabain. The Kd of ouabain binding to mutant or wild type pumps was the same (8-15 nM), indicating that the mutations did not introduce large-scale perturbations of protein structure. Mutations could be divided into four groups: those that had little effect on enzymatic activity (K480R, K480A, P588A, P587A); those that reduced the affinity of the pump for ATP (K480E, K501E); those that affected the formation or breakdown of E_2P (D586N, D586E); and those that interfere with formation of E_1P from ATP (K501R). The apparent affinity of wild type Na,K-ATPase for ATP (0.13 μM) was reduced in the K501E mutant (1.4 μM) and in the K480E mutant (17.8 μM). In contrast to the K501E mutant, which could be phosphorylated by either ATP or Pi, the K501R mutant did not form a phosphoenzyme from ATP.

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W-Pos467

Na⁺/K⁺-ATPase SUBUNIT EXPRESSION AND LOCALIZATION IN CELLS IN CULTURE: REGULATION BY INSULIN.

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Rat skeletal muscle expresses Na^+/K^+-pump α1, α2, β1 and β2 subunits: α1 exclusively in plasma membranes (PM), all other subunits in PM and intracellular membranes (IM). Insulin rapidly causes translocation of α2 and β1 subunits from IM to PM (JBC 267:5040,1992). Studies on the regulation of the Na^+/K^+-pump would benefit from cell cultures where the acute and chronic effects of insulin could be tested during cell differentiation, independently of variables coexisting in animal studies. Expression and subcellular localization of ATPase isoforms were analyzed in membranes from L6 and C2C12 muscle cells, and 3T3-L1 and rat adipocytes, using isoform-specific antibodies on Western blots. L6 myoblasts and myotubes did not express α2 or β2 ATPase isoforms; α1 content increased modestly and β1 dramatically during L6 myogenesis, both largely in PM. C2C12 cells expressed α1, α2, and β1 subunits in PM, α2 only after cell fusion. At this stage cells responded to insulin with activation of ouabain-sensitive $^{86}Rb^+$ uptake but without subunit translocation. This involved only the low [ouabain] inhibitable component, presumably α2. In 3T3-L1 adipocytes mRNA transcripts of α1, α2 and β1 (but not β2) were detected by Northern blots. The α1, α2 and β1 proteins were found in PM, but α2 and β1 were also present in IM. 3T3-L1 adipocytes responded to insulin with rapid translocation of α2 subunits from the IM to PM. In contrast, rat adipocytes expressed all four subunits of the Na^+/K^+-pump mostly in the PM, and no change in their distribution was seen in response to insulin. Thus, 3T3-L1 adipocytes are a unique cellular system to investigate the regulation and subcellular distribution of Na^+/K^+-pump subunits during insulin treatment and cell differentiation. ***Supported by the Medical Research Council of Canada.***

W-Pos468

AN EXTERNAL ACCESS CHANNEL DETERMINES THE VOLTAGE SENSITIVITY OF BOTH THE Na/Na EXCHANGING AND BACKWARD-RUNNING MODES OF THE SODIUM PUMP.

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The Na/K pump in the absence of K_o sustains ATP- and ADP-requiring electroneutral Na/Na exchange which is voltage sensitive, being enhanced to saturation by hyperpolarization (Biophys. J. 59:341a; 1991). Kinetic analysis of the V_m and $[Na]_o$ dependence of this transport mode shows (J. Gen. Physiol. 100:69a; 1992) that the sole V_m-sensitive step in the process is binding, but not release, of Na_o -- compelling evidence for an external binding locus inside a high-field access channel. The electrogenic backward-running pump is similarly enhanced by Na_o or hyperpolarization (Biophys. J. 53:223a; 1988). If Na_o stimulates both modes at the same locus, they should share a kinetic equivalence between $[Na]_o$ and V_m, i.e. the apparent access channel depths should be identical. We verified this prediction on internally dialyzed, voltage-clamped squid giant axons; Na/Na exchange was measured as dihydrodigitoxigenin (H_2DTG)-sensitive ^{22}Na efflux, and reverse pumping as H_2DTG-sensitive inward current. The kinetics of both modes are very well fit by $v = V_{max}/\{1 + (K_{0.5}^{\delta} \exp(\delta V_m F/RT)/[Na]_o)^n\}$, a Hill equation (coefficient $n \simeq 1.5$) whose apparent $K_{0.5}$ depends exponentially on V_m with a steepness governed by the apparent access channel depth δ. We conclude that the Na_o release/rebinding site is at the bottom of a deep, narrow channel about % across the membrane field from the external boundary.

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W-Pos469

Ca/CALMODULIN KINASE II (CaMKII) INHIBITS AND PHOSPHORYLATES THE Na,K-ATPase.

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Mouse brain CaMKII expressed in baculovirus phosphorylated the α subunit of the purified Na,K-ATPase from dog kidney in the presence of Ca and calmodulin (CaM). CaMKII had no effect without CaM and Ca, and neither Ca nor CaM had any effect in the absence of CaMKII. Under the conditions in which phosphorylation was observed, CaMKII inhibited the activity of the Na,K-ATPase. Activity was tested by preincubating the Na,K-ATPase (5 min) ± 100 nM CaMKII plus 0.4 mM ATP, 1 μM CaM, 0.5 mM Ca, 10 mM Mg, 50 mM Hepes, and 17 μg/ml Na,K-ATPase. Samples were diluted 10 fold and the activity measured in 55 mM NaCl, 30 mM KCl, 1 mM ATP, 5 mM EDTA, 8 μM free Ca, and 870 μM free Mg. The Na,K-ATPase with CaMKII was inhibited 33% compared to the same conditions without CaMKII. CaMKII had no effect on the Na,K-ATPase in the absence of Ca and CaM. This is the first report that a kinase both phosphorylates the Na,K-ATPase and alters its activity. These studies were undertaken based on our observation that KN-62, a specific inhibitor of CaMKII, blocks CaM inhibition of the Na,K-ATPase in human red blood cells. Thus, the effects of recombinant CaMKII on the purified Na,K-ATPase are consistent with the results obtained in a cellular membrane. We suggest that CaM and CaMKII could be part of a previously undescribed mechanism to regulate the short term activity of the Na,K-ATPase. (NSF grant DCB-8817269; CaMKII was a gift from T. Soderling & D. Brickey of Oregon Health Sciences U. and the Na,K-ATPase was kindly provided by J. Kaplan & J. Arguello of the U. of Penn.)

W-Pos470

MEMBRANE TOPOLOGY OF THE α SUBUNIT OF NA,K-ATPASE.

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Transmembrane folding of the α subunit of Na,K-ATPase was probed by immunoelectron microscopy to determine whether monoclonal antibodies with defined epitopes bind to extracellular or cytoplasmic surfaces. Purified, membrane-associated Na,K-ATPase was double-labelled employing protein A-gold particles of two different sizes as markers. Antibody M10-P5-C11 (WJ Ball) and wheatgerm agglutinin were used as controls for cytoplasmic and exoplasmic surfaces, respectively. Three antibodies, VG4, VG2 and IIC9, unambiguously bound to the extracellular surface. Previously, IIC9 had been assigned to the cytoplasmic surface based on immuno-fluorescent staining of intact and detergent-permeabilized cells. Therefore, a third assay for sidedness, competition binding in solution to right-side-out vesicles, was used. IIC9 bound to untreated vesicles, although binding was affected by experimental conditions. An extracellular disposition for all three antibodies is not compatible with existing folding models. The implications for Na,K-ATPase topology will be illustrated.

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W-Pos471

PROTEOLYTIC DIGESTION AND TOPOLOGY ANALYSIS OF THE Na,K-ATPase

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Purified canine renal Na,K-ATPase was exhaustively digested with trypsin in the presence of ligands which are known to stabilize different enzyme conformations. Three types of electrophoretic patterns were observed: (1) In the presence of ouabain and Mg, the so-called 19 kD membranes are produced. These have previously been obtained in the presence of Rb or other occluded cations and contain a minimal ion-occluding unit. Evidently, this structure is not only stabilized by monovalent cation occlusion. Removal of ouabain from the proteolysis results in the degradation pattern (2) below. (2) In the presence of Mg and Pi or Na, Mg and ATP, characteristic fragments between 6-14 kD only are obtained. Under these conditions, the β-subunit is completely cleaved into two fragments. (3) In the presence of ADP a major fragment of 32 kD is obtained with N-terminal sequence begins at Q^{701} GAIVA. The results of these and parallel studies performed in sealed right-side-out microsomal vesicles will be discussed where conclusions can be drawn about the rearrangement of the α-subunit intramembrane domain during enzyme functioning. [Supported by GM 39500]

W-Pos472

CURRENT-VOLTAGE RELATIONSHIPS OF THE Na/K PUMP IN *XENOPUS* OOCYTES ANALYZED USING AN ACCESS CHANNEL MODEL. ((A. Sagar, V. Wallner and R. F. Rakowski)) Univ. of Health Sciences/Chicago Med. Sch., N. Chicago, IL 60064.

The effect of extracellular [Na] on the voltage dependence of Na/K pump current was studied in *Xenopus* oocytes using a two microelectrode voltage clamp. All solutions contain (in mM) 20 tetraethylammonium Cl, 5 $BaCl_2$, 2 $NiCl_2$, 5 Tris/Hepes, and 10 KCl (pH =7.6, temp.=22 °C). [Na] was varied between 120 and 7.5 mM (tetra- methylammonium Cl substitution for NaCl). Current voltage relationships were measured in Na-loaded oocytes over the range -160 to 0 mV with a down-up-down voltage staircase from a holding potential of -40 mV. Pump current was measured by subtraction of the current in K-free solution from that in 10 mM K. The normalized pump current is given by the equation: $i/i_{max} = 1/\{1+ ([Na]/K_i(0))^n \exp(-n\delta\Psi)\}$, where n is the Hill coefficient for Na, $K_i(0)$ is the apparent inhibitory constant for Na at 0 mV, δ is the fractional depth of an external access channel and $\Psi = FV/RT$. The above equation was fitted to the current-voltage data and gave: $n = 1.06 \pm 0.04$, $\delta = 0.75 \pm 0.02$ and $K_i(0) = 3260 \pm 670$ mM. $K_i(V)$ is given by $K_i(V)^n = K_i(0)^n \exp(n\delta\Psi)$. K_i calculated at -120 mV=93 mM in close agreement with 90 mM reported by Nakao & Gadsby (*J.Gen.Physiol.* 94:539,1989). Supported by NIH Grant NS-22979.

W-Pos473

EQUALITY OF ON AND OFF CHARGE MOVEMENT BY THE Na/K PUMP IN *XENOPUS* OOCYTES. ((M. Holmgren and R. F. Rakowski)) Univ. of Health Sciences/ Chicago Med. Sch., N. Chicago, IL 60064.

Pre-steady state charge translocation by the Na/K pump in *Xenopus* oocytes has the characteristics of membrane charge movement: voltage dependent relaxation rate, saturating sigmoid voltage dependence and equality of on and off charge (except for extreme negative pulses) (Rakowski, *J. Gen. Physiol.*, in press). This on/off discrepancy has been re-examined. Voltage clamp experiments were done in K-free solution containing (in mM) 90 NaCl, 5 $BaCl_2$, 20 tetraethylammonium Cl, 2 $NiCl_2$, 5 MOPS (pH = 7.6, Temp. 22°C). Transient currents were measured by subtraction of current records obtained before and after the addition of 10 μM dihydroouabain or after Na_o removal (replaced by tetramethylammonium). The charge moved (Q) was determined by direct numerical integration. Q(on) and Q(off) were approximately equal at all voltages for the three holding potentials examined. Reanalysis of current records previously analyzed by extrapolation confirmed the equality of Q(on) and Q(off). At holding potentials of 0, -40 and -100 mV, the midpoint voltage V_q was -25.6±1.4, -40.9±0.07 and -42.5±1.5 mV, and the apparent valence Z_q was 1.16±0.07, 1.36±0.05 and 1.18±0.07, respectively. The mean value of the total amount of charge moved was 990 ± 60 pC. Given a linear capacity of 0.18 μF per oocyte, we calculated a pump site density of 340 ± 20 μm^{-2} (n=34), in good agreement with 330 to 360 ouabain binding sites per μm^2 (Vasilets et al., *JBC* 266:16285, 1991). Supported by NIH grant NS-22979.

W-Pos474

PHOTOLABILE AMILORIDE ANALOGUES AS CATION SITE PROBES OF THE NA,K-ATPASE

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Treatment of purified canine renal Na,K-ATPase with a range of substituted amilorides inhibits enzyme activity. Inhibition by amilorides bearing substituents on the terminal guanidino group is not prevented by the presence of any of the enzyme ligands. In contrast, most (70%) of the inhibition due to 5-amino amiloride substituted analogues can be prevented by the presence of Na^+, K^+, or ATP. Incubation of the enzyme with 100 μM probe for 10 min at 37°C results in complete inhibition, *without photolysis*. Maximal inhibition at lower probe concentrations or 25°C requires a longer incubation period. Pelleting, resuspension, and washing does not restore any enzyme activity, this suggests that very tight binding of the amiloride derivatives is produced by a slow on-rate and an extremely slow off-rate. Irradiation of enzyme inhibited with 5-(N-ethyl-N-[2'-methoxy-4'-nitrobenzyl])-amiloride results in covalent incorporation of the probe into the α-subunit of the Na pump as shown by Western blots using polyclonal anti-amiloride anti-bodies. Supported by GM39500

W-Pos475

FUNCTIONAL CONSEQUENCES OF ALTERATIONS TO PRO328 AND LEU332 IN THE α-SUBUNIT OF Na^+ K^+-ATPase.

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cDNA encoding the ouabain resistant α_1-subunit of the rat kidney Na^+,K^+-ATPase was cloned and site-specific mutagenesis used to replace the residues Pro328 and Leu332 located in the conserved PEGLL motif of the predicted transmembrane helix M4. The cDNAs encoding either of the Na^+,K^+-ATPase mutants Pro328→Ala and Leu332→Ala, and wild type, were transfected into COS-1 cells. Ouabain resistant clones growing in the presence of 10 μM ouabain were isolated, and the Na^+, K^+, ATP and pH dependencies of the Na^+,K^+-ATPase activity measured in presence of 10 μM ouabain were analysed. Under these conditions the exogenous expressed Na^+,K^+-ATPase contributed more than 95% of the Na^+,K^+-ATPase activity. The Pro328→Ala mutant displayed a reduced apparent affinity for Na^+ ($K_{0.5}(Na^+)$ 13.04 mM), relative to the wild type ($K_{0.5}(Na^+)$ 7.13 mM). By contrast, the apparent affinity for Na^+ displayed by the Leu332→Ala mutant was increased ($K_{0.5}(Na^+)$ 3.92 mM). Either of the mutants exhibited lower apparent affinity for K^+ relative to the wild type ($K_{0.5}(K^+)$ 2.46 mM for Pro328→Ala and 1.97 mM for Leu332→Ala, compare with 0.78 mM for wild type). Both mutants exhibited higher apparent affinity for ATP than wild type ($K_{0.5}$(ATP) 0.086 mM for Pro328→Ala and 0.042 mM for Leu332→Ala, compare with 0.287 mM for wild type). The influence of pH was in accordance with an acceleration of the E2(K)→E1 transition in the mutants relative to the wild type. These data are consistent with a role of Pro328 and Leu332 in stabilization of the E2 form and of Pro328 in Na^+ binding.

W-Pos476

KINETICS OF Na PUMP INHIBITION BY VANADATE ARE SIMULATED BY A POST-ALBERS MODEL OF THE Na,K-ATPase. ((A.J. Fielding, N. Ishizuka and J.R. Berlin)) Bockus Research Institute, Philadelphia, PA 19146.

The kinetics of Na pump current (I_{pump}) inhibition by intracellular vanadate were examined in rat ventricular myocytes voltage-clamped with a single patch electrode at 36°C. I_{pump} was activated by rapid application of K^+ (0.2 - 15 mM) in the superfusion solution. In Na^+ containing superfusion solutions, the Na pump was not inhibited by vanadate (0.01 - 1 mM in the electrode solution) in the absence of extracellular K^+ (K_o); however, in the presence of K_o (0.7 - 15 mM), I_{pump} was inhibited with pseudo-first order kinetics. After application of K_o, the peak I_{pump} was proportional to K_o concentration, similar to vanadate-free conditions. Steady-state I_{pump}, however, showed a biphasic dependence on K_o with a maximum at 2 mM K_o. The time constant for inhibition of I_{pump} was 2.5 ± 0.2 s (Mean ± S.D., n=9) with 100 μM vanadate in the electrode solution and was not significantly affected by K_o (0.7 - 15 mM). Similarly, the rate of recovery from vanadate inhibition was not significantly affected by K_o. These data were not consistent with expected first order kinetics. In nominally Na^+- and K^+-free superfusion solution, the Na pump was slowly blocked (time constant = 107 ± 3 s, n=3) with 100 μM electrode vanadate, but this inhibition was blocked by low concentrations of Na^+ in the superfusion solution ($K_{1/2}$ = 2.4 mM). Thus, low concentrations of extracellular Na^+ antagonized inhibition of the Na pump by vanadate. The kinetics and ion-dependence of I_{pump} inhibition by vanadate could be completely reproduced by computer simulations using a simplified Post-Albers scheme for the Na,K-ATPase in which vanadate interacted only with the E_2*2K conformation of the enzyme. Additional ion binding regulatory sites were not required in these simulations. *Supported by NIH and the Southeastern Pennsylvania affiliate of AHA*

W-Pos477

CONSTRUCTION AND EXPRESSION OF THAPSIGARGIN-, OUABAIN- AND Ca^{2+}-SENSITIVE CHIMERIC E1E2-ATPAses.

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Chimeric chicken cDNAs encoding the amino-terminal portions of the Na,K-ATPase and the carboxy-terminal of the SR Ca-ATPase were constructed and expressed in mouse L cells, according to the strategy described previously [Biophys. J. 62: 227-234, 1992]. The chimera ([n/c]CC), in which the amino terminal amino acids (Met1 to Ile_{163}) of the SR Ca-ATPase was substituted for the corresponding portion of the Na,K-ATPase, retained thapsigargin- and Ca-sensitive ATPase activity which was inhibited by ouabain, although the activity was lower than the wild-type SR Ca-ATPase. The chimera ([n/n]CC), in which the amino terminal amino acids (Met1 to Gly_{354}) of the SR Ca-ATPase was substituted for the corresponding portion of the Na,K-ATPase, lost the Ca-ATPase activity seen in [n/c]CC. These results demonstrate that the segment, Ile_{163} to Gly_{354}, of the SR Ca-ATPase is important for the efficiency of cross-talk between Ca-binding sites and ATP utilization sites, and that the N-terminal 200 amino acids are sufficient to exert ouabain-dependent inhibition. [Supported by grants from AHA (91002590) and NIH (GM44373).]

W-Pos478

CALCIUM PROMOTES E-P FORMATION BY INORGANIC PHOSPHATE OF A PROKARYOTIC CALCIUM ATPASE

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The SR Ca^{2+}-ATPase, which has been used as a general model for cation transport, can be phosphorylated directly by inorganic phosphate. The phosphointermediate is formed by reaction of Pi with the E2 form of the enzyme. Pi phosphorylation is half-maximally inhibited by 1 uM calcium. Pi labelling of the SR Ca^{2+}-ATPase in the presence of calcium has only been observed in mutant constructs in which the putative high-affinity calcium binding sites have been altered to uncharged amino acids, or when the protein is labeled first with NCD-4, an analog believed to interact with and block the calcium binding sites. However, the first study of this reaction in a prokaryotic ATPase reveals critical differences. Ca^{2+}-ATPase of *F. odoratum*, which is fully functional in both its calcium-transporting and ATP hydrolyzing activities, is preferentially phosphorylated by [^{32}P] Pi in the presence of calcium. The calcium-dependent phosphointermediate formed is sensitive to alkaline conditions and hydroxlamine, indicating formation of the expected acylphosphate. Vanadate inhibits the calcium-dependent Pi phosphorylation, but does not appear to have any effect upon phosphorylation in the absence of calcium. Turnover of the calcium-dependent phosphointermediate is rapid, as demonstrated by ADP and Pi chase experiments, but ADP has little effect upon the phosphorylated species formed in the absence of calcium. These results demonstrate that the prokaryotic ATPase, though functionally similar, is mechanistically distinct from the SR Ca^{2+}-ATPase, and may represent a novel transport reaction cycle.

W-Pos479

LOCALIZATION OF A POSSIBLE MG2+ BINDING SEQUENCE IN THE LARGE CYTOPLASMIC LOOP OF THE SARCOPLASMIC RETICULUM Ca2+-ATPase.

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Several regions of the main cytoplasmic domain of the SR Ca-ATPase exhibit a high degree of homologies with other ATPases of the P-type family. The region of highest degree of conservation is located between residues 700 and 712. We have found that this part of the sequence presents significant similarities with EF-hand structures of the calcium binding proteins. We have tested the capacity of this sequence to bind divalent cations by synthesizing a 38-residue polypeptide identical to amino-acids 682 to 719 of the Ca-ATPase.
The properties of this polypeptide were studied by circular dichroism, intrinsic tyrosine fluorescence and fluorescence energy transfer between terbium and tyrosine. Results show that it can form an helical structure in moderate concentration of trifluoroethanol and that it can bind divalent cations with an affinity around 10 mM.
Sequence variants on either one or two amino acids show significant reduction of the divalent cations binding properties. We propose that this sequence is involved in the binding of Mg2+ ions on the Ca-ATPase with a possible implication in phosphate and/or ATP binding.

W-Pos480

RAPID RELEASE OF Ca FROM SKELETAL SARCOPLASMIC RETICULUM THROUGH THE Ca-PUMP. L.G. Mészáros and J. Bak, Dept. Physiol. Endocrinol., Med. Coll. Georgia, Augusta, GA 30909.

In La^{3+} -quench experiments, a biphasic Ca^{2+} release from sarcoplasmic reticulum (SR) vesicles, preloaded with millimolar $^{45}Ca^{2+}$ by overnight incubation on ice, was observed upon dilution into solutions of pCa=8. Both the rapid (K_1=0.1-0.2 s^{-1}) and the slow (K_2=0.03 s^{-1}) phases of $^{45}Ca^{2+}$ release were sensitive to thapsigargin, but insensitive to micromolar ryanodine, submicromolar ruthenium red and millimolar Mg^{2+}, indicating that the efflux of Ca^{2+} is mediated by the Ca-pump and not by the ryanodine receptor channel. The rate of Ca^{2+} efflux during the rapid kinetic phase showed a dependence on the intravesicular Ca^{2+}, which was best described with the sum of the Hill equation (K_d=0.7 mM) and a linear function.

These findings seem to contradict any alternative access models generally used to describe the mechanism of the SR Ca-pump reaction, but support a model, which would assume that Ca^{2+} moves through a channel-like structure within the Ca-pump polypeptide, whose gating is regulated, in part, by the low-affinity internal Ca^{2+} -sites of the Ca-pump.

W-Pos481

CATALYSIS OF AN ATP ANALOGUE TETHERED TO LYS-492 OF SARCOPLASMIC RETICULUM Ca-ATPase.

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Irradiation of SR Ca-ATPase at alkaline pH in the presence of [γ-^{32}P] 2',3'-O-(2,4,6-trinitrophenyl)-8-azido-ATP and EGTA resulted in the covalent labeling of Lys-492, with retention of the γ-^{32}P group. Addition of Ca^{2+} to the derivatized ATPase accelerated the hydrolysis of the γ-^{32}P group up to 50-fold. The initial rate of hydrolysis was approx. 1000-fold slower than the normal hydrolysis of ATP. Ca^{2+} transport was not detectable. At alkaline pH and in the presence of dimethyl sulphoxide, phosphorylation of the ATPase was measured through the sensitivity of the latter and not the γ-^{32}P group to $NaBH_4$.
The native ATPase catalysed a similar slow Ca^{2+}-dependent hydrolysis of [γ-^{32}P]TNP-8-N_3-ATP (K_m=0.9 μM) with a similar pH dependence. Maximal levels of phosphorylation were equivalent to 0.5 mol/mol ATPase.
The results support the positioning of the tethered nucleotide and Lys-492 at the catalytic site and put a maximal distance between the carboxyl group of Asp-351, which is phosphorylated, and the amino group of Lys-492 of approx. 16 Å during transfer. The slowness of the latter appears to be related to the TNP group.

W-Pos482

Ca^{2+} BINDING TO SARCOPLASMIC RETICULUM ATPase REVISITED: INFLUENCE OF H^+ AND Mg^{2+} ON ITS COOPERATIVITY.

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H^+ and Mg^{2+} are known to inhibit Ca^{2+} binding to the transport sites of SR-ATPase. Evaluation of the affinity for the Ca^{2+} binding sites requires measurement of the amount of Ca^{2+} bound to ATPase as a function of the free Ca^{2+} concentration imposed by a Ca^{2+} chelator. The choice of the chelator is crucial as it determines the precision of the free Ca^{2+} concentration. At pH>7, the EGTA affinity for Ca^{2+} is higher than that of ATPase, inducing artefacts that alter the shape of the binding curves. Thus, we have used BAPTA, whose affinity is unchanged at pH$\geq$7. Ca^{2+} binding was studied at equilibrium, at pH 6-8 and 0-10 mM Mg^{2+}, using EGTA or BAPTA and [^{45}Ca]Ca^{2+}. Under all conditions, the stoichiometry was 2 Ca^{2+}/ATPase. At variance with previous studies, the Hill coefficient was 1.1-2, and higher at pH 6 than at pH 8. In addition, it decreased in the presence of Mg^{2+}. The Ca^{2+} binding curves were analyzed according to a model in which they result from a sequential binding of 2Ca^{2+}, each binding step being modulated by H^+ and Mg^{2+}. The effect of H^+ is described by two steps involving 2H^+ and 1H^+, at pK7 and 8, respectively. At pH6, ATPase must lose 2H^+ for the first Ca^{2+} to bind, and a third H^+, for the second Ca^{2+} to bind, whereas at pH9 both Ca^{2+} bind without any H^+ exchange. Mg^{2+} can bind to all species, except to that saturated with Ca^{2+}. The species having lost 2H^+ has a higher affinity for Mg^{2+} ($\leq$1mM) than the species having bound 3H^+ (4mM). The above model allows us to analyze the effects of H^+ and Mg^{2+} at each Ca^{2+} binding step, and to explain the changes in the apparent affinity and cooperativity.

W-Pos483

FUNCTIONAL ROLES OF AMINO ACIDS IN TRANSMEMBRANE SEQUENCE M4 OF SERCA1.

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The fourth transmembrane sequence (M4) of skeletal muscle Ca^{2+} ATPase (SERCA1) contains Glu^{309}, previously implicated in Ca^{2+} binding. This residue is surrounded by Pro^{308}, Gly^{310}, and Pro^{312}, previously implicated in conformational changes. A unique feature of this transmembrane sequence is the presence of alternating or juxtaposed small and bulky hydrophobic amino acids. Site directed mutagenesis was used to explore the significance of this arrangement Generally, the effects of mutations near the centre of the domain were more severe. Mutation of Ala^{305} to Val, Ala^{306} to Val, and Gly^{310} to Val resulted in complete inhibition of Ca^{2+} uptake activity. These mutants could be phosphorylated by ATP only in the presence of Ca^{2+}, and by P_i only in its absence, indicating that the Ca^{2+} binding sites were still intact. However, the phosphorylated intermediate was not ADP sensitive and was long-lived. Double mutants of Val^{304} Ala^{305} and Ala^{306} Ile^{307} that reversed the order of the pair showed the same phenotype. These mutations, therefore, result in an E_2P-E_2 conformational block. These results confirm that the M4 domain not only contributes to a high affinity Ca^{2+} binding site but also plays a pivotal role in the conformational changes required for Ca^{2+} transport.

W-Pos484

IDENTIFICATION OF SITES OF INTERACTION BETWEEN PHOSPHOLAMBAN AND SERCA TYPE CA^{2+} PUMPS THROUGH MUTAGENESIS. ((Toshihiko Toyofuku[1], Michihiko Tada[2] and David H. MacLennan[1])) [1]Banting and Best Department of Medical Research, University of Toronto, Toronto M5G 1L6 Canada and [2]Division of Cardiology, Department of Medicine and Pathophysiology, Osaka University School of Medicine, Osaka 565 Japan (Spon. R. Reithmeier)

Coexpression of wild type phospholamban with the Ca^{2+} pump isoform, SERCA2a, in HK293 cells in culture lowered the Ca^{2+} affinity of SERCA2 (measured as Ca^{2+}-dependence of Ca^{2+} transport), providing a functional assay for interaction between the two proteins. Coexpression of phospholamban with chimeric Ca^{2+} pumps, in which domains were swapped between SERCA2a and SERCA3, demonstrated a requirement for the nucleotide binding/hinge domain of SERCA2a, which provides high affinity Ca^{2+} binding, and for residues 370 to 400 in the phosphorylation domain of SERCA2a, which provide a site for phospholamban interaction. Positively charged residues Lys^3, Arg^9, Arg^{13} and Arg^{14} and phosphorylation site residues Ser^{16} and Thr^{17} in the cytoplasmic domain of phospholamban have also been shown to be essential, since their mutation to acidic or neutral residues led to loss of functional interaction between phospholamban and SERCA2a. Mutation of Cys^{41} to Phe in the transmembrane domain of phospholamban, destabilizing the pentameric structure of phospholamban, did not affect functional interaction. Further investigation should identify all interacting residues between phospholamban and SERCA2.

W-Pos485

EPR STUDIES OF Mn^{2+} BOUND TO SR CaATPase Ca^{2+} AND Mg^{2+} SITES FOR DIFFERENT ENZYME INTERMEDIATE STATES

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Three types of EPR measurements can be used to monitor the stoichiometry and identity of the sites to which Mn^{2+} is bound for different intermediate states of the CaATPase from sarcoplasmic reticulum. Mn^{2+} binds to both Ca^{2+} and Mg^{2+} sites on the SR CaATPase molecule. Scatchard plots of total bound Mn^{2+} determined from free Mn^{2+} EPR measurements can be fit to a model in which there are two cooperative sites (K_2/K_1 = 1.4 for the native enzyme and 117 for the CrATP-inactivated enzyme) and one to two additional sites which may correspond to enzyme Mg^{2+} sites.

Mn^{2+} bound at calcium transport sites can be detected through its effect on the EPR signal of iodoacetate spin label covalently bound at cys 670 and 674. The effect of bound Mn^{2+} on the ISL EPR signal is comparable to the effect seen upon calcium addition.

Mn^{2+} bound at a putative enzyme Mg^{2+} site results in a diminution of the EPR signal intensity of malemide spin label covalently bound at cys 344 and cys 364. The signal diminution is more pronounced at pH 6 than at pH 8. Calcium addition alone has no effect at either pH nor does calcium addition reverse the Mn^{2+} effect. This effect may be due to a dipolar magnetic interaction between the paramagnetic Mn^{2+} and the nitroxide spin label and may be an indication that the bound Mn^{2+} ion is close in space to either or both cys 344 or cys 364.

W-Pos486

DETERMINING THE SIZE OF THE ACTIVE SPECIES OF THE RBC Ca^{2+}-ATPase. D. L. Sackett, and D. Kosk-Kosicka*. NIH, NIDDK, Bethesda, MD 20892 and *The Johns Hopkins University, Dept. Anesth., MD 21287.

Previously we have determined that the Ca^{2+}-ATPase from human red blood cells, an enzyme crucial for maintaining Ca^{2+} homeostasis in the cell, undergoes reversible oligomerization (Kosk-Kosicka et al., J. Biol. Chem. 33, 19495; Biochemistry 29, 1875; 3772). The oligomerization process produces a highly cooperative Ca^{2+} regulated activation of the enzyme at physiologically relevant concentrations, and is independent of activation by calmodulin. Using fluorescence energy transfer technique we were able to differentiate two active species of the plasma membrane, which we defined as"oligomers" and "monomers with bound calmodulin".

Presently we have succeeded in determining the size of the two active species by equilibrium ultracentrifugation. Using short column, rapid equilibrium conditions for centrifugation and .nonlinear regression analysis for fitting to the data, we obtained a best-fit single weight-average molecular mass for the oligomeric form of 260 kDa (90% confidence interval=215-305) which indicates that the oligomer consists of two 140 kDa enzyme molecules. For the calmodulin dependent species the best fit gave 180 kDa (90% confidence interval 155-225) in agreement with this species being a monomer with bound calmodulin. We are in the process of refining the data and defining enzyme size under several experimental conditions that could be related to the in vivo situation.

W-Pos487

INHIBITION OF THE CALCIUM ATPASE OF SARCOPLASMIC RETICULUM BY MAGNESIUM, BERYLLIUM AND FLUORIDE. ((A.J. Murphy and R.J. Coll)) Biochemistry Dept., University of the Pacific, San Francisco, CA 94115.

Incubation of the sarcoplasmic reticulum (SR) calcium ATPase with magnesium, beryllium, and fluoride ($MgBeF_x$) resulted in time-dependent loss of activity which was faster than the rate previously reported for magnesium and fluoride (Murphy & Coll [1992] J. Biol. Chem. 267, 5229, 16990). Unlike $MgBeF_x$ inhibition of many other enzymes (e.g., G proteins and myosin), nucleoside diphosphate is not required. The rate of inhibition is higher at lower pH, while the presence of calcium has a protective effect. Even at low ratios of beryllium to CaATPase, the extent of inhibition tends toward completeness, consistent with the inhibited complex containing about 1-2 Be per ATPase. The beryllium-containing complex of the enzyme is not as stable as the one containing only magnesium and fluoride. Like the latter, the former complex is reactivated by addition of calcium. (Supported by NIH GM310083).

W-Pos488

H^+ COUNTERTRANSPORT AND ELECTROGENICITY OF THE SARCOPLASMIC RETICULUM CA^{2+} PUMP IN RECONSTITUTED PROTEOLIPOSOMES*. ((X. Yu, S. Carroll, J. Rigaud and G. Inesi)) Biological Chemistry, Univ. MD Sc. Med., Baltimore, MD 21201.

The Ca^{2+} transport ATPase of sarcoplasmic reticulum was reconstituted in unilamellar liposomes prepared by reverse phase evaporation. The proteoliposomes sustained ATP dependent Ca^{2+} uptake at rates proportional to the protein content (1-2 μmol Ca^{2+}/mg prot/min), reaching asymptotic levels corresponding to a lumenal calcium concentration of 10-20 mM. The low permeability of the proteoliposomes permitted direct demonstration of Ca^{2+}/H^+ countertransport and electrogenicity. Inhibition of the Ca^{2+} pump by lumenal alkalinization, consequent to H^+ countertransport, was relieved by the H^+ ionophore FCCP. In spite of H^+ countertransport, net positive charge displacement was produced by a Ca^{2+} transport, as revealed by a rapid oxonol VI absorption rise which was highest when SO_{42}^- was the prevalent anion, lower in the presence of Cl^-, and lowest in the presence of the lipophilic anion SCN^- The absorption rise was rapidly collapsed by addition of valinomycin in the presence of K^+. Experimentation with Ca^{2+} and H^+ ionophores was consistent with a primary role of Ca^{2+} and H^+ in net charge displacement. The estimated value of the steady state electrical potential was 50 mV. Our experiments demonstrate that the Ca^{2+} pump is electrogenic, and they are consistent with previous measurements indicating that one ATPase cycle involves countertransport of 2 Ca^{2+} and 2 H^+.

W-Pos489

COMPARATIVE STUDIES OF SKELETAL AND CARDIAC SARCOPLASMIC RETICULUM CA^{2+} PUMP INHIBITORS, THAPSIGARGIN, THAPSIGARGICIN, CYCLOPIAZONIC ACID, AND 2,5-DI(T-BUTYL)HYDROXYQUINONE ((Yutaka Sagara and Giuseppe Inesi)), Biological Chemistry, Univ. MD. Sch. Med., Baltimore, MD. 21201; ((L.R. Jones)), Dept. of Med. & Krannert Inst. Cardiol., Indiana Univ. Sch. Med., Indianapolis, IN 46202

The effects of the Ca^{2+} ATPase inhibitors, thapsigargin (TG), thapsigargicin (TGC), cyclopiazonic acid (CPA), and 2,5-di(t-butyl)hydroquinone (DBHQ), were compared in skeletal and cardiac SR vesicles. Half maximal inhibition of ATP hydrolytic activity of the skeletal and cardiac ATPase was obtained in the presence of 10 nM, 15 nM, 222 nM, and 485 nM added TG, TGC, CPA, and DBHQ, respectively. Ca^{2+} binding in the absence of ATP was inhibited by all the inhibitors. However, a higher Ca^{2+} concentration (50 μM) protected the ATPase completely from CPA and DBHQ inhibition, while TG and TGC inhibition was only partially reduced. When ATP was added in the presence of sufficient Ca^{2+}, the level of the phosphorylated intermediate was also reduced with a pattern suggesting preferential interaction of the inhibitors with a Ca^{2+} free enzyme state produced during the catalytic cycle. Solubilization of the Ca^{2+} ATPase with $C_{12}E_8$ decreased the affinity of CPA and DBHQ by 40 and 60 fold, respectively, while TG and TGC inhibition were affected to a lesser degree (2 fold for TG and 8 fold for TGC). While TG and TGC inhibition is apparently irreversible, CPA and DBHQ inhibit the ATPase reversibly. With respect to cardiac SR Ca^{2+} ATPase, we found that TG retains the same affinity and stoichiometry to the ATPase, independent of the influence of phospholamban. (NIH supported)

W-Pos490

SPECIFIC CA^{2+} ACTIVATION AND THAPSIGARGIN INHIBITION OF CA^{2+} AND NA^+,K^+ ATPASE CHIMERAS

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Two chimeric cDNA constructs comprising the amino- and carboxy-terminal portions of the Ca^{2+} transport ATPase and the central portion of the Na^+,K^+ ATPase α subunit, were expressed in mammalian cells. The ATPases were recovered in microsomal fractions, and demonstrated with monoclonal antibodies. The first chimera (Cp-N-C), in which segments Met1-Thr355 and Lys712-Ala994 of the Ca^{2+} ATPase flanked segment Leu379-Lys724 of the Na^+,K^+ ATPase, retained the phosphorylation site (p=Asp351) of the Ca^{2+} ATPase. In the second chimera (C-Np-C) the phosphorylation site (p=Asp 374) of the Na^+,K^+ ATPase (rather than that of the Ca^{2+} ATPase) was retained. In either case, ATP utilization was strictly Ca^{2+} dependent in analogy to the wild type Ca^{2+} ATPase. ATP utilization by the chimeric ATPases was also prevented by thapsigargin, cyclopiazonic acid and DBHQ. Our experiments demonstrate that specific Ca^{2+} dependence and inhibition can be conferred to the catalytic site of the Na^+,K^+ ATPase by the presence of Ca^{2+} ATPase transmembrane segments which are involved in Ca^{2+} binding. Inspite of Ca^{2+} dependent ATP utilization, net Ca^{2+} accumulation of both chimeric ATPases was very low, suggesting less than optimal folding. (NIH and AHA supported)

W-Pos491

PURIFICATION OF A PROKARYOTIC P-TYPE CALCIUM ATPASE.

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Low intracellular calcium levels are maintained in *Flavobacterium odoratum* primarily by a P-type ATPase. This vanadate-sensitive Ca^{2+}-ATPase has been purified approximately 1000-fold from membrane vesicles. Purification steps include: extraction of membrane vesicles with $C_{12}E_8$; ammonium sulfate fractionation followed by centrifugation through a 30-50% glycerol gradient; and DE52 ion-exchange chromatography. The purified Ca^{2+}-ATPase consists of a single polypeptide with an apparent molecular mass of 60 kDa. Importantly, this single polypeptide is fully functional and capable of all soluble activity of the Ca^{2+} pump. Maximal hydrolysis activity occurs at pH 7.6 with a specific activity of 30 μmol of ATP hydrolyzed per minute per mg of protein. The purified Ca^{2+}-ATPase has an apparent Km for calcium of 1.3 μM and for ATP of 130 μM. Vanadate strongly inhibits activity with an IC_{50} of 1.6 μM. It is rapidly phosphorylated by [γ-^{32}P] ATP in a calcium dependent, vanadate-inhibitable manner. The phosphointermediate is sensitive to alkaline conditions, a characteristic of the acylphosphate linkage found in P-type ATPases. This is the first report of the purification of a prokaryotic Ca^{2+}-ATPase. (Supported by NIH HL44202.)

W-Pos492

FLUORESCENCE PROPERTIES OF THE SINGLE TRYPTOPHAN IN THE F_1 ATPASE FROM ALKALIPHILIC *BACILLUS FIRMUS* OF4

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The deduced amino acid sequence of the F_1 ATPase in the F_oF_1 synthase complex of alkaliphilic *Bacillus firmus* OF4 indicates only one tryptophan (Trp) residue, γ171, for the entire $\alpha_3\beta_3\gamma\delta\epsilon$ F_1 complex. We have isolated homogeneous F_1 and studied its Trp fluorescence (295 nm excitation). The emission is blue-shifted compared to Trp in water, indicating that the F_1 Trp residue is in a nonpolar environment. The time-dependence of the fluorescence has also been studied. The fluorescence intensity decay of the F_1 Trp is multiexponential and emission-wavelength dependent. The intensity-weighted mean lifetime increases from 1.5 ns at 305 nm to ~4 ns at 400 nm. [Loss of enzymatic activity can be correlated with a red shift in emission and changes in the intensity decay parameters.] This multiexponential, wavelength-dependent behavior for a single Trp residue could result from: *i)* multiple, spectrally-distinct species due to the Trp in different environments; or *ii)* an excited-state 'solvent' relaxation of the indole ring by the protein. The multiple species model is feasible since global analysis finds three wavelength-independent lifetimes, and the resulting decay associated spectra are different. The relaxation model is currently being examined and will be discussed. Supported by NIH grants GM-28454 and GM-39750.

W-Pos493

F-TYPE ATP SYNTHASE: CHARACTERIZATION OF A SYNTHETIC PEPTIDE CORRESPONDING TO THE A CONSENSUS REGION OF THE α-SUBUNIT.

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The mitochondrial F-type ATP synthase contains at least two types of nucleotide binding sites within the F_1 moiety; catalytic purine nucleotide binding sites on the β-subunits and adenine nucleotide specific sites on the α-subunits. It has been suggested that the α-subunit sites are regulatory in nature and bind nucleotides with greater affinity than the catalytic sites. The structural basis for the increased affinity is unknown. The α-subunit primary sequence, like the β-subunit, contains the Walker A and B consensus homology regions. Whereas the primary sequence homology of the two subunits within these regions is not exact and the A consensus sequence provides the bulk of the binding energy in the β-subunit, we asked whether the sequence differences in the A region can account for the affinity differences. A 56 amino acid peptide was synthesized using t-Boc chemistry and purified by HPLC. Determination of the secondary structure by CD indicates that the peptide is predominantly in a coiled conformation in the absence of nucleotide. The peptide binds TNP-ATP > > TNP-ADP > > TNP-AMP. The binding of TNP-ATP is pH dependent with an apparent K_d of 2 μM at pH 6 and below and of 10 μM at pH 6.7 and above. ATP at concentrations of 2 mM competes with TNP-ATP for the binding site but AMP does not. In comparison with a homologous 50 residue β-subunit peptide, the α-subunit peptide has a less ordered secondary structure and binds adenine nucleotides with less affinity, arguing that residues within the α-subunit A consensus region do not provide for the additional affinity observed at the regulatory site within the intact F_1. (Supported by NIH CA10951)

W-Pos494

OVER-EXPRESSION AND CHARACTERISATION OF THE CYTOPLASMIC FRACTION OF THE PLASMA MEMBRANE H+-ATPase OF THE YEAST SACCHAROMYCES CEREVISIAE.

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We report here the expression in E. coli, the purification and the binding properties of the Saccharomyces cerevisiae plasma membrane H+-ATPase cytoplasmic domains fused with glutathione S-transferase (GST). The fusion protein (GST-L) contains the ATPase cytoplasmic large loop from Alanine 340 to serine 660, which has been proposed to be involved in ATP, Pi and Mg++ binding. Another protein (GST-SL) was prepared that contained in addition the small cytoplasmic loop from glutamate 162 to valine 275. The refolded GST-L interacts with the ATP analogue TNP-ATP whose binding was measured by fluorescence. Results were identical with GST-SL indicating that the small loop is not involved in the formation of the ATP binding site. No binding was observed with GST alone. ATP displaces TNP-ATP from GST-L competitively. Dissociation constants for TNP-ATP (4 μM) and ATP (4 mM) are very close to that found for a non-glucose activated native H+-ATPase.

W-Pos495

THE BOVINE HEART MITOCHONDRIAL H^+-ATPASE IS AN IRON TRANSPORTER ON THE INNER MEMBRANE.

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In order to determine if the mitochondrial H^+ATPase could transport iron similar to the vacuolar enzyme, the ATPase was reconstituted in phospholipid (1:2:9/PS:PE:PC) liposomes (1:25, ATPase:lipid) containing 0.5 mM of ^{59}Fe (1:4, Fe:NTA). To reconstitute the ATPase in soybean phospholipids, the liposomes were pretreated to remove most or all of the absorbed surface iron, mitochondrial ATPase was mixed with the liposome preparation by swirling, and incubated on ice for 30 min or more. Ascorbate was added to the Fe-NTA solution before encapsulation in order to observe Fe(II) transport and 1 mM Ferrozine was present in teh extravesicular solution to chelate the transported iron. Ascorbate is not an essential requirement for iron transport. If the soybean phospholipids are not pretreated, a significant amount of iron is found to be associated with the external surface and showed an apparent transport of 26.6% total Fe(III) and 23% total Fe(II). Using pretreated phospholipids for liposome preparation, Fe(II) transport was obsaerved from 0.98±0.20% (zero time) to 44.0±1.8% (2.5 hours) and Fe(III) transport from 0.40±0.20% to 51.0±0.8%. Kinetic experiments showed the rate of Fe(III) transport was 0.0176± 0.00295 μmole of Fe/mim/mg of ATPase. The rate of transport decreased 66.3±9.5% with 50 μM DCCD, and increased by 72.3±10.5% and 118.3±8.2% with 25 μM NEM or 40 μM oligomycin respectively. The rate of Fe(II) transport was about 75% of Fe(III). Overall, these studies demonstrate that the mitochondrial H^+-ATPase can serve as an Fe transporter but performs this function with an Fe(II)/Fe(III) specificity that is different from the vacuolar enzyme.

W-Pos496

THE VACUOLAR H^+-ATPASE FROM RABBIT RETICULOCYTE ENDOSOMES ACTS AS AN IRON TRANSPORTER. ((J. Glass, C-Y. Li, and J.A. Watkins)) Center of Excellence in Cancer Research, Treatment, and Education, LSUMC-S, Shreveport, LA, 71130.

To determine if the ATPase can act as an Fe transporter the purified enzyme was reconstituted into liposomes by sonication (0.1 mg/ml protein, 24 mg/ml phospholipids, 1PS:2PE:9PC) and Fe transport assayed by either of two methods. First, liposomes were reconstituted in 34 μM Fe:citrate (1:1500), the free Fe removed by gel filtration, and diluted into 1 mM FerroZine (an Fe chelator) in a buffer consisting of 50 mM MES, 50 mM Tris, 50 mM KCl, 1 mM EGTA, 1 mM $MgCl_2$, pH 7.0. Upon the addition of 10 mg/ml ascorbate, efflux was detected by formation of a Fe(II)-FerroZine complex with an absorbance at 550 nm, with an initial rate of efflux of approximately 8.0 μmoles Fe/mg ATPase/min. Both thiosulfate and ferrocyanide could substitute for ascorbate. The efflux was inhibited by 28% using 100 μM of the proton channel blocker DCCD, stimulated by 150% with 70 μM NBD-Cl, and not effected by the presence of ATP or 0.5 mM NEM. In the second method, ^{59}Fe:ascorbate was trapped within the vesicles, separated from non-encapsulated ^{59}Fe by gel filtr4ation, and after incubation at 37°C, in the MES-Tris buffer, the transported ^{59}Fe was separated from liposomes by gel filtration. After a 90 min incubation in the presence of FerroZine or citrate, 56±6.9% and 59.5±10.5% of the total internal ^{59}Fe was transported out of the liposomes respectively, and transport was inhibited by 40% by 100 μM DCCD. In the absence of an external chelator, tranport decreased to 43.3±0.2%. The presence of reconstituted ATPase and intraliposomal ascorbate were required for transport indicating a specificity for Fe(II). These results suggest that the H^+ from endosomes may serve as an iron transporter.

W-Pos497

SECONDARY AND TERTIARY STRUCTURE CHANGES OF NEUROSPORA CRASSA PLASMA MEMBRANE H^+-ATPASE PROBED BY INFRARED SPECTROSCOPY. ((E. Goormaghtigh, L. Vigneron and J.M. Ruysschaert)) Free University of Brussels, Campus plaine CP206/2, B-1050 Brussels, Belgium.

The plasma membrane H^+-ATPase from Neurospora crassa is member of a large family of ion-translocating ATPases which is characterized by an aspartyl-phsophoryl-enzyme intermediate. Susceptibility of the enzyme to trypsin proteolysis demonstrates that different ligands are able to lock the enzyme in different conformations (1,2). When an active lysophosphatidylglycerol-ATPase complex (3) in aqueous solution or prepared as a hydrated films is studied by attenuated total reflection (ATR) FTIR spectroscopy, the analysis of the shape of amide I (4) indicates very little changes (less than 3%) in the secondary structure content in the presence of these different ligands. On the other hand the kinetic of H/D exchange followed by measurement of the amide II and amide II' makes differences clearly appear. Data collected from 20 seconds to 160 min indicate the following sequence for the rate of exchange:

no ligand>>Mg^{++}-Vanadate>Mg^{++}-ATP-vanadate>Mg^{++}-ADP

The same ligands have no effects on the exchange kinetic of unrelated proteins. Since the pH and the secondary structure are identical, a change in the accessibility of amide proton is likely to cause the change in the kinetics. Opening and closure of a cleft could be the prime cause of the change of accessibility.

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MATHEMATICAL MODELS AND BIOPHYSICAL THEORY

W-Pos498

IN VIVO "FEEDBACK" OF INFORMATION TO THE BONE MARROW: POSSIBLE CLUES FROM AGNOGENIC MYELOID METAPLASIA. ((R.P. Spencer)). Univ. Connecticut Health Center, Farmington, CT 06030.

Bone marrow function depends upon at least 3 factors: supply of nutrients (such as iron), intactness of the system (not replaced by tumor or infection) , and effects of hormones such as erythropoietin and other molecules. An opportunity to study these latter factors is presented in agnogenic myeloid metaplasia or AMM in which the marrow (being replaced by fibrosis) can often be stimulated by removal of the spleen (splenectomy, SX). Brenner and coworkers (Hemostasis 17:141, 1987) presented assay of circulating hemoglobin (HB) values, white blood cells (WBC)& platelets (PL) before and after SX in 10 patients with AMM. We have compared results before and after the SX, for all 3 of the formed blood elements. Correlation was best for platelets and WBC ($p < .01$) and least for HB ($p = .05$). Dividing patients into those with the 5 highest and 5 lowest initial values, the post-SX correlations were only significant for PL in those with the highest initial values and HB in those with the lowest initial results ($p < .05$). Hence, there were somewhat "discrepant" results for these 2 elements.This may be related to the specific "feedback" systems involved. Erythropoietin is a recognized stimulant to red cell/HB production. With PL, mechanisms may be more complex with both stimulants and inhibitors. Continued assay in larger series of cases may yield further evidence in the search for "feedback" of information to the bone marrow.

W-Pos499

MAXIMUM LOAD-BEARING CAPACITY AND FORCE-VELOCITY CURVES FOR CANINE TRACHEAL SMOOTH MUSCLE (TSM). ((J. Wang and N. L. Stephens)) Dept. of Physiol. U. of Manitoba, Winnipeg, Manitoba, Canada R3E 0W3

It has been reported in a variety of striated muscles, that at high loads, the force-velocity (F-V) curve deviated from the rectangular hyperbola defined by Hill's equation at both single fibre and tissue levels. We have recently derived a non-hyperbolic F-V equation, that better fits the data points. From this curve we could calculate the maximum load-bearing capacity (LB_{max}) of the muscle, which had a value of 1.27 P_o (P_o = maximum active isometric force at optimal length l_o), 1.35 P_o and 2.06 P_o for canine TSM during 2, 6 and 10 second electrical stimulations, respectively, compared with the reported 1.6 P_o for skeletal muscle. The difference in the LB_{max} at different time points suggested that the latchbridges could bear a much heavier load than early, normal cycling crossbridges. Even though our earlier studies of instantaneous stiffness had shown the number of active crossbridges is greater in the latter, *vis-a-vis* the former. Since the LB_{max} represents the force limit at which all crossbridges are mechanically broken and the speed of elongation of the muscle tends to infinity, we postulate that at high loads, even though the loads are lighter than the maximum isometric force P_o, a small number of crossbridges is detached so that the remaining individual bridge is forced to bear heavier loads than in the absence of such detachment. Therefore, the shortening velocities decrease at those loads, and are less than those predicted by Hill's equation. To measure the number of crossbridges at these different loads, we applied a second force step to measure stiffness, an indirect index of the number of crossbridges, 220 ms after the first quick-release that was applied to obtain the first velocity measurement. The stiffness elicited by the second force clamp increased nonlinearly and the rate of change decreased with loads, which experimentally supported our hypothesis. (Supported by an operating grant from the Council for Tobacco Research. J. Wang is the recipient of a Manitoba Health Research Council Studentship Award).

W-Pos500

A MONTE CARLO APPROACH TO THE SIMULATION OF A WEAK ELECTROLYTE SOLUTION. ((C.Y. Shew and P.A. Mills)) Department of Chemistry, Hunter College, 695 Park Avenue, New York, NY 10021.

We have modified the Metropolis Monte Carlo algorithm to simulate a weak electrolyte solution. Our solution contains a uni-univalent electrolyte (A^+ and B^-), an uncharged binary molecule (AB) and an aqueous solvent represented by a uniform dielectric constant ε. The potential energy of the system includes: 1) a Coulombic potential among all charged species, 2) no interaction among uncharged molecules, 3) no interaction between a molecule and an ion and 4) a harmonic potential representing the AB vibrational, internal energy. At equilibrium, our system can be described by an equilibrium constant for the association and dissociation processes represented by $A^+ + B^- <--> AB$. We incorporate the dynamical processes into the Monte Carlo algorithm by 1) randomly choosing an ion, an ion pair or a molecule to attempt a trial move, 2) computing a trial distance between A^+ and B^- if an ion pair or a molecule is chosen, 3) assuming that if the trial distance between the ion pair is less than a specified value (dmax), the ion pair has formed a molecule, and if the distance is greater than dmax, the molecule has dissociated, 4) assigning the energy of the molecule from the harmonic oscillator potential and 5) accepting the trial move according to the usual Metropolis Monte Carlo criterion. The trial distance for the association and dissociation processes are obtained from Newton's equation which includes a random, solvent initiated force that can induce the association or dissociation process. We present the results of this simulation technique and speculate about applying the method to the study of multi-valent ligand interactions with oligonucleotides.

W-Pos501

B_{Mg}, MAGNESIUM BUFFER COEFFICIENT: A NEW METHOD FOR DETERMINING MAGNESIUM BUFFERING IN VENTRICULAR MYOCYTES. ((K.L. Koss, R.W. Putnam, and R.D. Grubbs)) Dept. of Physiol./Biophys., Univ. of Cincinnati, Cincinnati, OH 45267; Dept. Physiol./Biophys. & Dept. of Pharmacol./Toxicol., Wright State Univ., Dayton, OH 45435.

A new expression for intracellular Mg buffering (B_{Mg}) was derived which is analogous to the formal definition of H^+ buffering power. The Mg buffer coefficient, B_{Mg}, is a unitless indicator defining the amount of Mg that must be added to a cell to raise the free Mg (αMg^{2+}_i) of that cell by 1 mM. In the present study, B_{Mg} was utilized to evaluate the intracellular Mg buffering of ventricular myocytes. Liberation of Mg^{2+} into the ventricular myoplasm was accomplished by metabolic inhibition causing the depletion of cellular Mg-ATP. Cellular adenine nucleotide levels were determined by HPLC, myoplasmic αMg^{2+}_i using the Mg^{2+}-sensitive fluorescent indicator, mag-fura2, and αCa^{2+}_i using the Ca^{2+}-sensitive dye, fura2. Within 5 minutes of exposure to the metabolic inhibitors IAA/CN, ATP was depleted, liberating a net Mg^{2+} of 1.26 mM and causing an increase in αMg^{2+}_i of 0.9 mM. Using these measurments, a B_{Mg} value of 1.4 was determined (in the presence of physiological $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$). In the absence of extracellular Ca^{2+}, 5 min of IAA/CN exposure liberated a net 1.72 mM Mg^{2+} and increased αMg^{2+}_i by 0.69 mM. From these values, a B_{Mg} of 2.5 was calculated. Metabolic inhibitors caused an increase in αCa^{2+}_i in the presence of $[Ca^{2+}]_o$ but not in its absence. These data indicate that Mg buffering in ventricular myocytes can be modulated by changes in myoplasmic αCa^{2+}. This coefficient can be used to evaluate buffering of other ions such as Ca, Na and K.[NSF DCB-9018677 & AHA, OH Aff.]

W-Pos502

THE HIGH-ORDER KINETICS OF HYPEROSMOTIC CYTOLYSIS

((Robert J. Williams, Allen G. Hirsh, Tsuneo Takahashi and Harold T. Meryman)) American Red Cross Holland Laboratory, Rockville, Maryland USA and Japanese Red Cross Research, Sapporo, JAPAN

A paradox of cryopreservation is that there is a range of temperatures below freezing but above the glass transition temperature at which injury is accelerated by temperature lowering. An Arrhenius expression in which osmotic stress supplied an "activation energy" failed to reflect our observations of the kinetics of cytolysis. The data were well modelled ($r_2 > 0.95$) by a generalized Johnson-Mehl-Avrami equation: $\ln N_0 / N = (t/\tau)^n$ Two processes were seen to occur in osmotically stressed unfertilized sea urchin eggs. A slow process with a time constant τ = 660 secs was preceeded by a rapid process τ = 270 secs, with Avrami coefficients, n =1 and 7, respectively. In human red cells undergoing thermal shock hemolysis the time constants were 80 and 25 secs; the Avrami constants were 1 or less and 9 or greater, respectively. These data suggest that nuclei form which grow dendritically, most probably in the cytoskeleton. Our hypothesis is that the actin filaments undergo a stress failure when the stress exceeds a critical value. Supported in part by NIH Grant BRSG 2 507 RR05737.

W-Pos503

The Effect of Geometrical Constraints in Design of Dendritic Trees.

I. Aharon *Dept. of Neurobiology, Hebrew University, Jerusalem, Israel.*

Early work investigators have proposed various models for branching angles in arteries and rivers. These models operate by minimizing some parameters which are known as "cost". These parameters include frictional and metabolic power losses as well as drag. Depending on the level of agreement between observed and expected angles, one can reject or accept the hypothesis that a given cost principle might control the angle of branching for a set of junctions. Our work assumes, that for physiological reasons, the dendritic tree itself controls the total surface area of the tree, and tries to "build" a tree that has a given total surface and minimum volume (the brain has a finite volume contains many dendritic trees). This work presents an analytical discussion of the problem of locating the junction point between three branches, so that the sum of the total volume of the branches will minimum when the total branch surface is given. We conclude that there are only a few degrees of freedom in the growth mechanism of the dendritic tree, that give it the ability to grow randomly, and at the same time "design" a tree with a determined surface as well as minimum volume, by controlling only one parameter . In this study the cost penalty of nonoptimum branching is also calculated to determine how far from optimum these trees are actually.

W-Pos504

CREATION OF LONGITUDINAL DISSIPATIVE STRUCTURES IN MODELED CYLINDER-SHADED CELLS.

((S.M. Korogod and L.P. Savtchenko)) Lab. of Biophysics & Bioelectronics, Dniepropetrovsk State University, 320625, Dniepropetrovsk, the Ukraine.

In a mathematical model of cylinder-shaped cell it was observed creation of dissipative structures (DSs) in the form of steady state spatially non-uniform, in particular spatially periodical, longitudinal distributions of the transmembrane potential, those of cation intracellular concentration and of density of the open channels conducting inward passive electric current of these cations. DSs were created under following conditions: 1) the channels were subject to potential dependent conformation transitions between open and closed states and able to diffuse laterally of in the membrane without electrophoretic drift; 2) the diffusivity of the open channels was greater than that of the closed ones; 3) an outward active or pump current in the steady state was a sigmoid function of the local intracellular cation concentration. It was suggested that DSs maintained due to consuming the energy of dephosphorylation of the ATP molecules.

W-Pos505

DESCRIPTIVE ENLARGEMENT OF INTRACRANIAL ANEURYSMS TO A CRITICAL THICKNESS FOR RUPTURE.

((Austin, G.*, Gong X.**, and Suo, Z**)) Aneurysm Research Institute*, and Dept. of Mechanical Engineering**, UCSB, Santa Barbara, CA

Most intracranial aneurysms (IA) are thinner at the apex; and rupture at the apex. (Weir 1987; Crompton, 1966). Minimum thickness of an unruptured aneurysm is reported 20 microns (Suzuki, et at 1980). We have developed a model of aneurysm enlargement starting from a vascular membrane using the membrane elasticity equations of Green and Adkins, 1960 and Bogen & Mc Mahon, 1978, and a computer solution with a new Fortran program by X. Gong. The homogeneous wall enlarges with suprathreshold pressure increments and is assumed incompressible. Parameters include initial neck diameter, initial membrane thickness, material constant, relative Young's Modulus, relative initial apex inflation, and curvature at the apex. The enlarging aneurysm is nonspherical with cylindrical symmetry. Growth of the aneurysm shows increased thinning toward the apex and a predictable minimum volume for the threshold thickness at rupture. Graphic results are displayed to show pressure-volume, thickness-apex distance, and thickness-pressure relations. The graphic and pictorial plot of aneurysm volume versus wall thickness and diameter, predicts proximity to rupture for given initial paramenters.

BIOTECHNOLOGY AND BIOENGINEERING

W-Pos506

THE EFFICIENCY OF ELECTROPORATION-MEDIATED DNA UPTAKE BY NIH3T3 FIBROBLASTS DOES NOT DEPEND ON DNA TOPOLOGY.

((T. D. Xie[1] and T.Y. Tsong[1,2])) Dept of Biochem, [1]Univ of Minn, St. Paul, MN 55108 and [2]Hong Kong Univ of Sci & Tech, Hong Kong.

Neumann and coworkers [EMBO J. 1:841-845 (1982)] have reported that the efficiency of electrotransfection of mouse L-cells was much higher for the linear form than for the circular form of DNA. However, with the bacterium E. coli, we have found that electroporation-mediated uptake of plasmid DNA did not depend on DNA topology, although the transfection efficiency was much lower for the linear (ln-) DNA than that for the circular relaxed (cr-) and the supercoiled circular (sc-) DNA [Biophys. J. October 1992 Issue]. We report here the transfection of NIH3T3 mouse fibroblasts by the plasmids, $pRSV_{cat}$, $pRSV_{neo}$, and $pRSV_{gpt}$. [^{3}H]-labelled plasmids were used to monitor surface binding before, and cell uptake of DNA after, electroporation. Transfection efficiency was evaluated by the transient expression of antibiotic resistances by these plasmids and the appearance of permanent antibiotic-resistant transformants. Our results indicate that all three topological forms of DNA bound to the cell surface with equal affinities. Electroporation-mediated cell uptake of the three forms of DNA was also identical under a broad range of experimental conditions, including varying [Mg^{++}] and electric parameters. However, cr-DNA and sc-DNA were at least 10 times more efficient than ln-DNA in assays for transient and permanent expression. These results agree with our previous work with E. coli but disagree with the work of Neumann et al. These results also point out that electrotransfection involves several steps: the cation-dependent cell surface binding of DNA, the electric field-driven entrance of DNA into cells, and the expression and the integration into the host chromosome, of the loaded DNA. [Supported by Office of Naval Research].

W-Pos507

EXPRESSION OF RECOMBINANT HUMAN PANCREATIC POLYPEPTIDE IN *E.coli*.

((M.D. Kapanadze, Yu.V. Griko*)) Johns Hopkins University, Baltimore, MD 21218, USA* and Institute of Protein Research of the Russian Academy Sciences, Russia.

Human pancreatic polypeptide (hPP) is a small hormone, consisting of 36 amino acid residues, that plays a critical role in regulation of secretion of many physiologicaly active peptides from the pancreas. The region of DNA encoding human pancreatic polypeptide was obtained by polymerase chain reaction (PCR) and subcloned into an expression vector. The pancreatic polypeptide gene was expressed in *Escherichia coli* in two versions: as a cleavable fusion protein with IgG-binding synthetic ZZ domains of protein A from *Staphylococcus aureus* and with the 1-55 fragment of λ Cro repressor. Site-specific hydrolysis by hydroxylamine was used to cleave the fusion protein, releasing the human polypeptide. This hPP differs from the natural one by non-amidation of the C-terminal tyrosine. Both fusion systems can be efficiently used for protein preparation and site-specific mutagenesis.

W-Pos508

BASIS FOR PROTEIN SEPARATIONS BY MATRIX COPRECIPITATION. Rex Lovrien, Mark Conroy and Tim Richardson. Biochemistry Dept., Univ. of Minnesota, St. Paul, MN 55108

Matrix coprecipitation uses organic ionic ligands to coprecipitate proteins and peptides. Suitable ligands have ionic heads that promote strong binding, and tail groups of nonpolar ring systems like naphthalene that stack to pull the matrix together. The matrix method commonly coprecipitates enzymes from 0.01 to 0.10% solution. It is protective against thermal, pH, oxidative, bioprocessing shock. Suitable ligands are readily trapped out (exchange resins) to release enzymes and retrieve their activities. We work with about 15 enzymes. Matrix reactions are "pulling" reactions which form coprecipitates in contrast to salting out which probably pushes proteins out of solution. Energy drives for forcing coprecipitation start from electrostatic forces, with anionic ligands attracted to cationic proteins. Simultaneously there is displacement of water, and insertion of lower dielectric organic material which reinforces Coulombic attraction. The organic parts of such ligands attract one another to cement the matrix and scavenge sought-for proteins from very dilute crudes. Coprecipitation and cocrystallization in this way is sensitive to organic structures of such ligands dependent on how well they stack to make the matrix host. Displacement of water, replacement with ligands gives a relatively dry, dense product which centrifuges with low gravities, helping processing and scaleability. Ref.: Conroy and Lovrien, J. Crystal Growth 122, 213-222 (1992).

W-Pos509

PERMEABILITY OF IMPLANTED-TUMOR VASCULATURE TO POLYMER-GRAFTED LIPOSOMES ((D. Needham, D. Da and T. L. Rudoll, H. P. Ting-Beall, N. Wu and M. W. Dewhirst)) Dept. Mechanical Engineering and Materials Science, the Center for Biochemical Engineering and Dept. Radiation Oncology, Duke University, Durham, NC.

Polyethylene glycol bearing lipids (PEG-lipids) have recently been incorporated into Stealth® liposomes (® trademark, Liposome Technology) for use in antitumor drug delivery. The rate of extravasation of Stealth liposomes through leaky tumor microvasculature and their accumulation and distribution in tumor tissue are, as yet, not well quantified. Therefore, in order to characterize, in-situ, drug dosimetry and distribution in tumors we have begun studies that use a dorsal flap rat widow preparation containing an implanted mammary adenocarcinoma. Fluorescently labelled liposomes (0.2mls; 10mM lipid; 97nm diam, made by Lipofast extrusion) were injected through the femoral vein, and the tumor microvasculature was viewed intermittently for 90 minutes by fluorescence videomicroscopy. The average of 9 Stealth (Egg PC/Cholesterol 2/1, 5 mol% PEG^{2000}-lipid) and 7 conventional (Egg PC/Cholesterol 2/1) liposome experiments showed that: (1) 90 mins after injection, the concentration of Stealth liposomes in the tumor interstitium was 2 times higher; (2) the tumor vascular permeability coefficient of Stealth liposomes was 3.42×10^{-7} cm/s versus 1.75×10^{-7} cm/s for conventional; and (3) blood circulation half-life was 2 hrs for Stealth versus 0.6 hrs for conventional. Also, electron microscopy showed conventional liposomes to be more aggregated in buffer. Thus, the greater accumulation of Stealth liposomes in tumors is promoted by a sustained concentration of well dispersed liposomes in the blood and an increased permeability coefficient for extravasation through the tumor vasculature.

W-Pos510

ACCURACY OF THE BUBBLE PRESSURE METHOD (BPM) IN ESTIMATING MICROPIPETTE DIAMETER ((Charles L. Bowman and Abdul M. Ruknudin)) Department of Biophysical Sci. SUNY-Buffalo, Buffalo, NY 14214.

BPM was evaluated for estimating the diameter of micropipettes used in patch clamp studies of a variety of cells. Micropipettes were immersed in filtered ethanol and pressure was applied using a syringe. The minimum pressure needed to release bubbles from the tip was measured with a digital manometer. The diameter was calculated from Laplace's law, and compared to measurements using scanning electron microscopy. BPM generally resulted in a diameter 150 nM greater than ID. Differences in diameters estimated from BPM were smaller for pairs of micropipettes made from the same capillary (mean 0.031μm), but larger for micropipettes pulled in succession (mean 0.570μm). As expected, threshold for bubble pressure was sensitive to the surface tension of the liquid. Siliconizing the pipette decreased the bubble pressure by 10%, but exposure to nitrogen glow discharge was without effect. Threshold pressure is dominated by the surface tension of the air-ethanol interface. BPM generally gives a more accurate estimation of micropipette ID than light microscopy.

W-Pos511

VERSATILITY OF A FIBER OPTIC BIOSENSOR.

((E.James, L. Shriver-Lake, R. Ogert, W. Wiesmann, A. Cross, F. Ligler)) NRL, Wa.DC 20375, WRAIR, Wa.DC 20307.

The NRL fiber optic biosensor is based on changes in fluorescence intensity. As a fluorescently tagged molecule binds to the fiber optic probe it is excited by the evanescent wave, resulting in an increase in fluorescence emission intensity. Small molecules (TNT), large proteins (13,000-160,00 MW) and a glycolipid (endotoxin) were detected using the NRL fiber optic biosensor. An antibody or specific binding protein was covalently immobilized on a fiber optic probe. For detection of small molecules, a displacement immunoassay was sensitive to < 1 ng/ml. For the larger molecules, binding was observed using the sandwich immuno assay technique. In this assay an unlabelled molecule of interest is allowed to react with the immobilized capture molecule. This is followed by a second fluorescently labelled recognition molecule. Limits of detection for this type of protein assay are <5 ng/ml. Using the evanescent wave fiber optic biosensor, we have detected concentrations in the picomolar range. This method of detection allows rapid detection times, high sensitivity and selectivity.

ELECTROPHORESIS OF DNA

W-Pos512

2-DIMENSIONAL MOTION OF DNA BANDS DURING PULSED-FIELD GEL ELECTROPHORESIS((M. Shane Hutson, Lynn M. Neitzey,and G. Holzwarth)) Department of Physics, Wake Forest University, Winston-Salem, NC 27109

The position and velocity of a band of double-stranded, linear G DNA(670 kb) were measured in 1% agarose during 120° PFGE, using a video micrometer, for switch times T = 5 to 240 s, which span the antiresonance time T^*. The path traced in the xy(gel) plane was in excellent accord with Southern's ratchet model, in which the two ends of a chain of fixed length alternate in leading the chain through the gel. However, the measured instantaneous velocity v_x showed a sharp backward spike each time the field changed direction, with amplitude about twice the mean drift velocity, whereas v_y showed a sharp positive spike with amplitude more than 3 times the plateau velocity in the y direction. The velocity spikes are consistent with the idea that, for $T > T^*$, most DNA chains are stretched into U-shaped or herniated configurations. When the field changes direction, the U's and hernias recoil backward rapidly in response to intramolecular DNA chain tension. The velocities are inconsistent with models which assume a constant curvilinear velocity of DNA in a tube and with the biased reptation model without fluctuations. The dependence of 2D velocity on M and field angle has also been studied.

W-Pos513

TWO-DIMENSIONAL ELECTROPHORESIS SOUTHERN BLOT METHOD TO DETECT HUMAN GENOME POLYMORPHISMS. ((G. F. LeBlond, H. Nakashima, M. Yi, and P. O. P. Ts'o)) Biochemistry Dept., Johns Hopkins SHPH, Baltimore, MD 21205-2179.

A high resolution Southern blot method to resolve the distribution of interspersed, repetitive sequences in the human genome has been developed. The method consists of two separate restriction enzyme digestions, including in situ digestions, with large scale (40cm X 50cm) agarose gel two-dimensional electrophoresis followed by Southern transfer of the size separated DNA fragments onto a membrane. The very 5'-end of the LINE-1 DNA sequence was used as a probe to compare several genomic DNA samples from different people. About 900 LINE-1 signals were resolved on a two-dimensional plane for each sample. We detected 15 polymorphic signals via visual inspection. Additional rounds of in situ digestions and electrophoresis separations, of unresolved regions of the gel, yielded about 400 more fragment signals. These methods have been applied to monitor polymorphisms or rearrangements adjacent to the interspersed sequences in the human genome.

Supported by DOE grant DE-FG02-88ER60636 and by an American Cancer Society grant CN66.

W-Poe514

CONSTANT ACTIVITY GEL ELECTROPHORESIS (CAGE) - A NEW METHOD TO CHARACTERIZE THE BINDING OF NONIONIC LIGANDS TO NUCLEIC ACIDS ((T.L. Trapane and P.O.P. Ts'o)) Biochemistry Dept., Johns Hopkins SHPH, Baltimore, MD 21205.

Complex formation by nucleic acids has been studied by methods in which thermal dissociation/ association affords a T_m value which can be analysed to derive thermodynamics. It is also possible to evaluate complex formation of similarly charged species (e.g. an oligonucleotide and its complement) through the differential mobilities of free versus bound species observed during gel electrophoresis. However, when the charge on the target nucleic acid and its intended ligand are widely different, electrophoretic methods generally fail due to the difference in mobility of the interacting species. We describe a method designed to study complex formation between nonionic ligands and oligonucleotides. Polyacrylamide gels are constructed in which the nonionic species is maintained at constant activity within the gel matrix. The labeled target is introduced into the gel in the well and then electrophoresed into several zones containing ligand at various concentrations. The formation of a complex is observed as a decrease in mobility of the target versus its mobility in the absence of ligand. At conditions of constant temperature, binding constants can be derived. Duplex formation between the charged oligonucleotide, $d(AG)_8$, and its complementary uncharged ligand, $d(\underline{CT})_8$ (having nonionic methylphosphonate backbone linkages), is investigated. Various results and experimental and theoretical issues on CAGE will be addressed. (Supported by NCI)

W-Poe515

DEPENDENCE OF BASE PAIR MISMATCH STABILITIES ON NEAREST NEIGHBOR SEQUENCE: DETERMINATION BY TEMPERATURE-GRADIENT GEL ELECTROPHORESIS USING DNA FRAGMENTS (S-H. Ke and R.M. Wartell) School of Biology, Georgia Tech, Atlanta,GA 30332.

The thermal stabilities of DNA fragments that contain single base pair mismatches have been examined by temperature-gradient gel electrophoresis (TGGE). Homologous 373 bp DNA fragments differing by single base pair substitutions were employed. Heteroduplexes containing single base pair mismatches were formed by melting and reannealing pairs of DNAs. Product DNAs were separated based on their thermal stability in a temperature-gradient polyacrylamide gel containing urea and formamide. Results indicate that all fragments containing mismatches are destabilized by about 2.4 to 4.2 °C with respect to homologous DNAs with complete Watson-Crick base pairing. Both the bases at the mismatch site and neighboring stacking interactions influence the destabilization caused by a mismatch. For the GXT/AYC sequence (X/Y being the mismatch), the order of stability was G/G,A/G > G/A > G/T,T/G > A/A > T/T,C/A,A/C > T/C,C/T > C/C. For the sequence TXT/AYA, the ranking of stability was G/G,A/G,G/T > T/G,G/A > C/T,A/A,T/T,T/C > C/A,A/C,C/C. Our results are in good agreement with available data from solution studies of short DNA oligomers. The TGGE approach allows for the rapid study of base pair mismatch stability at different sites within a long DNA fragment. (Supported by N.I.H.).

MACROMOLECULAR CROWDING IN CELLULAR BIOCHEMISTRY AND BIOPHYSICS

Th-AM-Symi-1

HIPPOCAMPAL COMPUTATION: THEORETICAL AND NEUROPHYSIOLOGICAL ANALYSIS ((Edmund T. Rolls)) Univ Oxford, Dept Exptl Psychol, Oxford OX1 3UD, England.

The primate hippocampus is needed for spatial memory tasks in which the location of objects must be remembered, and in which the location of places where responses must be made are to be remembered. It is also involved in some non-spatial memory tasks such as recognition memory for visual stimuli. Neurophysiological and computational investigations lead to the hypothesis that the importance of the hippocampus in spatial and other memories is that it can rapidly form "episodic" representations of information originating from many different areas of the cerebral association cortex. A key aspect of the theory is that the CA3 pyramidal cells with their 4% interconnectivity and Hebb-modifiable synapses implement an autoassociation memory which provides the basis for "episodic" memories (Rolls, 1989, 1990). It has been possible to develop an analytic model for such a system which shows that storage and retrieval are efficient even with neurons with continuously variable firing rates (Treves and Rolls, 1991). Moreover, the non-linearity in an NMDA-based learning rule can enhance the number of memories that can be stored in such a network. It has also been suggested that a function of the mossy fibre inputs to the CA3 cells is to force during learning a new pattern of activity onto the CA3 cells by virtue of strong synapses, thus minimising during learning the randomizing effect produced by the recurrent collaterals as a result of the storage of other memories. It has also been suggested that the direct perforant path input to the CA3 cells is associatively modifiable and is required to initiate retrieval (Treves and Rolls, 1992).

Rolls,E.T. (1989) Ch. 13, pp. 240-265 in Neural Models of Plasticity: Experimental and Theoretical Approaches, eds. J.H.Byrne and W.O.Berry. Academic Press: San Diego.
Rolls,E.T. (1990) Cold Spring Harbor Symposia in Quantitative Biology 55: 995-1006.
Treves,A. and Rolls,E.T. (1991) Network 2: 371-397.
Treves,A. and Rolls,E.T. (1992) Hippocampus 2: 189-199.

Th-AM-Symi-2

USE OF CALCIUM AND SODIUM IMAGING TO STUDY MECHANISMS OF CALCIUM ENTRY INTO HIPPOCAMPAL NEURONS

Nechama Lasser-Ross, Dept. of Physiology, New York Medical College, Valhalla, NY 10595

Activity related increases in dendritic calcium concentration ($[Ca^{2+}]_i$) are believed to be essential for the induction of both NMDA and non-NMDA forms of LTP. $[Ca^{2+}]_i$ changes are also involved in LTD, cytotoxicity and exocytosis. It is therefore important to understand the mechanisms by which these changes occur.

We have used a high speed CCD camera to monitor changes in $[Ca^{2+}]_i$ and $[Na^+]_i$, correlated with electrical activity, in hippocampal pyramidal cells in slices. Neurons filled with fura-2 or the Na^+ indicator SBFI were stimulated intracellularly or synaptically.

Results indicate : (a) The highest change in overall dendritic $[Ca^{2+}]_i$ is triggered by Na^+ spikes (evoked synaptically or by a depolarizing pulse); the spatial pattern reflects the extent of Na^+ action potential propagation. (b) A much smaller change in $[Ca^{2+}]_i$ follows subthreshold synaptic activation. Most of this signal is due to Ca^{2+} entry via voltage-gated channels, activated by the synaptic potential.(c) Under conditions where the NMDA synaptic current was maximized and current via voltage-gated channels largely reduced, Ca^{2+} entry through the NMDA channels could not be resolved with certainty.

Experiments supporting these findings will be described and their relevance to LTP will be discussed.

Th-AM-Symi-3

TWO MODIFICATIONS CONTRIBUTE TO LONG-TERM SYNAPTIC POTENTIATION IN CA1 HIPPOCAMPUS

Roberto Malinow

University of Iowa, Iowa City, IA 52242

The modification responsible for the long-term synaptic potentiation (LTP) that follows a brief conditioning period is not known. To elucidate this change, we have looked for quantal levels of transmission before and after induction of LTP. Statistical, analytical and experimental tests indicate that quantal transmission can be resolved. During LTP we find an increase in both the number of quanta released and in quantal amplitude, consistent with combined pre- and postsynaptic modifications. On average, about 60% of LTP can be accounted by presynaptic enhancement. The increase in either quantal amplitude or quantal content varies significantly among different experiments, but is inversely correlated with its initial value. The increase in the two modifications are not correlated. This suggests that there are two mechanisms contributing to LTP, that can be regulated independently. These results may help to reconcile the different views concerning the site of LTP expression.

Th-AM-Symi-4

CaM-KINASE AS A MEMORY STORAGE DEVICE. ((J. Lisman)) Biology Dept., Brandeis University, Waltham, MA 02254

A cornerstone of neurobiology is the idea that memory can be stored in nerve networks by activity-dependent changes in the efficacy of synapses. Dr. Roll's talk will review this concept. Much has been learned about the activity-dependent process itself; Dr. Malinow has used quantal analysis to show that there are both pre and postsynaptic changes; Dr. Lasser-Ross has used imaging methods to study dendritic Ca entry, an entry that triggers changes in efficacy. My own work deals with how efficacy is stored--i.e. the physical basis of memory. Central to this question is how proteins can provide long term information storage despite protein turnover. I have explored whether Ca/calmodulin protein kinase II, a protein localized in postsynaptic densities (PSD), can store long-term information. Calculations show that the known positive feedback autophosphorylation of this molecule gives it bistable switch-like properties with remarkable stability. Furthermore, this mechanism resists protein turnover. The group of kinase molecules in the PSD could store graded information through changes in the fraction on. These kinase molecules could control efficacy by modulating the nearby glutamate receptors that mediate synaptic transmission. Tests of this model will be reviewed.